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## Oral Abstracts

### Session: Additive Manufacturing of Polymer Melts for Scaffold-Based Tissue Engineering

Date and Time: Wednesday, September 9, 2015,  
10:30 AM - 12:00 PM

#### Patterned and Photo-cross-linked Fibrous Scaffolds Via Melt Electrospinning Writing

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Melt electrospinning writing (MEW) enables the design and fabrication of micrometer-thin fibrous scaffolds with highly controllable architectures and patterns. So far, MEW has been mainly applied using a low melting point thermoplastic, poly( $\epsilon$ -caprolactone). However, scaffolds prepared from such thermoplastics exhibit a large reduction in modulus upon absorption of water in application. It was reasoned that cross-linking would result in a hydrated scaffold that retains a high modulus. The objective of this study was to manufacture and characterize a scaffold using a photo-cross-linkable polymer (poly(L-lactide-co- $\epsilon$ -caprolactone-co-acryloyl carbonate), P(LLA-CL-AC)) using the MEW process and assess its modulus following hydration. The P(LLA-CL-LA) was successfully melt electrospun in a direct writing mode without inducing crosslinking, as confirmed from sol measurements. The average fiber diameter of the scaffold was 30  $\mu$ m and the fibrous layers of the scaffold were oriented at 90°. The MEW scaffolds were efficiently cross-linked with UV light. The uncross-linked scaffolds possessed lower modulus (66.3 MPa) compared to cross-linked groups (92 MPa). Interestingly, the uncross-linked scaffolds exhibited a marked reduction in modulus following hydration, to 6.3  $\pm$  0.5 MPa, while the modulus of the hydrated cross-linked scaffolds was significantly higher, at 23.2  $\pm$  3 MPa. This work demonstrates for the first time the melt electrospinning writing of a photo-cross-linkable polymer. This scaffold preparation approach is of potential interest for ligament or tendon tissue engineering applications. Future studies will examine the fatigue properties of the scaffolds, the change in their mechanical properties following *in vitro* degradation, and cellular response within a dynamically loaded bioreactor.

#### Melt Electrospinning Writing of Suspended Fiber Arrays

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**Introduction:** Additive manufacturing is extending design capabilities, allowing complex shape fabrication. Challenges currently facing fused deposition modelling include: 1) achieving small filament diameters and 2) filaments spanning voids, without underlying support structure. Melt electrospinning-writing (MEW) solves both challenges: generating well defined arrays of 3  $\mu$ m diameter fibers that were suspended across voids, at least 6 mm wide. This allows the fabrication of defined, suspended fiber arrays as scaffolds.

**Methods:** MEW was performed with poly(caprolactone) (PCL) on a custom-built direct writing machine. Nozzle to collector distance was 6 mm, with a high voltage of 5 kV and -1.5 kV applied to the nozzle and collector respectively.

**Results/Discussion:** Fiber diameters were consistent and much smaller than for melt extrusion; ranging from 3–15  $\mu$ m (variations of less than 10% for each diameter investigated). Initially, a frame of PCL fibers were generated with repeated deposition (5–25 times) at a spacing between 150  $\mu$ m–6 mm. Suspended fibers were then depos-

ited across the void between the PCL supports with interfiber distance of 100  $\mu$ m. Repetition of this format creates the 3D spacing required to build defined, orientated scaffolds that could be handled *in vitro* while mechanically protecting the suspended fiber array.

**Conclusions:** Defined, fiber arrays were suspended across millimeter gaps using MEW. The highly controlled alignment and spacing in all axes will assist in the development of 3D constructs for investigating numerous aspects of cell-substrate interactions, as well as in regenerative medicine as tissue engineered scaffolds for inducing repair and regeneration after traumatic nervous system injuries.

#### Gradient Scaffolds for Tissue Engineering and Regenerative Medicine

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As our tissues have been developed following biophysical, mechanical, and biological gradients, the aim of this study was to engineer 3D scaffolds displaying these kind of gradients to actively influence stem cell activity for skeletal regeneration. We implemented different gradients in the design of scaffolds fabricated by additive manufacturing: (i) structural, through the variation of fiber pattern; (ii) physico-chemical, through the deposition of different biomaterials; and (iii) biological through polymer-brushes mediated binding of growth factors.

The fabricated scaffolds showed to support early differentiation of adult bone-marrow derived stem cells into the osteogenic and chondrogenic lineages. Osteogenesis was favored by an increase in pore size and pore rhomboidal shape at a structural level, by a hydrophilic and softer polymer composition at a physico-chemical level, and by the binding of BMP-2 at a biological level. Conversely chondrogenesis was favored by a decrease in pore size and pore rhomboidal shape at a structural level, by a more hydrophilic and stiffer polymer composition at a physico-chemical level, and by the binding of TGF- $\beta$ 3 at a biological level.

Ultimately, we present here the creation of a new library of active scaffolds able to steer stem cell activity by engineering cell-material interactions in three-dimensions.

#### Microfabrication and Perfusion Culture of New Tissue Elements Possessing Hollow Structures with High Mechanical Strength towards Scaling-up to Implantable Liver

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Addressing the issue of insufficient mass transfer in scaling-up to implantable liver tissue equivalent, we designed a new three-dimensional (3D) microstructure with integrated channel-like structures supporting greater medium distribution. Fabricated from biodegradable poly( $\epsilon$ -caprolactone) (PCL), the module of cylindrical microstructure is produced via selective laser sintering (SLS) aiming at sufficient mechanical strength and higher design accuracy with 60% porosity. The modules (1100  $\mu$ m in diameter; 1500  $\mu$ m in

height) possessing three-perpendicular hollow structures (300  $\mu\text{m}$  in diameter) are initially inoculated by hepatocarcinoma (Hep-G2), and then randomly packed together in a bioreactor (0.06  $\text{cm}^3$ ) at a high-density for 14 days of perfusion culture as one of the methodologies of bottom-up tissue engineering. Together with the interstitial gaps generated by the random packing, the hollow structures of each micro scaffold acting as a macro-channel ensured and smoothed the accessibility of cell suspension and perfused culture medium. This fundamental advantage demonstrated not only more efficient cell attachment but also the sufficient and uniform nutrient supply maintaining high cellular growth, viability, and cellular function. The embedded micro scaffold promised the attachment efficiency by  $5.2 \pm 1.7 \times 10^4$  cells/module and confirmed the sustained growth reaching  $2.56 \pm 0.26 \times 10^7$  cells/ $\text{cm}^3$ , with 1.75 times higher albumin production compared to that in conventional cylindrical module as a control. The packed bioreactor system with interstitial gap as medium distribution channels is conveniently scaled up towards larger liver tissue construction.

### Additive Biomanufacturing in Tissue Engineering and Regenerative Medicine

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The application of Additive Biomanufacturing represents one of the most rapidly advancing areas of biomedical sciences in which engineers, scientist and clinicians are contributing en masse to the future of human health care and more specifically to the areas of medical devices, tissue engineering & regenerative medicine (TE/RM) and *in vitro* disease model development. Current challenges in TE/RM research lie in identification of emerging rules for organ regeneration and translation of these rules into scaffold design parameters as simply and as effectively as possible in order to achieve measurable clinical translatability. To achieve widespread routine clinical application, firstly the TE constructs must be reduced to basic crucial properties to make them as easy to handle as possible, secondly involve minimal post-processing after fabrication and lastly must be able to be manufactured in a reproducible, controlled process at economical costs and speed. To fulfill these requirements a number of automated fabrication methods have been employed to create scaffolds with well-defined architectures and AM methodologies lie at the forefront of such automated fabrication methods. Hence, this talk will postulate the quest that the 21 century scaffold for any tissue has a multiphase design that should be based on the accumulated knowledge from studying the relevant scaffold literature and implementing the key design parameters into the design of a architecture and morphology which allows cell migration, proliferation and subsequently vascularized tissue formation.

### Session: Advanced Therapy Medicinal Products - Challenges and Opportunities in the Next Decade

**Date and Time: Wednesday, September 9, 2015, 10:30 AM - 12:00 PM**

#### Bone Marrow Concentrate and Platelet Rich Plasma differ in Cell Distribution and Interleukin 1 Receptor Antagonist Concentration

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The aim of this study was to determine the cellular composition and cytokine concentrations of bone marrow concentrate (BMC) and platelet-rich plasma (PRP) and furthermore to compare two commercially available BMC systems used in the same patient cohort.

**Methods:** Patients (29) undergoing orthopedic surgery were enrolled. Bone marrow aspirate (BMA) was processed to generate BMC from two commercial systems (BMC-A and BMC-B). Blood

was obtained to make PRP utilizing the same system as BMC-A. Bone marrow derived samples were cultured to measure colony forming units and flow cytometry was performed to assess mesenchymal stem cells (MSCs) markers. Cellular concentrations were assessed for all samples. Catabolic cytokine and growth factors important for cartilage repair were measured using multiplex ELISA.

**Results:** Colony forming units were significantly increased ( $p < 0.0001$ ) in BMC compared to BMA. Flow cytometry markers were consistent with MSCs (CD73, CD271 positive; CD45, CD34 negative). Platelet counts were not significantly different between BMC and PRP ( $p = 0.46$ ). Platelet-derived TGF $\beta$ 1 and PDGF were not significantly different between BMC and PRP ( $p = 0.05$ ). IL-1ra in BMA (4,510 pg/mL  $\pm$  2,994) was twice previously reported values for autologous conditioned serum (ACS). In BMC, IL-1ra (13,432 pg/mL  $\pm$  8,588) was an order of magnitude greater than ACS. The ratio of IL-1ra:IL-1 $\beta$  in all BMC samples was above the ratio reported to inhibit the catabolic cytokine IL-1 $\beta$ .

**Conclusions:** Cellular and cytokine composition differ between BMC systems. Bone marrow concentrate and PRP have distinct molecular compositions with BMC offering stem cells and increased concentrations of IL-1ra compared to PRP.

### Controlling Cellular Bioelectric Signaling for Regenerative Medicine, Cancer, and Bioengineering

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An important and as yet largely-untapped component of biological regulation is developmental bioelectricity: endogenous, voltage-based signals that are exchanged by all cells (not just excitable nerve and muscle). We created the first methodology to track and rationally modify the bioelectrical conversations that keep individual cell activity orchestrated towards specific anatomical outcomes. Voltage potentials regulate the proliferation, differentiation, migration, and shape of somatic and stem cell populations. Voltage-sensitive fluorescent dyes enable detection of special cell populations (like pre-cancer) *in vivo*. Importantly, these gradients function non-locally, integrating body-wide information among cells to dictate organ identity, size information, and geometrical organization of a growing or remodeling organism. By learning to manipulate bioelectric gradients using misexpression of ion channels and application of channel-targeting drugs in animal models (e.g., frog), we make alterations in large-scale growth and form. Beyond the control of individual cell identity, we can reprogram various body regions into whole eyes or legs, control the patterning of the developing face, induce leg regeneration, and trigger melanoma-like body-wide metastasis in the absence of genetic damage. Most interestingly, our approach demonstrates a roadmap for superseding genetically-specified information by re-wiring bioelectrical networks. Current applications include the suppression of tumorigenesis despite presence of strong oncogenes, repair of brain defects despite genetic mutations in master neurogenesis genes, induction of spinal cord regeneration, and more. The use of ion channel drugs, many already approved for human use, as electroceuticals beyond the nervous system, suggests clear strategies for transformative advances in regenerative medicine, cancer, and synthetic bioengineering.

### Macromolecularly Crowding *in vitro* Microenvironments Maintain Human Corneal Fibroblasts Phenotype and Function

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Tissue engineering by self-assembly suffer from longer culture time which results in loss of cell phenotype and therapeutic potential of regenerated tissue. It is reported that the addition of inert macromolecules in culture medium; macromolecular crowding (MMC) significantly accelerate the extracellular matrix (ECM) deposition within two days of culture. Herein, the role of MMC on human

corneal fibroblasts (HCFs) culture was assessed using the neutral (Ficoll™, FC) and negative charged (dextran sulphate 500 kDa; DxS and carrageenan; CR) macromolecules. SDS-PAGE and complementary densitometry confirmed the significant increase in collagen I deposition ( $p < 0.005$ ). The immunofluorescence further confirmed the more aligned organization of ECM in the presence of MMC. The MMC further promoted the development of HCFs cell sheet using cell sheet technology without compromising the light transmittance. AFM and histological analysis using van-Geison, Picro-serius and Trichrome's staining demonstrated the enhanced ECM deposition in HCFs cell sheets in presence of MMC. No change in mRNA expression for collagen type I, fibronectin or  $\alpha$ -SMA was evidenced at day 6 of FC and CR treatment ( $p < 0.05$ ), confirming the phenotype maintenance. The proteomics data showed increase in total proteins and other ECM peptides deposition, whilst,  $\alpha$ -SMA; a myofibroblastic marker remains unchanged. Thus, MMC provides an alternative approach in cell-based therapies for corneal stromal repair with considerable clinical implications.

## References

Kumar P *et al.*, Macromolecularly Crowded *In Vitro* Microenvironments Accelerate the Production of Extracellular Matrix-rich Supramolecular Assemblies. *Scientific Reports* 5, 8729, 2015.

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## Translational Challenges in Applying Gene Therapy to Regenerative Orthopaedics

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There has been substantial pre-clinical progress in applying gene therapy to the field of regenerative orthopaedics. As gene transfer approaches to bone healing, cartilage repair, and other such indications continue to advance, they will encounter the need for translation into the clinic. This talk discusses the scientific, regulatory, economic and organizational issues surrounding this process. In general terms, the process is long and expensive and, ideally, future translational issues should be addressed early in the development of the research, rather than late in the pre-clinical phase of development, as is frequently the case. The process is greatly facilitated by institutional infrastructure to provide multifactorial support. Safety concerns are particularly important for applications in regenerative orthopaedics, where the target conditions are not life threatening. Progress, however, is being made and a gene therapy for cartilage repair is undergoing human clinical trials in South Korea

## A Regulatory Body-Approved Methodology for Localizing and Quantifying Human Mesenchymal Stem Cells Administered to Murine Pre-Clinical Models

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Determining mesenchymal stem cell (MSC) fate after transplantation is an essential part of characterizing its mechanism of action and safety. Preclinical biodistribution studies are therefore a regulatory requirement prior to clinical trial initiation.

This study's objective was to determine the distribution and persistence of locally administered human MSCs in murine models. A primer/probe qPCR assay was developed to quantify the presence of human Alu (hAlu) DNA sequences without detecting background murine DNA (mDNA). The resultant assay was more sensitive than the current state-of-the-art, detecting the DNA equivalent of 1 human cell in 200,000 mouse cells. Using this assay human MSC biodistribution was evaluated in two different preclinical models (i) three months after a single intramuscular injection into Balb/c nude mice

and (ii) two months after an intrafemoral injection into diabetic C57/B6 mice.

The genomic DNA was extracted from isolated organs and hAlu sequences were quantified by qPCR analysis. No hAlu sequences were detected in the brain, heart, lungs, kidneys, spleen, pancreas, small intestine, large intestine or liver of mice that received hMSCs. However, hAlu was detected in the calf and thigh muscles of Balb/C nude mice that received hMSCs intramuscularly. Between 0.1%-4.9% of the administered DNA persisted at the time of sacrifice.

hDNA (indicating hMSC localization) was detected three months after cell administration in immune compromised animals only at the administration site and not in distal tissues. In conclusion, this assay is reproducible, inexpensive and effective at detecting hDNA to the level required by regulatory agencies.

## Beyond Cell-Based Therapy: Paracrine Action of Human Adipose Derived Stem/Stromal Cells

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Mesenchymal Stem Cells (MSCs) therapeutic properties are believed to be related to the secretion of bioactive molecules in response to micro-environment. Since hASCs (human Adipose-derived Stem/Stromal Cells) are largely applied in regenerative medicine and their secretome has found to be enriched in trophic and immunomodulatory factors [1], we evaluated the ability of these cells and their conditioned medium (CM) to revert pain-related behaviors in two preclinical models of peripheral neuropathy. Previously, we demonstrated the rapid, dose-dependent and lasting analgesic effect of systemically administered  $10^6$  hASCs in the sciatic nerve Chronic Constriction Injury (CCI) murine model [2]. Here we show that iv injected CM derived from  $2 \times 10^6$  hASCs exerted a comparable antihyperalgesic effect, which lasted about 10 days and was restored by further treatments. Similarly, in the murine model of streptozotocin-induced diabetic neuropathy, both  $10^6$  hASCs and CM from  $2 \times 10^6$  hASCs showed a comparable anti-allodynic effect, which was very rapid (3–24h) and long-lasting (21–30 days). Moreover, diabetic animals regained weight after both hASC and CM treatment. The significant therapeutic effect of both hASCs and CM involves their immunomodulatory features which counteract the chronic inflammation [2], although we believe that in the long run hASCs could also mediate a regenerative effect, since a trophic effect of both hASCs and hASC-CM has been observed *in vitro*. Further analyses are required to elucidate the mechanism of action of this cellular therapeutic approach.

1. Franchi S, Castelli M. *Biomed Res Int.* 2014;2014:470983.

2. Sacerdote P, Niada S. *Stem Cells Dev.* 2013 Apr 15;22(8):1252–63.

## Session: Advances in Heart Valve Tissue Engineering

**Date and Time: Wednesday, September 9, 2015, 2:15 PM - 3:30 PM**

### Self-Assembled Tubular Heart Valves from Human Fibroblasts

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Recently, the tubular shape has been suggested as a simple and effective heart valve geometry, allowing easy fabrication, fast implantation, and minimal crimped footprint from a transcatheter delivery perspective. This minimalistic design is well-suited for the self-assembly method, with which the only support to the cells is the

extracellular matrix they themselves produce, allowing the tissue to be completely free from exogenous materials during its entire fabrication cycle. The objective of this research is to adapt the self-assembly tissue engineering method to the design of tubular heart valves. Tubular constructs were produced by rolling human fibroblastic self-assembled sheets on solid mandrels. After maturation, the tubes were installed at the root of an artificial aorta, fixed along their entire circumference on the ventricular side and with only three single point attached commissures (SPACs) on the aortic side. This allows the tube to collapse under back-pressure, adopting a shape similar to that of a tri-leaflet heart valve during diastole. A custom pulsed flow bioreactor was used to evaluate the valves performances. Preliminary results show good functionality with excellent leaflet coaptation and opening area. Aortic flow conditions have yet to be tested and durability has to be assessed. Histology confirmed abundant collagen content, good cell distribution and homogeneous tube fusion. This research shows that the self-assembly method, which has already proven its potential for small diameter vascular grafts, can be used to achieve functional tubular heart valves.

### Fluid-Structure Interaction Computational Modelling of the Mitral Valve Apparatus

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Currently there is a drive towards more personalised surgical interventions concerning valve repair and replacement. The mitral valve (MV) is a complex anatomical structure and its proper function depends on the delicate force distribution and synchronised function of its components. The aim of this project was to develop a novel prognostic/forecasting computational simulation tool that will provide patient-specific pre-operative optimisation of MV replacement/repair. MicroCT images of the intact porcine MV were segmented and the whole MV apparatus was reconstructed. In preliminary computational models, the atrial surfaces of the leaflets were simplified as membranes and the chordae as tension strings. Component-specific stress-strain data from uniaxial and biaxial tension tests were used in constitutive models to describe the regional biomechanics of the MV. LS-DYNA was used for Fluid-Structure Interaction (FSI) simulations of the MV for one cardiac cycle. The atrioventricular differential pressure was given as inlet boundary condition. The MV apparatus demonstrated significant regional and directional mechanical anisotropy. The simulations predicted regions of both leaflets with elevated stress concentration during the cardiac cycle, in accord with failure regions observed clinically. These regions were associated with regions in the fluid domain where vortices were developed. This study has indicated that different components of the MV experience different levels of stress and strain, which has an implication in the selection of appropriate repair materials for MV reconstruction. Future work will include development of blood-structure interaction models of the native 3D geometries incorporating surrounding structures. This study was supported by the REBIRTH cluster of Excellence.

### Biomaterial-based *In Situ* Tissue Engineering of Heart Valves

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*In situ* tissue engineering using biodegradable synthetic scaffolds is being explored to create living heart valve replacements inside the

human body. This breakthrough technology entails the implantation of a cell-free scaffold that gradually transforms into a living valve at the site of implantation.

Our approach is based on the notion that the natural inflammatory response to a scaffold can be harnessed to induce physiological healing and neo-tissue formation. The challenge is to develop instructive scaffolds capable of host cell repopulation and that provide biochemical and/or biophysical cues for a stable cellular phenotype and load-bearing extracellular matrix formation. We use electrospun scaffolds consisting of supramolecular polymers that can be functionalized with bioactive moieties to elicit specific responses of host cells, typically recruited from the blood stream. Biomimetic *in vitro* models and computational analyses are being used in direct comparison with *in vivo* experiments to optimize scaffold biochemical, biophysical, and degradation properties. Using *in vitro* models we demonstrated the modulation of inflammatory responses via the release of bioactive moieties to favor the recruitment of beneficial macrophage subpopulations. This modulation resulted in functional vessel formation by circulating cells during 3-month *in vivo* follow up in small animals. Optimization of scaffold structural-mechanical properties resulted in the creation of heart valves that can maintain mechanical and biological function up to 6 months in a sheep model.

(Project iValve of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs. Financial contribution of Netherlands Heart Foundation is gratefully acknowledged.)

### Improved Biofunctionalization of Cardiovascular Implants Following a Detergent-Free, Non-Proteolytic Decellularization Approach

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**Objectives:** Low immunogenicity and high repopulation capacity are crucial determinants for the functional and structural performance of decellularized cardiovascular grafts. The present study evaluates a detergent-free, non-proteolytic regimen for decellularization of heart valve and vessel grafts, particularly focusing on their biofunctionality after implantation into the systemic circulation.

**Methodology:** Rat aortic conduits (rAoC; n=70) were decellularized using detergents (group DET) or by a biological, detergent-free, non-proteolytic regimen (group BIO) applying latrunculin B, high ionic strength salt solutions and DNase. Structural aspects and cytocompatibility were analyzed *in vitro*, while biofunctionality and *in vivo* degeneration of rAoC under severely procalcific conditions were examined over 12 weeks in a rat implantation model (n=12).

**Results:** Decellularization of rAoC in group BIO resulted in more efficient removal of DNA (>99% with p<0.001) and cellular proteins as compared to group DET, while the extracellular matrix structure was preserved. The architecture of rAoC in group BIO allowed for improved biofunctionalization with fibronectin, significantly increasing speed and amount of autologous medial cellular repopulation *in vivo* (medial cells per cross section after 8 weeks: 96±18 vs. 27±6 with p<0.001), and decreasing the formation of hyperplastic intima (intima-to-media-ratio: 0.6±0.1 vs. 1.1±0.1 with p<0.001), as compared to fibronectin-coated DET-decellularized grafts. Calcifying degeneration under severely procalcific *in vivo* conditions was not aggravated in group BIO. Moreover, there were no signs of infiltration with inflammatory cells.

**Significance:** The present biological, detergent-free, non-proteolytic regimen balances effective decellularization and extracellular matrix preservation in cardiovascular grafts, and allows for improved autologous cellular repopulation *in vivo*.

### Long-term Implantation of an Off-the-Shelf Tissue-engineered Aortic Valve in Sheep

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Diseased aortic valves often require replacement, with over 30% of the current aortic valve surgeries performed in patients who will outlive a bioprosthetic valve. While many promising tissue-engineered valves have been created in the lab, none have been successfully tested long-term in the aortic position of an animal. The high pressure gradients and dynamic flow across the aortic valve leaflets require engineering a tissue that has the strength and compliance to withstand high mechanical demand without compromising normal hemodynamics.

A long-term preclinical evaluation of an off-the-shelf tissue-engineered aortic valve in sheep model is presented here. The valves were made from a tube of decellularized cell-produced matrix mounted on a frame.

The engineered tissue matrix is primarily composed of collagen, with strength and organization comparable to native valve leaflets. *In vitro* testing showed excellent hemodynamic performance with low regurgitation, low systolic pressure, and large orifice area. The implanted valves showed large-scale leaflet motion and maintained effective orifice area throughout the duration of the 6-month implant, with no calcification. After 24 weeks implantation (over 17 million cycles), the valves showed no change in tensile mechanical properties or collagen content. In addition, histology and DNA quantitation showed repopulation of the engineered matrix with interstitial-like cells and endothelialization. New extracellular matrix deposition, including elastin, further demonstrates positive tissue remodeling in addition to recellularization and valve function.

Long-term implantation in the sheep model resulted in excellent functionality, matrix remodeling, and recellularization, unprecedented results for a tissue-engineered aortic valve.

### Session: Animal Based, Preclinical (research) Models (Part 1)

**Date and Time: Wednesday, September 9, 2015, 2:15 PM - 3:30 PM**

#### Fetal Regeneration versus Adult Repair - Lessons to Learn from Fetal Healing

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Injured tendon and cartilage tissues do not regenerate but form repair tissue with significantly inferior biomechanical properties. Fetal tissues, in contrast, have the inherent capacity to regenerate without repair tissue formation. Recapitulation of developmental processes is discussed as a potential pathway to tissue regeneration. Information gained from comparing fetal regeneration and adult healing may further elucidate the fibrotic response observed in mature tissue and potentially outline future treatment.

The aim of the presented study was to identify key factors responsible for regeneration in the fetus versus fibrotic repair in adults. The study was carried out in an ovine model. Standardized cartilage and tendon lesions were surgically induced in adult (aged 2–4 years) and fetal sheep (80 days gestation). Time points for sampling were chosen according to the three stages of healing (inflammatory phase, reparative phase, remodeling phase). Comparison was carried out at a gene expression (micro arrays), protein secretion (mass spectrometry) and histologic level with special emphasis on extracellular matrix composition, collagen organization and collagen types,

qualitative and quantitative composition of growth factors and cytokines, inflammatory response, chemotaxis, MMPs, cell proliferation and migration as well as senescence.

More than 2000 proteins for tendon tissue and 3000 for cartilage could be identified. For both tissues more than 30,000 peptides were localized each. The main identified factors were extracellular matrix proteins, growth factors and inflammatory mediators. The obtained data may include valuable information for the development of future regenerative therapies.

Funding for this project was generously provided by BMWFW (Sparkling Science).

### Application of Adult Stem Cells and Biomimetic Scaffolds for Articular Cartilage Repair

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Degenerative joint diseases affects up to 15% of the population, representing the most prevalent cause of physical disabilities, particularly in aged individuals. The intrinsically low reparative capacity of cartilage is a clinical challenge to effective treatment of diseases such as osteoarthritis (OA). Current treatments, including administration of non-steroidal anti-inflammatory drugs and hyaluronan injection, provide only short-term pain relief, with total joint arthroplasty as the only effective, but terminal, treatment. Tissue engineering and regenerative medicine, by applying cells, scaffolds, and biological signals, represents a potentially promising therapy. Adult mesenchymal stem cells (MSCs), harvested from sources such as bone marrow, adipose, skeletal muscle, and other adult tissues, have multi-lineage mesenchymal differentiation potential, including chondrogenesis, and are considered a promising candidate cell type for cartilage repair. A critical component to successful cell-based cartilage tissue engineering is the use of a biocompatible biomaterial scaffold that serves not only as a cell carrier, but ideally also enhances cell differentiation and maturation. We have developed and successfully applied biomimetic scaffolds, including electrospun nanofibers of biodegradable polymers and photocrosslinked hydrogels derived from natural extracellular matrix components, as effective components of MSC-based cartilage repair. These scaffolds demonstrate excellent cell retention, long-term cell viability, and differentiation. Our current efforts are also focused on developing 3D microtissues that may act as *in vitro* micro-physiological systems amenable to the study of OA pathogenesis and treatment, as well as skeletal malformations.

*Support:* Commonwealth of Pennsylvania Department of Health, NIH, U.S. Department of Defense, US Environmental Protection Agency.

### Remote Cell Activation for Bone Regeneration - a Pre Clinical Animal Study

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We aim to develop a cell-based injectable solution for treatment of non-union bone fractures. Our approach is based on the use of functionalised magnetic particles (MNPs) targeted to the TREK-1 mechano-sensitive receptor on the mesenchymal stem cell (MSCs) membrane. Attached MNPs respond to the application of an oscillating external magnetic field resulting in remote receptor activation and enhanced osteogenic differentiation. In this way, we can remotely deliver functional mechanical stimuli to therapeutic cell populations *in vivo* following injection either systemically or to the repair site. This concept has been validated through a series of *in vitro*, *ex vivo* and small animal studies and is currently being tested in a pre-clinical sheep model for bone repair.

A key element in the remote activation of MNP tagged cells is the application of an oscillating magnetic field applied externally. Here, we describe the design of a magnetic array used to stimulate cell populations *in vivo* in a large animal model. The minimum magnetic

field strength required to activate cells was identified as 0.16 KG by using a HEK-293 reporter cell line with field strengths ranging from 0 KG-3.5 KG. This was validated *in vitro* with ovine MSCs where enhanced osteogenesis was demonstrated in stimulated groups. Magnet shape, orientation and grade was simulated in 2D using FEMM software to identify optimal magnet arrangement and 6 prototypes fabricated for thorough 3D analysis. An *ex vivo* sheep model was established to compare the tissue penetration depth of the 6 prototype and the final design incorporated into a sheep harness.

### Localized Gene Expression Analysis in an Experimental Model of Osteoarthritis: A Focus on Regenerative Medicine Treatment

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Osteoarthritis (OA) is a common debilitating joint disorder worldwide, with an annual impact of over \$100 billion on healthcare costs in the U.S. alone. Although mainly described as a disease of cartilage, OA involves sclerosis of the subchondral bone and inflammation of the synovium. In many cases, osteophytes (bone spurs) are also formed at the end of the joints and are a significant source of pain. The evaluation of prospective tissue engineering and regenerative medicine approaches in osteoarthritis requires development of a reliable test bed. This study seeks to characterize local events in the medial meniscus transection (MMT) animal model, targeting cartilage, synovium, and osteophyte tissue.

Gene expression of the articular cartilage and synovial membrane from the medial tibial plateau in the MMT was compared to that of control tissue at 3 and 6 weeks post-surgery. Our results revealed a heightened expression of genes related to extracellular matrix (ECM) turnover (e.g. *coll1a1*, MMPs) in the medial articular cartilage at 3 weeks. Phenotypical changes in chondrocytes amassed over time (3 vs. 6 weeks). This may suggest that changes in the ECM precede chondrocyte changes. In the synovium, we observed elevated MMPs at an earlier timepoint (3 vs. 6 weeks). Although the MMT model is not an OA inflammation model per se, this preliminary data indicates that changes in the synovium are comparable to those in the human joint at early timepoints. Forthcoming data will include gene expression analysis at earlier timepoints (1 and 3 weeks) for articular cartilage, synovium, and osteophytes.

### Establishment of Genetically Stable Canine Liver Organoids for Translational Studies

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The Regenerative Medicine field is progressing rapidly and predictive pre-clinical animal models are now crucial to validate innovative approaches before clinical application. Over the last decade, we have shown that pathological and molecular pathways of liver diseases are highly similar in man and dogs. Spontaneous liver diseases in pet dogs are therefore the best validated translational models. Adult stem cell-based therapies, including liver organoids are a promising example. Our aim was to develop long-term genetically stable canine liver organoid cultures (healthy and Wilsons disease) for translational transplantation and disease modeling studies. Canine biliary duct fragments were isolated by enzymatic digestion and cultured in 3D using basement-membrane-extracts and RSPO1-based culture media. Fresh and (snap) frozen liver tissues obtained by wedge, Tru-cut or fine needle aspiration biopsy were used for isolation procedures. Gene expression analysis, immunohistochemistry/

fluorescence, hepatocyte-function-tests, EdU-incorporation and karyotyping were used to analyze the organoid characteristics and differentiation. The established canine liver organoids, grown from all types of liver tissue harvested, remained genetically stable (over 8 months) and expressed stem cells characteristics (self-renewal and differentiation), mimicking *in vivo* liver progenitor cell phenotype. Wnt-conditioned medium was important for the organoid proliferation, differentiation towards functional hepatocytes was mediated by removal of Wnt agonists and Notch-inhibition. Taken together, the stable canine liver organoid culture offers an important platform for fundamental and (pre-)clinical translational studies to bring regenerative hepatology towards human clinical application. The culture of organoids derived from (snap) frozen and (fine needle) biopsy-size liver tissue is of critical importance for (autologous) clinical use.

### Session: Biofabrication: Converging Technologies for Functional Regenerative Medicine

Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM

#### Osteochondral Regeneration using a Scaffold Free Three-Dimensional Construct of Swine Adipose Tissue-derived Mesenchymal Stem Cells

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This study was designed to investigate the regeneration of articular cartilage and subchondral bone using a three-dimensional construct of autologous adipose tissue-derived mesenchymal stem cells (AT-MSCs). A construct consisting of about 980 spheroids each containing  $5.0 \times 10^4$  AT-MSCs was implanted into an osteochondral defect (diameter of 6.8 mm, depth of 5 mm) at the right femoral medial condyle in five adult minipigs. The contralateral (left femoral) defect was the control. The radiolucent capacity (RC,  $\text{mm}^3$ ) of the defects were calculated in the multiplanar reconstruction of postoperative computerized tomography images. Magnetic resonance (MR) images and pathology were scored with modified-MR Observation of Cartilage Repair Tissue (MOCART) system and International Cartilage Repair Society (ICRS) system respectively at the 6th months post-operatively. The percentages of RCs at the 3rd and 6th months against those immediately after the surgery were significantly ( $p < 0.05$ ) decreased in the implanted defects ( $52.5 \pm 16.5$  and  $30.2 \pm 10.4\%$ , respectively) comparing to the control defects ( $74.5 \pm 8.0$  and  $50.5 \pm 13.6\%$ , respectively). The total scores of MOCART system were also significantly ( $p < 0.05$ ) improved in the implanted sites ( $36.0 \pm 17.8$ ) comparing to the controls ( $15.0 \pm 0.0$ ). Although there was no statistical difference in the averages of gross scores ( $2.3 \pm 1.5$  and  $1.0 \pm 0.7$  in the implanted and the control sites, respectively), the average of histological scores was significantly ( $p < 0.05$ ) higher in the implanted sites ( $1.7 \pm 1.0$ ) than the control sites ( $0.3 \pm 0.2$ ). These results suggest that implantation of a scaffold free 3D structure of AT-MSCs into osteochondral defect could regenerate the original structure of cartilage and subchondral bone for postoperative 6 months.

#### Novel Technology to Create 3D Anisotropic Hydrogel Materials with Precise Molecular Composition

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**Introduction:** Properties and functionality of most human tissues arise from their highly anisotropic three-dimensional (3D) organization. The purpose of this study is to develop a strategy to create multifunctional materials that will provide spatial and biochemical complexity to guide cells into the organization found in human tissues.

**Methods:** We have developed a novel patent-pending technique that enables the generation of more biomimetic cell culture platforms

exhibiting 3D patterns of multiple kinds of functional molecules. The strategy is simple, hydrogel-independent, versatile, and does not require sophisticated equipment or chemical reactions. The technique permits the fabrication of precise, multifunctional and well-defined patterns of different molecules (such as peptides and proteins) with micron scale resolution and up to centimetres in depth.

**Results:** Cell culture studies revealed selective molecular recognition inducing cell penetration into hydrogels, demonstrating that the patterned molecules are functional after the fabrication process.

**Discussion:** The capacity to manipulate and localize multiple proteins in their native state replicating geometrical configurations found in biological systems would benefit applications that require fine control of molecular and cell organisation. We believe that the device has the potential to grow into a novel material platform technology, enabling generation of hybrid multifunctional environments which can serve as a new standard in biomimetic cell culture studies.

### Bioprinting Technology as a Tool for Building Complex Tissues and Organs

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Advances in tissue engineering and regenerative medicine have led to the development of many clinical therapies. However, challenges still exist in developing complex tissue systems. One challenge that hampers rapid clinical translation is the lack of effective delivery methods for cells and biomaterials to build complex tissue constructs. Living tissues maintain inherent multi-cellular heterogeneous structures, and rebuilding of such complex tissue structures requires subtle arrangements of different cell types and extracellular matrices at their specific anatomical target sites. 3D bioprinting has emerged as an innovative technology that has the potential to address this endeavor. In this session novel and versatile approaches to building tissue structures using 3D printing technology will be discussed. Clinical perspectives unique to 3D printed structures will also be discussed.

### Biofabrication Strategies in Otosurgery: From the Outer to the Inner Ear

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Ear physiology occurs via minute highly-specialized and histologically diverse tissue components with a shape-dependent function, which ultimately allow a fine hearing. Such an anatomic site is thus a challenging application for tissue-engineers in which microfabrication techniques can make a difference. The aim of this study was to develop specific biofabrication strategies to replace the ear tissues, which could be useful in otologic surgery. 3D fiber deposition (3DF) was used as an additive manufacturing technique to obtain ear bone replacements, such as outer auditory canal wall and ossicular chain (OC), based on poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) copolymer. These scaffolds were cultured with human mesenchymal stromal cells entrapped in fibrin clots as a biological nanofibrous matrix. After 21–27 days of osteodifferentiation, cell viability, bone markers and microCT were performed to assess appropriate mineralization. The measured acoustic response of OC constructs was superior to those of commercial prostheses in the hearing ranges: 250–8,000 Hz frequencies and 50–

100 dB sound pressures. The tympanic membrane is a flexible and though connective tissue apt for vibration. Electrospinning was used in combination with 3DF to produce biomimetic PEOT/PBT dual and triple scale scaffolds provided with over-impressed patterning (radial, circular and reticular) able to localize cells and their synthesized biomolecules as in the native eardrum. The possibility of producing electrospun meshes that enable cell alignment was also investigated via a radial collector. Finally, other biofabrication strategies were investigated to produce thin ceramic/polymer composite scaffolds able to support the inner ear function, including spin coating, hot-press, and co-axial electrospinning.

### 3D Printed Nanocellulose Threads for Delivering Human Stem Cell inside Wounds

A. Sanz-Garcia<sup>1,2</sup>, H. Mertaniemi<sup>3</sup>, O. Ikkala<sup>3</sup>, M. Yliperttula<sup>1</sup>, C. Escobedo-Lucea<sup>1,2</sup>;

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One of the main issues in regenerative therapies is still the lack of appropriate vehicles for delivering cells into wounded areas effectively and safely. Additionally, cell application in surgery has traditionally relied on animal-origin constituents that may induce immune reactions or infections. To solve these problems, we propose 3D printed and cross-linked sutures of wood-derived nanofibrillar cellulose (NFC) to deliver immunomodulatory xenogeneic-free cells inside wounds.

GrowDex NFC, received as hydrogel, was printed into an ethanol bath by using a Fab@home 3D printer. After that, threads were cross-linked with a solution consisting in glutaraldehyde and zinc nitrate. The threads were sterilized before human adipose-derived stem cells (hASCs) (n=4) were seeded and cultured. Viability, toxicity of threads over the hASCs cells was analysed. Molecular and cellular *in-vitro* studies were performed in order to detect changes in the hASCs profile or bioactivity. Suture strength was also tested through skin layers in both *ex-vivo* and *in-vivo* pig model.

Our study showed cross-linked NFC threads presented high mechanical strength even under the wet surgery conditions. Moreover hASCs attached over the 3D printed threads without showing any evidence of toxicity or proliferation reduction. Cell morphology and undifferentiated state were also maintained as well as their bioactivity concerning cytokine's pattern.

As a proof of concept, we demonstrate herein the possibility to create functionalized surgical sutures using 3D printed cross-linked NFC threads decorated with hASCs. Our findings highlight the use of these bio-sutures as a promising tool for fighting against post-surgical inflammation and chronic wound situations.

### Non-viral Gene Delivery within 3D Bioprinted PCL-Reinforced Alginate Hydrogels for Bone Regeneration

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The combination of gene delivery technology with 3D-Bioprinting offers a promising platform for musculoskeletal tissue engineering. The overarching goal of this approach is to generate an "off-the-shelf", mechanically functional gene delivery system for bone repair. Specifically, this study investigated the efficacy of a bioprinted composite polycaprolactone (PCL)-alginate system, enriched with mesenchymal stem cells (MSCs) and gene delivery technology to drive mineralisation and bone formation *in vivo*. Non-viral gene delivery was achieved using nano-hydroxyapatite (nHA) particles to co-deliver two plasmid DNA (pDNA) vectors encoding for TGF-β3 and BMP-2; two genes which play a vital role in bone development and healing. These nHA-pDNA complexes were successfully printed within a RGD-modified alginate hydrogel, and co-printed with PCL



to form PCL-reinforced alginate constructs (6 mm diameter, 3 mm height). The study also explored the necessity for co-printing of MSCs into the constructs to induce bone formation. N=8 constructs per group were implanted subcutaneously into nude mice and analysed following 4 and 12 weeks *in vivo*. uCT analysis revealed enhanced levels of mineralisation at 12 vs. 4 weeks. The acellular construct printed to contain pDNA had significantly lower levels of mineralisation than the cellular group, indicating that the incorporation of MSCs enhanced bone formation *in vivo*. The results of this study point to the utility of 3D bioprinted gene delivery systems for the regeneration of musculoskeletal tissues.

### Session: Biofabrication: 3D Printing of Regenerative Medical Implants

**Date and Time: Thursday, September 10, 2015, 10:30 AM - 12:00 PM**

#### Mitigating Implant Failure Through Design and Manufacturing of Nitinol Fixation Hardware

M. Elahinia<sup>1</sup>, N. Shayesteh Moghaddam<sup>1</sup>, M. Taheri Andani<sup>1</sup>, R. Skoracki<sup>2</sup>, I. Valerio<sup>2</sup>, M. Miller<sup>2</sup>, D. Dean<sup>2</sup>;

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**Background:** Most skeletal fixation and joint replacement devices are prepared from Ti-6Al-4V which at 112 GPa is significant stiffer than bone (15–30 GPa). This mismatch increases the likelihood of device loosening or failure and bone stress shielding. An alternative material, nitinol (48 GPa), can be 3D printed to create stiffness-matched hardware for mandibular and other skeletal fixation. Our fixation hardware design and fabrication process begins with a patient's 3D CT-scan.

**Methods:** Porous Nitinol samples were fabricated from pre-alloyed powder and tested to complete a Finite Element Model (FEM) that in addition to the fixation hardware includes teeth, periodontal ligament, cortical and cancellous mandibular bone, masticatory muscles, and a 4 cm double-barrel fibular graft in the left molar region. In order to simulate chewing, a maximal bite force of 526 N on the right first molar tooth is considered.

**Results:** Our 3D printed porous nitinol parts demonstrated stiffness reduction from 48 GPa (no porosity) to between 35 and 6 GPa by imposing 3D printed porosity. Our FEM results confirm that stress on the grafted bone is increased and therefore the risk of stress shielding is reduced. And, as expected, stress concentrations on the fixation hardware are decreased.

**Conclusion:** Our FEM results confirm that stiffness-matched nitinol skeletal fixation hardware provides the immobilization necessary for bone healing and is expected to lead to reduced device failure, increased remodeling and strengthening of grafted or fractured bone, increased return of masticatory chewing muscle power, and increased likelihood that the reconstructed mandible will support dental implants.

#### 3D Printing Scaffolds for Craniofacial Regeneration

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The treatment of large craniofacial bone loss due to congenital defects, trauma or cancer resection remains a huge clinical challenge. There are approximately one million fractures requiring bone transplantation annually in the US and it is predicted that within the next 10 years this number will significantly increase, particularly in persons over 50 years of age. Tissue engineering provides a viable means of obtaining 'autologous' bone grafts for the treatment of large bone defects. Successful application of tissue-engineered grafts however requires that we can couple the formation of *de novo* vasculature in tandem with new bone growth. Our lab has developed techniques for 3D printing anatomically-shaped bone scaffolds and pre-vascularizing these scaffolds using adipose-derived stem cells (ASCs) to engineer vascularized bone grafts that can be used to repair craniofacial defects

#### 3D Cell Printing *In Vitro* Biological Models for Tissue Engineering

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3D Bio-Printing uses cells, biologics and/or biomaterials as building block to fabricate personalized 3D structures or functional *in vitro* biological models for regenerative medicine, disease study and drug discovery. This presentation will report our recent advances on 3D printing cells for tissue engineering application, micro-organ chips and *in vitro* 3D tumor models. An overview of 3D Bio-Printing will be given according to its technological development and application stages. Engineering printing process will be described. Examples of 3D Printing for tissue engineering models, drug metabolism models and disease models will be given, along with the study of printing effect on cell viability and 3D structural formation, and characterization of cell morphologies, proliferations, protein expressions and chemoresistances. Comparison of biological data derived from 3D printed models with 2D planar petri-dishes models will be conducted. Discussions on challenges and opportunities of 3D Bio-Printing will also be presented.

#### Additive Manufacturing Applied in the Biomedical Sciences - Bioprinting, Biofabrication, Cell printing...? The Need for Definitions & Norms

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The application of Additive Biomanufacturing represents one of the most rapidly advancing areas of biomedical sciences in which engineers, scientist and clinicians are contributing in large to the future of human healthcare and more specifically to the areas of medical devices, tissue engineering & regenerative medicine (TE/RM) and *in vitro* disease model development. The combined efforts of a large number of groups around the globe have developed a strong research thrust, which has resulted in a large number of publications. Reviewing the literature, there is an increasing trend that single groups or larger research consortia invent their own definitions and terminology, which makes it often difficult to find and compare the results of different research groups. Therefore, to move the field constructively forward it is a *conditio sine qua non* to clarify terminologies such as biomanufacturing, biofabrication, bioprinting, etc., which are often used interchangeably in the literature. ASTM International and the International Organization for Standardization (ISO) announced a cooperative agreement between ASTM International Committee F42 and ISO Technical Committee 261 on Additive Manufacturing. The agreement will reduce a duplication of effort. ASTM F42 released a standard terminology for coordinate systems and test methodologies in 2012. Based on this background, this invited lecture advocates tightening the terminology and has the objective to pen out definitions, which ultimately allow developing an official industry standard term such as ASTM and or ISO for technologies developed for TE/RM.

#### Session: Bioinspired Systems and Devices for Regenerative Medicine

**Date and Time: Wednesday, September 9, 2015, 10:30 AM - 12:00 PM**

#### Creation of Functional Hepatocyte Sheet Tissues by using Heparinized Thermoresponsive Surfaces

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Our laboratory has developed a unique approach to harvest cultured cell sheets only by lowering temperature from thermo-responsive poly(N-isopropylacrylamide) (PIPAAm)-grafted cell culture surface, which exhibits temperature-dependent hydrophilic/hydrophobic changes. Hepatocyte sheet-based tissue engineering is an attractive method for the treatment of liver diseases. However, hepatocytes rapidly lose their viability and phenotypic functions on isolation from the native *in vivo* microenvironment of the liver. In this paper, heparin-functionalized PIPAAm-grafted cell culture surface, which interacts with heparin-binding proteins such as heparin-binding EGF-like growth factor (HB-EGF), has been designed for maintaining hepatic functions during the cultivation. The addition of HB-EGF in the cell culture media was essential for the survival of hepatocytes. When the medium contained less than 10 ng/cm<sup>2</sup> of soluble HB-EGF, the hepatocytes were not able to adhere and form their cell sheets. Hepatocytes adhered well and formed their sheets on HB-EGF-bound heparin-functionalized thermoresponsive surface. The secretion of albumin was maintained and higher compared to that on PIPAAm-grafted surfaces with soluble HB-EGF. In addition, when lowering temperature to 20 °C, the cultured cell sheets were detached from the surface through the reduction of affinity binding between HE-EGF and immobilized heparin with increasing the mobility of heparin and the swollen PIPAAm. Therefore, functional hepatocyte sheets was prepared using heparinized thermoresponsive cell culture surfaces.

#### Closed Hybrid Hierarchical Reservoirs Based on the Deconstruction of the Cellular Native Microenvironment

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The stem cell niche organization and dynamics provide valuable cues for the development of mimetic environments that could have potential to stimulate the regenerative process. We propose the use of biodegradable biomaterials to produce closed miniaturised structures able to encapsulate different cell types or bioactive molecules. In particular, capsules are fabricated using the so-called layer-by-layer technology, where the consecutive (nano-sized) layers are well stabilized by electrostatic interactions or other weak forces. Using alginate-based spherical templates containing cells or other elements (e.g. proteins, magnetic nanoparticles, microparticles) it is possible to produce liquefied capsules that may entrap the entire cargo under mild conditions. The inclusion of liquefied microcapsules may be used to produce hierarchical compartmentalised systems for the delivery of bioactive agents. The presence of solid microparticles inside such capsules offers adequate surface area for adherent cell attachment increasing the biological performance of these hierarchical systems, while maintain both permeability and injectability. We demonstrated that the encapsulation of distinct cell types (including mesenchymal stem cells and endothelial cells) enhances the osteogenic capability of this system, that could be useful in bone tissue engineering applications.

#### A Microfluidic-based Platform for High-Throughput Screening of Stem Cell-Niche Interactions in 3D Gradient Hydrogels

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The niche in which stem cells reside is a complex, multi-factorial environment including biochemical and mechanical signals. While the importance of niche cues on stem cell fate has been well established, most work to-date has been performed on 2D substrates. How biochemical and mechanical signals interact together to regulate stem cell responses in 3D remain unclear. Furthermore, conventional strategies are typically low throughput; require large number of cells and materials. Previous high-throughput studies require a large number of microarray samples, and only select niche cues in a dis-

crete manner. To overcome the above challenges, here we report a microfluidic-based platform for fabricating microscale hydrogels as stem cell niche with gradients of insoluble biochemical and mechanical cues. To control hydrogel stiffness, 8-arm PEG-NB was chosen due to its bioinert nature, which can be crosslinked by PEG dithiol. Methacrylated chondroitin sulfate was used to mimic biochemical content of cartilage. Successful formation of gradient niche cues was verified using FITC-labelled crosslinker, and gradient hydrogels form within the microfluidic chamber upon light exposure. Importantly, our microfluidic design protects cells from shear force during encapsulation, which yields high cell viability. The gradient hydrogels reported here allow varying niche cues in a continuous manner using orders of magnitude less cells and materials. Such microfluidic-based platform for fabricating cell laden gradient hydrogels offers a novel tool to facilitate rapid discovery of optimal niche cues that promote desirable cellular fates tissue regeneration, and enable analysis of how complex niche cues influence stem cell fates in a high-throughput manner.

#### Use of Synthetic Peptides for the Delivery of Growth Factors for Tendon/Ligament Healing

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Adult tendons/ligaments have low oxygen and nutrient requirements, low cell density and poor regenerative capacity and often surgical intervention is needed to promote healing of these tissues. Delivery of soluble growth factors to these tissues is known to promote and enhance the healing process, however this is accomplished with supraphysiological concentrations of growth factors. One way to overcome this problem is to immobilize the growth factors in biomaterials, however very little research exploiting this strategy has been done for tendons and ligaments. Here we present for the first time a non-covalent method for the delivery of growth factors to promote and enhance the healing process of these tissues. In this work we functionalized polycaprolactone with synthetic peptides that display affinity for specific growth factors. Growth factor binding peptides were synthesized using Fmoc-Solid Phase Peptide Synthesis and purified using standard HPLC methods. We demonstrated that it is possible to specifically immobilize TGF- $\beta$ 1, BMP-2 and VEGF on PCL functionalized with the respective affinity binding peptide and confirmed this by antibody staining and ELISAs. Our data shows that the immobilized growth factors retained their bioactivity and activated the respective signaling pathways. Immobilized TGF- $\beta$ 1 induced Smad2/3 translocation to the nucleus, activation of tendon/ligaments related genes and led to a 2,5 fold increase in collagen protein content in human Hamstring cells while Immobilized BMP-2 activated Smad1/5/8 phosphorylation and induced ALP expression in C2C12 *in vitro*. Further experiments will be conducted *in vivo* and with combination of different growth factor binding peptides within the same material.

#### A Biomimetic Approach for Engineering Stratified Organization of Articular Cartilage by Recapitulating Biochemical, Biomechanical and Geometrical Factors Involved in Cartilage Tissue Development

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Articular cartilage is a multifunctional tissue with a highly organized structure which provides a lubricating surface for the gliding joint and a load bearing matrix attached to the underlying bone. During native cartilage tissue development, changes in biochemical, mechanical, and geometrical factors direct the formation of stratified

structure of articular cartilage. In this study we attempted to simulate the zonal organization of articular cartilage by recapitulating biochemical, mechanical and geometrical factors involved in the tissue development. To this end human mesenchymal stem cells (hMSCs) were encapsulated in acrylate-functionalized lactide-chain-extended polyethylene glycol (SPELA) gels simulating cell density, stiffness and geometrical properties of different layers of articular cartilage. hMSCs encapsulated in a soft gel (80 kPa) and cultivated with a combination of TGF- $\beta$ 1 and BMP-7 growth factors differentiated to the superficial zone phenotype of articular cartilage. hMSCs encapsulated in a gel with 2.1 MPa stiffness and cultivated with a combination of TGF- $\beta$ 1 and IGF-1 differentiated to the middle/deep zone phenotype. hMSCs encapsulated in a 320 MPa gel, reinforced with nanofibers aligned perpendicular to the gel surface and loaded with TGF- $\beta$ 1 and HA showed the hypertrophic phenotype of calcified zone.

The results of this work can potentially lead to the design of more clinically effective multilayer grafts mimicking the biochemical, biomechanical and geometrical properties of the native tissue for treatment of articular cartilage defects.

### Session: Biologically Derived Biomaterials from Natural Resources

**Date and Time: Thursday, September 10, 2015, 10:30 AM - 12:00 PM**

#### Tuning Three-dimensional Collagen Gel Stiffness Independently Modulates Endothelial Cell Behavior

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Numerous studies have described the effects of matrix stiffness on cell behavior using two-dimensional cell culture surfaces, but less is known about the effects of matrix stiffness on cells embedded in three-dimensional biomimetic matrices. A primary limitation in investigating the effects of matrix stiffness in three dimensions is the lack of materials that can be tuned to control stiffness independently of matrix density. Here, 0.3% (w/v) collagen gels, where the mechanical properties are tuned by different extraction methods, i.e. acid-soluble and pepsin soluble collagen, were used and extracted from epidermis. The effects of pH value and ionic strength on the kinetic fibrin self-assembly and corresponding properties of collagen gel were investigated. Using these collagen gels, our results demonstrate the ability to de-couple matrix stiffness from matrix density and structure in collagen gels, and that increased matrix stiffness results in decreased sprouting and outgrowth of blood-derived endothelial colony-forming cells *in vitro*.

#### Collagen Extraction from the Duck's Foot and Fabrication of Collagen Patch for the Repair of Acute Tympanic Membrane Perforations

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Collagen constituting the extra cellular matrix (ECM) has been widely used as biocompatible materials for human use. In this connection, collagen had been isolated from bovine, porcine and rat name to few. However, all these methods are proving to be economically unfeasible. Therefore, collagen is considered as most expansive biomaterial which had limited its use only in research labs and not at the place where patients requiring can be benefitted. In this study, we have carefully selected duck foot which is a livestock waste which can potentially solve this burning issue as it had totally no use in any other industry.

We investigated the effects of repairing large tympanic membrane (TM) perforations in rats with a good biocompatibility collagen patch compared with the commonly used paper patch. We performed bilateral myringotomies of 1.8 mm in diameter on adult Sprague-Dawley rats with intact TMs. The perforations in the right ears of rats

were treated with a collagen patch, and the perforations in the left ears of the same rats were treated with a paper patch. Ten rats acted as controls. The mean perforation closure times were significantly shorter in collagen-patch- and paper patch-treated ears than in the control animals. The endoscopic and histological findings of this study provide evidence that collagen-patch treatment accelerates wound healing and shortens TM perforation closure time. We suggest that the collagen patch may prove to be an effective material for repairing TM perforations in human patients in an outpatient clinical setting.

#### The Effect of Sulfation in Glycosaminoglycan (GAG)-Mimetic Scaffolds on Promoting Chondrogenesis

G. P. Huang, R. Menezes, G. Collins, T. L. Arinze;

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Articular cartilage has a limited ability to heal. Current surgical procedures to repair cartilage result in the formation of fibrocartilage instead of hyaline cartilage. The presence of fibrocartilage may suggest deficient bioactivity to promote the chondrocyte phenotype. Glycosaminoglycans (GAGs) have been shown to interact and maintain the bioactivity of growth factors due to their level and spatial distribution of sulfate groups. Sodium cellulose sulfate (NaCS), a semi-synthetic derivative of cellulose, is a sulfated polysaccharide with structural similarity to GAGs and has yet to be explored for cartilage repair. This study evaluated the effect of varying the degree of sulfation in NaCS on human mesenchymal stem cell (MSC) chondrogenesis and its effect on maintaining the bioactivity of TGF- $\beta$ 3 in comparison to other sulfated GAGs. NaCS was combined with bovine gelatin, electrospon to form fibrous scaffolds and characterized for fiber dimension, interfiber spacing, mechanical properties and hydrolytic stability. Partially sulfated (pSC) and fully sulfated (NaCS) were evaluated. NaCS had significantly higher amounts of complexed TGF- $\beta$ 3 than chondroitin 6-sulfate and pSC. Yet, MSCs on pSC-gel scaffolds had the highest production of collagen type II and ratio of collagen type II to I, greatest gene expression for chondrocyte markers (aggrecan, collagen type II, chondroadherin, Sox9 and Tenascin C) and the lowest expression for collagens type X and I as indicators of hypertrophic chondrocytes and MSCs, respectively, when compared to cells on NaCS-gel and gelatin alone. The findings suggest that pSC-gel scaffolds, having partial sulfation, may support a more homogeneous cartilage tissue formation.

#### Collagen Cross-Linking Modulates Scaffold Stability and Pro-inflammatory Macrophage Response

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Network of Excellence for Functional Biomaterials, National University of Ireland, Galway, Galway, IRELAND.

Collagen-based materials have the ability to enhance wound healing and to facilitate functional repair. Nonetheless, most of these collagen materials exhibit low resistance to degradation and mechanical stability. Therefore, exogenous cross-linking methods are widely used for collagen stabilisation. Unfortunately, chemical cross-linking is associated with impaired healing and foreign body reaction. Specifically, macrophage interactions with cross-linked collagen materials have recently been demonstrated to play an important role in tissue remodelling outcomes; however, little is known about the underlying biological mechanism involved. Hence, the overall objectives are to explore modified material properties by cross-linking and to look for alternative cross-linking methods, based on plant extracts and polyethylene glycol (PEG). It is hypothesised that collagen films can be optimally cross-linked with PEG and plant extracts to induce adequate stability, while modulating macrophage response. Collagen type I films were cross-linked with 0.625% glutaraldehyde, 50 mM carbodiimide, 1 mM 4S-StarPEG, 0.625% genipin or 0.1% oleuropein. Carbodiimide and oleuropein decreased free amine groups of collagen, whilst they did not increase enzymatic

stability. Only 4-arm branched-PEG and genipin provided an enzymatic stability equivalent to glutaraldehyde cross-linked collagen films without increasing toxicity, while inducing a similar release profile of pro-inflammatory cytokines to non-cross-linked films. Moreover, cell culture assays using pre-conditioned media strongly suggest that sub-products potentially released from cross-linked collagen films are not responsible for the macrophage alteration. This is most likely associated with modifications in the collagen surface by chemical cross-linking. Overall, this study advocates 4S-StarPEG and genipin as alternative cross-linkers for tissue engineering scaffolds.

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### Synthesis and Characterization of Injectable *In Situ* Enzymatically Plasma Hydrogel

Y. Chu, S. Mao, Y. Chen;

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A phenolic hydroxyl group was incorporated into albumin, using aqueous carbodiimide activation chemistry, to obtain *in situ* gellable and injectable albumin-based materials for customized tissue engineering applications. By this means, albumin derivatives that were gellable via a peroxidase-catalyzed reaction were obtained. The enzymatically cross-linked albumin gels showed tunable storage modulus and proteolytic degradability. The time necessary for gelation decreased with increasing content of the phenolic hydroxyl (Ph) group, peroxidase concentration and decreasing H<sub>2</sub>O<sub>2</sub> concentration. We further synthesized autologous plasma hydrogels that retained its integrity *in vivo* for more than one month and was eventually degraded due to hydrolysis. The synthesized plasma hydrogels exhibited little cytotoxicity and hemolysis; the acute inflammatory response after implanting the hydrogel was negligible, and the degradation products were biocompatible. A mouse model of sidewall defect-bowel abrasion was employed, and a significant reduction of post-operative peritoneal adhesion has been found in the group of *in situ* formed plasma hydrogels.

### Novel Nanocellulose Alginate Bioink for 3D Bioprinting of Soft Tissue

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The objective of this research has been to prepare bioink based on nanofibrillated cellulose (NFC). 3D bioprinting is an emerging technology which combines the use of hydrogels and living cells resulting in bottom-up printed constructs with complex and customized architecture. The biggest challenge in this exciting technology is the combination and the properties of the appropriate hydrogels which form the bioink. Hydrogels are favored in 3D bioprinting due to their biocompatibility and non cytotoxic properties. In this study nanofibrillated cellulose has been chosen for its outstanding shear thinning and mechanical properties. Alginate (ALG) was used in order to enhance the printing fidelity of the bioink and to enable crosslinking of the printed constructs. The optimization of the appropriate bioink resulted in a proportion of 80:20 (NFC:ALG) percentage with excellent results in viscosity, printability, mechanical properties and cell viability. The shear thinning properties of the bioink enabled printing of both 2D gridlike and complex 3D constructs like a human ear auricle and a sheep meniscus; both detailed cartilage structures. Cytotoxicity after bioprinting with living cells revealed high viability (73% viable cells) after 24 h in cell culture. These results demonstrate the use of this novel nanocellulose alginate bioink as a suitable hydrogel for 3D bioprinting of soft tissues with living cells.

**Acknowledgment:** EU program Eureka and Vinnova, Sweden are greatly acknowledged for financial support of the Project E!8355 CELLINK.

### Session: Biomimetic Bioreactors Integrated Monitoring

**Date and Time: Friday, September 11, 2015, 9:15 AM - 10:45 AM**

#### A Modular, Adaptable and Automated Bioreactor - A New Standard in Cardiovascular Tissue Engineering

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Up to now, no testing system, specifically addressing minimal invasive and TE-heart valves, is published or commercially available. Therefore, we developed a fully automated bioreactor, allowing us to mimic *in-vivo* conditions. As a key feature, a professional programmable logic control monitors and regulates all actuators and sensors. In the standard setting, temperature, pH, pO<sub>2</sub>, pCO<sub>2</sub> and pressures (pre- and postvalvular) are logged in real-time and provided via a graphical user interface. The implementation of additional sensors is possible. Moreover, all flow characteristics (frequency, flow-volume curve, compliance and resistance) can be configured. This allows us to examine and evaluate TE-valves while having control of all vital parameters to guarantee a physiologic environment. Valve behavior can be evaluated by slow-motion video analysis as well as particle image velocimetry. Due to its modular design, the device is easily adjustable and expandable to specific requirements. Another unique feature of the bioreactor is the possibility of minimal invasive deployment of heart valves during operation. Furthermore, exchangeable and freely designable landing zones can be used. By application of 3D-printed geometries, countless physiologic and pathologic anatomies, including calcifications, are educible. Additionally, the implementation of specific patient related data is possible. The system is equipped with a specific incorporated sterilization unit allowing an automatic sterilization during full assemblage.

In summary, the newly developed modular and industrial-grade bioreactor allows testing of all currently available types of heart valve, including TAVPs. By providing and maintaining sterile and physiologic conditions, this system specifically addresses the development and evaluation of TE-heart valves.

#### *In-Vivo* Bioreactor Design for Tissue Engineered Trachea Clinical Application

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A tissue engineered trachea (TET) was used to replace 4 cm long left main bronchus in a lung cancer patient. TET was made of decellularized porcine dermis seeded with autologous skin keratinocytes and peripheral blood mononuclear cell PBMCs. A continuous medium perfusion were created inside the implanted TET through two totally implantable access port (TIAP) that connected to two portable peristaltic pumps respectively. The perfusion maintained the living of seeded cells and washed the dead cells out. PBMCs harvested from 20 cc blood were injected into the TET every 4 days. The perfusion and PBMCs refresh lasted for four weeks post operation (PO). The patient is clinic well and discharged 3 weeks PO. Bronchoscopy showed no anastomosis stenosis two months PO. Through this design, we try to combine *in vitro* 3D cell culture with *in vivo* TET regeneration and treat the patient as the bioreactor for his own TET reconstruction. We named it "*in-vivo* bioreactor" which offers real-time monitor and control of TET regeneration.

#### An Integrated Biomechanical Platform for Fabrication, Dynamic Maturation and Biomechanical Evaluation of Vascular Engineered Tissues *In Vitro*

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The design and the development of a biomechanical *in vitro* platform conceived for the maturation and characterization of vascular tubular structures is here presented. The bioreactor, consisting of a culture chamber, a hydraulic circuit and a programmable monitoring/control system, allows at the same time to:

i. fabricate vascular engineered tissues cast in tubular shape directly inside the device by means of a molding technique, and perform *in vitro* culture of smooth muscle cells (SMCs) within a controlled and reproducible physiological- or pathological-like hemodynamic environment, mimicking *in vivo*-like shear stress or pressure-related cyclic strain (culture mode).

ii. live-monitor the biomechanical properties of hosted samples (testing mode), in non-disruptive tests during the culture.

Testing mode was validated by compliance tests performed on porcine coronary arteries, obtaining values ( $5.0 \pm 0.7 \times 10^{-3}$  mmHg<sup>-1</sup>) comparable with literature.

Concerning the culture mode, preliminary tests, carried out on SMCs-populated collagen-based gel constructs, demonstrated the feasibility of using the device since the early fabrication step of the constructs. Ongoing experiments are aimed at investigating the effects of biomechanical conditioning on the architecture and maturation of the constructs both in terms of cellular behaviors and tissue organization.

In summary, the developed *in vitro* platform represents a novel device where 3D cellularized engineered tissues can be fabricated, cultured and characterized, altogether. Providing high level monitoring and control of the biomechanical parameters and properties involved in vascular tissue regeneration, this device will facilitate the investigation of the mechanobiology of vascular tissue models during development, remodeling and in pathological-like conditions.

### Metabolite Monitoring as a Method of Real-Time Nondestructive Analysis of Bone Tissue Engineered Constructs

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Flow perfusion bioreactors have been proposed as ideal vehicles for the culture of cell seeded scaffold constructs for bone tissue engineering, due to their ability to provide improved nutrient transport and expose the cultured cells to shear stress that has been shown to stimulate osteoblastic differentiation. In order to better understand the mechanosensation and nutrient transport, we are analyzing the effects of scaffold architecture and surface chemistry on the shear forces and cellular/tissue developments and also identifying oxygen and nutrient concentration gradients at different stages of the culture. For this study, we used three types of 85% porous PLLA scaffolds: spunbonded, solvent-cast/particulate leached, and 3D printed. The scaffolds were dynamically seeded with rat MSCs. After seeding, the constructs were cultured for up to 16 days. During the culture period, we measured the levels of oxygen, glucose, and lactate, and validating the outcome of these measurements with the results obtained from numerical simulations. By taking nutrient readings at different time points during culture, we were able to monitor the metabolic state of the cells in the porous scaffolds as the constructs mature into bone tissue-engineered constructs. For the macroscopic nutrient levels, we used a convection-dispersion model to represent the consumption of oxygen at various scaffold locations, assuming that oxygen in the porous medium, is being consumed by uniformly distributed particles which represent the cells. As for the microscopic CFD models, we were able to create a three dimensional view of the localized oxygen and glucose levels within the scaffold.

### Flow-loop Circulation System for Conditioning, Evaluating and Monitoring of Tissue-Engineered Heart Valves

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The significant progress made in the development of tissue-engineered heart valves has accelerated the requirement for an all-encompassing bioreactor system where valves with different designs can be i) conditioned with increasing hydrodynamic load; ii) monitored in real time to evaluate the performance and optimize the conditioning protocol; iii) finally tested under physiological flow and pressure conditions according to ISO guidelines.

We developed a computer-controlled, pneumatically driven flow-loop circulation system with tunable compliance and resistance to obtain adjustable flow and pressure profiles. In this way, we are able to test the different typologies of tissue-engineered valves developed in our lab (mitral, pulmonary and aortic, for surgical and transcatheter implantation) under physiological hydrodynamic conditions. The valve performance is evaluated in terms of effective orifice area, transvalvular pressure drop and regurgitation via processing of recorded data measured by means of pressure gauges and an external flow meter.

The chamber where the valve is accommodated enables real-time monitoring of the valve's closing and opening behaviour optically and by ultrasound imaging. Ultrasound visualization simulating the transthoracic parasternal long-axis view allows for the evaluation of the leaflets' coaptation during *in-vitro* remodeling, a crucial aspect for the valve's functionality.

Other design considerations include the uncomplicated fixation of the different heart valves, the ease of assembly under sterile conditions, the minimized medium volume needed for operation and the user-friendly interface of the Labview program.

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### Bioreactor Preconditioning for Tissue Engineered Skeletal Muscle Repair

G. Christ;

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Skeletal muscle possesses a robust capacity for regeneration and repair. Nonetheless, there are injuries that exceed the endogenous regenerative capacity of skeletal muscle leading to permanent functional and cosmetic deficits. Such injuries are referred to as volumetric muscle loss (VML) injuries, and these injuries commonly result from trauma, infection, congenital anomalies, or surgery. All current treatment options have significant limitations, and as such, tissue engineering approaches may offer a potential therapeutic solution. We have been developing a Tissue Engineered Muscle Repair (TEMR) technology platform that utilizes muscle derived cells (MDCs) seeded on a decellularized extracellular matrix delivery vehicle. Moreover, we have demonstrated that both the rate and magnitude of functional recovery from VML injury can be enhanced by bioreactor preconditioning of the TEMR constructs prior to implantation in rodent models. Recently we have begun development of next generation bioreactors in order to improve cellular seeding protocols for augmented phenotypic modulation of the implanted constructs. This talk will describe the utilization of bioreactor preconditioning to further expand the clinical applicability of our TEMR technology platform for the treatment of VML injuries to Wounded Warriors and civilians.

**Session: Bone Marrow on the Bench: The New Frontiers of Bioengineering Tools for Studying Hematopoiesis and Production of Functional Blood Components *ex vivo***

**Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM**

**Towards Industrialization: Development of Ips Cell-derived Platelet Production System**

**K. Harimoto**, Y. Tsuda, Y. Ito, H. Endo, S. Nakamura, K. Eto;

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In order to obtain huge amount of transfusion product, platelets from rare HLA or HPA (human platelet antigen) donor-iPS cells, our most task is to develop how to expand megakaryocytes (MKs), platelet precursors, *in vitro*. While there has been no evident way to sustain long-term self-repopulating MK progenitors *in vitro*, it was a bit surprising report that MK progenitors can be directly generated from murine hematopoietic stem cells (HSCs) within bone marrow (BM) *in vivo* (Cell, 2013). Similarly, we have established megakaryocytic cell lines (imMKCLs) derived from human iPS cells to grow continuously for up to 5–6 months, which was achieved by inducible overexpression (O/E) system with c-MYC, BMI1, and BCL-XL to control either MK expansion or platelet yield in 2D feeder-dependent culture (Cell Stem Cell, 2014). To further address definite protocols including expansion of imMKCLs in a large scale > 20 L liquid culture independently of adherent feeder cells, we developed 3D culture method. Moreover, towards clinical scale platelet production, condensation of platelets to 200 mL package for transfusion in bedside, or quality control even if culture is performed at 37°C (but platelets must be maintained at 20–24°C *ex vivo*), newly developed various technologies including hollow fiber-based filtration system have been proposed in our system.

**Towards Developing a Process for Efficient *Ex Vivo* Platelet Production: Megakaryocyte Synchronization and Shear-Stress-Enhanced Platelet Release**

**M. T. Duncan**, T. A. DeLuca, A. C. Schlinker, A. Martinez, J. Magura, R. McMahon, W. M. Miller;

Chemical and Biological Engineering, Northwestern University, Evanston, IL.

*Ex vivo* platelet production has been a long-standing goal within the biotechnology/hematology communities. Despite advances demonstrating robust *ex vivo* production of megakaryocytes (MKs, the precursors to platelets) from CD34+ hematopoietic stem and progenitor cells, stimulating MKs to efficiently generate platelets in culture remains a major challenge. We have developed microfluidic bioreactors to evaluate the positive effects of shear stress on platelet release. Using a 2-compartment microfluidic bioreactor, we were able to culture mature MKs for 16 h while maintaining high viability. This system retains nucleated cells via a microporous membrane, even at high flow rates (100 µl/min), which should enable collection of a pure platelet fraction. We are optimizing the design and operating conditions to achieve efficient platelet release. In parallel, we are developing two microfluidic systems for single-cell capture to allow tracking of individual mature MK responses to well-defined flow conditions and the presentation of extracellular matrix (ECM) proteins. These microfluidic bioreactors will serve as robust platforms for identifying conditions that optimally promote proplatelet formation (PPF). We are particularly interested in identifying ECM proteins that mediate loose MK adhesion, as we observed that spread MKs forming stress fibers do not form proplatelets. However, culturing MKs on ultra-low attachment (ULA) surfaces that resist protein adsorption also inhibits the formation of proplatelets, suggesting some level of adhesion is required for PPF. Interestingly, MKs matured on ULA surfaces maintain the ability to rapidly generate proplatelets when shifted to a supportive surface, suggesting that ULA surfaces may be useful in synchronizing PPF.

**A Tissue Engineering Approach to Mimic the Megakaryocyte Niche: A Route Towards *In Vitro* Platelets Production**

**G. Bouet**<sup>1</sup>, M. Arumugam<sup>1</sup>, M. Colzani<sup>1</sup>, D. Howard<sup>1</sup>, T. Moreau<sup>1</sup>, W. Afshar Saber<sup>1</sup>, J. Shepherd<sup>2</sup>, S. Best<sup>2</sup>, R. Cameron<sup>2</sup>, C. Ghevaert<sup>1</sup>;

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Platelet transfusions to thrombocytopenic patients are increasing by 7–10% per year. We are currently entirely reliant on donor-derived platelets, which have limitations: short shelf-life, risk of donor-derived transmitted infections and issues of mismatch for patients with HLA class I antibodies. An alternative is to produce platelets *in vitro* derived from megakaryocytes (differentiated from either primary hematopoietic stem cells or, ultimately, pluripotent stem cells). However the production of functional platelets on a large scale from cultured megakaryocytes is hampered by our inability to recreate the complex physico-chemical characteristics of the bone marrow niche which signals positively to the megakaryocytes in order to release platelets.

We have thus developed a collagen-based three-dimensional (3D) scaffold, which is subsequently specifically functionalised with recombinant proteins in order to reproduce the cell surface landscape of the bone marrow sinusoids to give a direct contact signal to megakaryocytes to produce proplatelets.

We show that we boost platelet production of megakaryocytes seeded on 3D collagen scaffolds up to 6 times compared to the 2D classical system.

Out of a library of 50 ectodomain recombinant proteins derived from surface expressed protein of vascular cells, we identified two which positively regulate platelets formation. Platelets production is further enhanced when these two proteins are immobilised on the collagen scaffolds.

Finally the functionalized scaffolds are incorporated into custom made two chamber bioreactors to recreate the shear stress found in the bone marrow sinusoid lumen to facilitate platelet release and harvest into storage solutions compatible for human use.

**Development of a Hematopoietic Microenvironment for the Production of Red Blood Cells (RBCs) in a Novel 3D Hollow Fibre Bioreactor**

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Successful *ex vivo* production of RBCs from human umbilical cord blood (hUCB) for transfusion would relieve limitations in the blood donation system, but remains impractical due to the current costly and inefficient suspension culture systems. We previously developed a 3D hollow fibre bioreactor to promote long-term, scalable expansion of red cells using smaller volumes and physiological cytokine concentrations. We extend these studies to further characterise the hematopoietic microenvironment for erythroid expansion and maturation.

The collagen-coated polyurethane scaffold (5.25 mL, 87.4% porous, 130 µm pore size) was formed using thermally induced phase separation around extruded ceramic hollow fibres (59.9% porous, 440 nm pore size). Mononuclear cells from hUCB (20 × 10<sup>6</sup>/mL) were cultured for 28 days with perfusion of 650 mL serum-free medium supplemented with 1% penicillin-streptomycin, and an average of 4.9 ng/mL stem cell factor (SCF) and 0.45 U/mL erythropoietin (EPO).

Flow cytometric, confocal and electron microscopy analyses indicated total hUCB cell expansion of 8-fold (3 × 10<sup>8</sup> cells/mL) by day

21 achieving near tissue-like cell density, and included erythroid (2.4% c-Kit<sup>+</sup>, 3.4% EPO-R<sup>+</sup>, 32.5% GYPA<sup>+</sup>) and osteoid cell types (32.2% OSx<sup>+</sup>, 8.6% OPN<sup>+</sup>). OPN<sup>+</sup> populations were distributed closer (77% between 1.1–1.8 mm) to the hollow fibres whereas OSx<sup>+</sup> cells were located further away (74% 2.5–3.6 mm). EPO-R<sup>+</sup> expressing cells clustered together, with 61% EPO-R<sup>+</sup> cells within 75 μm of other EPO-R<sup>+</sup> cells. The data suggest that the 3D hollow-fibre bioreactor spontaneously sustained a multilineage microenvironment, the understanding of which could facilitate the cost-effective production of functional RBCs.

### Mimicking Physiology and Nature's Design Principles to Generate Donor-independent Platelets

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To produce donor-independent blood platelets, we have recently generated a bioreactor that recapitulates the bone marrow cellular environment and vasculature under physiologically relevant shear forces. Using this model, we have demonstrated that physiologically relevant shear forces stimulate proplatelet production, reproduced *ex vivo* proplatelet production, and generated functional platelets from stem-cell derived megakaryocytes.

To achieve a total yield of 1x10<sup>8</sup> platelets per 300 μL needed to assess bioreactor-derived platelet quality/safety *in vitro*, and perform function studies *in vivo*, we have: (1) Scaled our original bioreactor 100-fold to trap more megakaryocytes, (2) improved shear-stress exposure and surface interaction to increase platelet yield, and (3) modularized our design to support device parallelization for future scale up. Our next-generation bioreactor consists of two chambers (a top infusing chamber and a bottom collecting chamber) that are separated by a porous membrane. Devices are manufactured from polydimethylsiloxane using soft lithography, cost less than \$100/device to manufacture, and are both reusable and disposable/biodegradable. Megakaryocytes are infused into the top chamber using a microfluidic pump. Media is directed through the porous membrane causing megakaryocytes to release platelets through the pores and out the bottom chamber. Porous membranes can be coated with different proteins and ligands to increase yield.

We are approaching 1x10<sup>8</sup> platelets needed to begin *in vivo* studies in humanized mouse models. Our next-generation bioreactor-derived platelets are structurally and functionally consistent with donor platelets. Perfusion of human stem cell-derived megakaryocytes through our scaled bioreactor should yield safer, longer-lived, functional human platelets that can be produced on-site and on-demand.

### Functional Platelet Generation *Ex Vivo* using Bioengineered Silk Protein Hematopoietic Bone Marrow Niches

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Bone marrow is a complex tissue protected by bones and dedicated to the production of all blood cells. Bone marrow failure is the result of diseases, trauma and cancer treatments, leading to ineffective hematopoiesis. Millions of platelets, specialized cells that regulate haemostatic and inflammatory functions, are transfused worldwide every year and while demand for functional cells is growing constantly, supply is limited. Platelets are produced by megakaryocytes extending long filaments, called proplatelets, directly into the bloodstream. Bone marrow structure and extracellular matrix composition, together with soluble factors, are key regulators of this process by supporting cell differentiation and platelet release. Despite this knowledge, very little is known about the mechanisms involved in platelet production. To address these needs, we present our results using natural silk fibroin as a biocompatible and non thrombogenic material to engineer a 3D bone marrow system to house megakaryocytes for functional platelet production *ex vivo*. Using primary human megakaryocytes, platelet generation was recorded inside

this model in response to variations in surface topography, stiffness, co-culture with endothelial cells and shear forces. A critical feature of the system was the possibility to control silk functionalization with cytokines, extracellular matrix components and endothelial-derived proteins. Millions of human platelets were produced and shown to aggregate and participate to thrombus formation. Furthermore, using adult hematopoietic progenitor cells our system demonstrated the ability to reproduce alterations observed in diseased states.

In conclusion, we developed a versatile new laboratory tool to study mechanisms of physiologic and pathologic thrombopoiesis in the bone marrow.

### Session: Bone Tissue Engineering - Novel Developments

Date and Time: Wednesday, September 9, 2015,  
10:30 AM - 12:00 PM

#### Healing of Osteoporotic Bone Defect by Genetically Engineered Osteoporotic BMSCs Expressing MicroRNA Sponge

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Repairing bone defects following fracture in osteoporotic patients remains challenging. MicroRNAs (miRNAs) regulate osteoclastogenesis, osteoblast differentiation and bone formation. Here we aimed at exploiting miRNAs for repairing osteoporotic bone defects and uncovered that bone marrow-derived mesenchymal stem cells (BMSCs) harvested from rats with long-term estrogen deficiencies (osteoporotic rats) over-expressed certain miRNAs (e.g. miR-140 and miR-214). We hypothesized that suppressing the expression of miR-140 or miR-214 might promote the osteogenesis of osteoporotic BMSCs and developed gene delivery vectors for prolonged expression of the sponges (i.e. decoys) of miR-140 or miR-214. Engineering the osteoporotic BMSCs with the vectors expressing miRNA sponges persistently down-regulated the miR-140/miR-214 expression, enhanced the osteogenic differentiation and mitigated osteoclast maturation via a paracrine fashion, yet expression of miR-214 sponge exhibited stronger effects. To augment the *in vivo* bone healing, we further engineered the osteoporotic BMSCs with the vectors expressing miR-214 sponge and BMP2. Allotransplantation of the BMP2/miR-214 sponges-expressing osteoporotic BMSCs into the critical-size defect (3 mm in diameter) at the femur metaphysis of ovariectomized rat potentiated the bone healing and remodeling, filling ≈ 28% of bone volume/total volume (BV/TV) at 4 weeks in comparison to non-operated healthy group (≈33% of BV/TV in sham group). The BMP2/miR-214 sponges-expressing osteoporotic BMSCs not only accelerated the healing, but also ameliorated the bone quality (density, trabecular number, trabecular thickness and trabecular space), as evaluated by micro computed tomography, histology and immunohistochemical staining. Altogether, this study paved a new avenue to treatment of osteoporotic bone defects using miRNA-modulated BMSCs.

#### Enhanced Metabolism & Osteochondrogenic Differentiation of Human Periosteal-derived Cells upon Microaggregation: A Biomimetic Approach towards Tissue Engineered Constructs

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Stem cell-based strategies play an important role in bone tissue engineering. However, traditional 2D cultures lack the metabolic and proliferative gradients that are present in the body, hence the physiological conditions that are relevant for translational approaches from *in vitro* to *in vivo*.

Therefore, human periosteal-derived cells (hPDCs), a major stem cell source for postnatal fracture healing, were cultured in microwells (150 μm in diameter) to self-assemble into microaggregates, thereby

mimicking the mesenchymal condensation of progenitor cells during early limb bud development.

Compared to 2D culture, microaggregation of hPDCs resulted in an enhanced cell cycle activity, evidenced by a 4-fold and 3-fold increase in S and G2/M phase, respectively. Furthermore, aggregates showed a consistently higher ratio between the rate of glucose uptake and of lactate production, suggesting a less efficient aerobic fermentation process. This finding correlates with the transmission electron microscopy images showing accumulation of elongated mitochondria close to the nuclei. Interestingly, aggregation of hPDCs activated cellular differentiation, evidenced by decreased expression of the stemness markers (CD73 and CD90), and transcript levels of both chondrogenic (COL2, ACAN and SOX9) and osteogenic (OSX and OCN) genes. This effect was further significantly intensified when cells were exposed to BMP-2. Strikingly, BMP-2 stimulated cells upon aggregation showed abundant cartilage matrix formation after 1-week in nude mice.

In conclusion, our data revealed that microaggregation of hPDCs activated their cell cycle, altered metabolic activity, and induced osteochondral differentiation. These findings suggest that a microaggregation strategy supports a biomimetic, developmental approach towards tissue engineered constructs.

### Vascularized Bone Tissue Engineering under Xenogeneic-Free Conditions in a Large Animal Model as a Basis for Early Clinical Applicability

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For decades, researchers have been working successfully on bone tissue engineering. However, going from bench to bedside one major problem remains the traditional use of xenogeneic substances in cell cultures, which makes direct clinical application impossible. Beyond that, vascularization is the key challenge in tissue engineering for critical size defects.

In this study the large animal intrinsic arteriovenous loop model was further developed using perforated titanium chambers for acceleration of vascularization and evaluated using histological and 3D-imaging methods. A xenogeneic-free system for culturing mesenchymal stem cells (MSC) was established *in vitro* using autologous sheep serum instead of FCS and evaluated regarding gene and protein expression (real-time PCR/flow cytometry), cell proliferation (MTT) and osteogenic differentiation. MSC grown under different culture conditions were implanted subcutaneously in sheep with a nanostructured bone substitute in a stable block and a moldable putty form.

Combination of intrinsic and extrinsic vascularization promotes tissue ingrowth and remodeling of the bone substitute. Extrinsic vessels contribute to faster vascularization resulting in shorter pre-vascularization periods. *In vitro* and *in vivo* results indicate that autologous sheep serum is suitable for cultivation of MSC for bone tissue engineering purposes. While vascularization *in vivo* was better in the putty, bone formation was only visible in the block group.

This study paves the way for further pre-clinical testing using the sheep tibia defect model. Based on the results obtained in this strictly xenogeneic-free cell culture system in combination with a large autologous animal model, transfer into clinics will become possible in the near future.

### Critical Long Bone Defect Treated by Magnetic Scaffolds Fixed by Permanent Magnets and Vascular Endothelial Growth Factor Injection

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Bone tissue structure and function depend on ultrastructural organization of its components: cells, extracellular matrix (ECM) and inorganic components. Bone regeneration was evaluated in a critical diaphyseal defect after implantation of a magnetic scaffold fixed by hybrid system (magnetic and mechanical), supplied through magnetic-nanoparticle (MNP) functionalized with Vascular-Endothelial-Growth-Factor (VEGF) and magnetic-guiding.

In 8 sheep metatarsus diaphysis we created a critical bone defect (length 20.0 mm), then a Hydroxyapatite scaffold that incorporates magnetite (HA/Mgn:90/10) was implanted, proximally fixed by two cylindrical permanent NdFeB-magnets (one 6.00 mm diameter magnetic rod incorporated into scaffold and one 8.00 mm diameter fitted into proximal medullary canal, both 10.00 mm long); screws and plate were used as a bridge. Scaffolds biocompatibility previously assessed *in vitro* using human osteoblast-like cells. Magnetic forces through scaffold were calculated by finite-element software.

One week after surgery, VEGF-MNP were injected at scaffold mid portion in 4 sheep. After sixteen weeks, sheep were sacrificed to perform macroscopical, radiological, microCT and histomorphological examination.

Bone tissue formation was seen inside scaffold pores with complete scaffolds coverage. X-rays showed scaffold integration and good healing process, without mobilization. MicroCT showed new bone formation inside scaffolds, in particular at magnetized bone-scaffold interface. Histomorphological evaluation confirmed greater bone regeneration at magnetized interface, in both groups. Comparing groups bone regeneration was greater when VEGF-MNP were injected.

These results showed how magnetic forces can stimulate bone formation, as attested by *in vitro* and *in vivo* models, improve fixation at bone-scaffold interface, as calculated by finite element software, and guide targeted drug-delivery without VEGF-MNPs dissemination in all body.

### Different Ribonucleic Acid (RNA) Molecules as Therapeutic Tools for Tissue Engineering

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Tissue regeneration requires a series of orchestrated events leading to changes in cellular behavior. Growth factors may guide this cellular behavior. They have been used in tissue engineering (TE). However, they present important limitations. Supraphysiological concentrations or repetitive applications are needed in order to exhibit a physiological effect. This may lead to undesirable side effects. Thus, there is a clear need for alternative approaches to induce tissue regeneration. Gene therapy (GT) could have clear advantages over protein delivery. With GT, the therapeutic protein can be produced and expressed for extended periods locally. Additionally, the doses of therapeutic proteins used during treatment are reduced. Conventionally, pDNA has been used for GT. Viruses are most efficient to introduce pDNA into the cell. However, they are associated with safety concerns and non-viral vectors are limited by low gene transfer efficiency. An attractive alternative to pDNA is messenger RNA (mRNA) delivery. Since mRNA exerts its function in the cytoplasm, limitations related to the transport across the nuclear membrane are overcome. Thus, therapeutic proteins could be more efficiently expressed. mRNA can be chemically modified to improve immunogenicity and stability. On the other hand, overexpression of unwanted proteins may jeopardize tissue regeneration. The inhibition of these signals by small interfering RNA may be an effective therapeutic strategy. Another alternative is microRNA. They modulate regulatory circuits governing tissue repair. They can be either activated or inhibited by microRNA mimics respectively inhibitors.

Special attention will be given to molecules that are capable of stimulating bone regeneration.



### Chemically Modification of NELL-1, an Osteogenic Factor, as a Novel Systemic Therapy for Osteoporosis

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**Objective:** NELL-1 is a pro-osteogenic protein shown to successfully reverse osteoporotic bone loss through local intramedullary injection in rats. However, the short half-life limits its application as a practical systemic therapy. Thus, the purpose of this study is to modify NELL-1's structure via PEGylation, one of the most biocompatible technologies to prolong half-life of protein, to improve its pharmacokinetics and maintain its bioactivity.

**Methods:** PEGylated NELL-1 was prepared using three types of PEG: linear 5 KDa, 20 KDa, and branched 40 KDa. The conjugates were characterized by GPC and thermal-shift assay. Bioactivity was determined by cell-based assays, and pharmacokinetics was determined in mice. The osteogenic capacity was investigated by systemic administration in mice (1.25 mg/kg) for 4 weeks. Femurs and lumbar vertebrae were analyzed by DXA, microCT, histology and immunohistochemistry.

**Results:** NELL-PEG-5K was found to yield the most thermally stable conjugate while maintaining comparable bioactivity to unmodified NELL-1 *in vitro*. The half-life of NELL-PEGs significantly improved from 5.5 h up to 31.3 h, and more protein (2–3 fold) was distributed to bone tissues. DXA analysis showed a statistically significant increase in BMD compared to control. MicroCT data revealed significant improvements in trabecular BMD and structural values. Furthermore, histology confirmed increased bone formation and trabeculation, while TRAP staining and OCN expression revealed decreased osteoclastic activity and increased osteoblastic activity in NELL-PEG-5k group.

**Conclusion:** PEGylation significantly improves NELL-1's pharmacokinetics while preserving its bioactivity *in vitro* and systemic osteogenicity *in vivo*. Our findings demonstrate NELL-PEG's potential as an innovative and further accessible approach for systemic treatment of osteoporosis.

### Session: Clinical Translation Strategies for Respiratory, Urologic and Gastrointestinal Tissue Engineering

**Date and Time: Wednesday, September 9, 2015, 2:15 PM - 3:30 PM**

#### Long-term Outcomes of Endoscopic Transplantation of Oral Mucosal Epithelial Cell Sheets to Prevent of Esophageal Stricture After Endoscopic Submucosal Dissection

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**Introduction:** We developed a regenerative medical treatment to prevent esophageal stricture after endoscopic submucosal dissection (ESD) of esophageal carcinoma using tissue-engineered oral mucosal epithelial cell sheets, and reported in a short term. In this study, we report long-term outcome of this treatment.

**Purpose & Methods:** The purpose of this study is to reveal the long-term outcome of oral mucosal epithelial cell sheets transplantation.

Epithelial cells, isolated from the patient's oral mucosal tissue, were cultured for 16 days using temperature-responsive culture dishes. Then, the harvested epithelial cell sheets were endoscopically transplanted onto the site of the esophageal ulcer after ESD. Courses of 10 patients, who underwent endoscopic transplantation of oral mucosal epithelial cell sheets from April 2008 through September 2010, were recoded.<sup>(1)</sup> We analyzed the outcomes.

**Results:** All patients were precisely being followed-up. No stricture was detected in any of the patients after a brief interval. One

patient was deceased because of advanced pancreatic cancer. One patient underwent chemo-radiotherapy according to pathological finding of a specimen after esophageal ESD. One patient underwent surgery due to metastasis of mediastinal lymph nodes. He had survived until now without tumor.

**Conclusion:** Transplantation of cell sheets has been proven to be a safe and effective method in long term. No patients showed any controlled esophageal stricture. This treatment was only an exploratory clinical research. Furthermore, prospective randomized trials will be needed.

#### Reference

- Ohki T, *et al.* Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. *Gastroenterology* 143, 582, 2012.

#### Adipose Stromal Cells Seeded on Hydrogel Protects Against Colonic Radiation-Induced Damage

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**Introduction:** Radiotherapy aims to reduce tumor whilst preserving surrounding tissues. Pelvic irradiation causes loss of intestinal integrity and leads to gastrointestinal complications. Intravenous injection of Adipose Stromal Cells (ASC) reduces radiation-induced damage but a big amount of cells and multiple injections are needed due to cell death. Our aim was to develop a cell-biomaterial strategy to improve the effects of ASC treatment on radiation-induced lesions.

**Methods:** SD rats were subjected to 29 Gy colorectal irradiation. Three weeks after irradiation, ASC from GFP-SD rats were administered intravenously (5M of cells) or locally (1M +/- hydrogel (Si-HPMC) by colonoscopy). Seven days after injection, microscopic damage (MD), measurement of colonic paracellular/transcellular permeability (CPP/CTP), engraftment of cells, macrophages, apoptosis and proliferation assays were performed.

**Results:** Four weeks after irradiation, CPP and CTP were increased compared to controls. IV and local injections of ASC did not change CPP and CTP compared to irradiated rats. MD decreased in rats injected with 5M IV but not 1M locally. However, injection of ASC with hydrogel decreased, compared to irradiated group, MD, CPP and CTP ( $P < 0.05$ ). The beneficial effects of ASC + hydrogel were associated to the increased GFP-ASC found in the submucosa. ASCs locally injected attract macrophages whilst hydrogel seems to protect them. Proliferation/apoptosis assay demonstrated that cells are not proliferating but not apoptotic.

**Conclusion:** We developed an ASC/hydrogel injectable treatment to reduce the severity of damage induced by colorectal irradiation. This approach improves the viability of ASC and allow their engraftment to the site of the lesion leading to better therapeutic benefit.

#### An Optimized Strategy for Artificial Urinary Conduits in a Porcine Model

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A readily available artificial urinary conduit will prevent the use of bowel tissue and its associated complications in standard diversions. However, implementation of large constructs remains challenging, due to the lack of cells and/or vascularization. We investigated the effect of

pre-implantation of large reinforced collagen-based tubular constructs, compared to (a)cellular constructs in a urinary diversion pig model. Constructs (l= 12 cm,  $\phi$ = 15 mm) were prepared from type I collagen and biodegradable Vicryl meshes, through standard freezing, lyophilization and cross-linking techniques. In the first surgery, either a bladder biopsy was taken or simple constructs were subcutaneously pre-implanted. In the second surgery, the right ureter was used to create a diversion with a skin outlet of  $\phi$ =2.5 cm using either i) seeded constructs with urothelial and smooth muscle cells (n=5), ii) unmodified constructs (n=7), or iii) pre-implanted constructs (n=4). A catheter was inserted for 10 days. All pigs were sacrificed at one month. Implanting the reinforced collagen-based constructs resulted in a conduit which allowed urinary flow in most cases (13/16). However, different degrees of hydronephrosis were macroscopically and microscopically observed. An unmodified construct (ii) without cells resulted in a better outcome than a cell-seeded scaffold (i), possibly due to a massive inflammatory response caused by the cellular, non-vascularized component. Pre-implantation (iii) and creation of a urinary conduit resulted in better results with only moderate hydronephrosis, mild inflammation and contraction. Providing an existing vasculature by pre-implanting an unmodified construct resulted in an improved outcome with less hydronephrosis and contraction.

### Regenerative Medicine of the Larynx and Cervical Trachea

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Airway reconstruction after resection of inflammatory lesions or malignancies remains a difficult surgical challenge. Our concept “*in situ* tissue engineering” is designed to mediate the healing and natural tissue re-growth in the body. The purpose of our study is to present new perspectives for regenerative medicine of the larynx and cervical trachea.

A tissue scaffold made from non-absorbable polypropylene mesh tube covered by absorbable porous and microcellular collagen sponge was developed and implanted into the partial defect of the cricoids and trachea in beagle dogs. Postoperative endoscopy during the observation period from 3 to 60 months showed no airway obstruction. Good epithelialized ciliary mucosa was regenerated on the luminal surface. The implanted material of polypropylene mesh was incorporated with connective tissue without inflammation and the airway framework was firmly supported. With informed consent based on approval of IRB, the tissue scaffold was implanted for repair of the airway defect in 12 patients with laryngeal and cervical trachea stenosis or with cancer invasion. Postoperative endoscopy and CT scan showed well epithelialized airway lumen during the observation period from 9 to 96 months. Our concept “*in situ* tissue engineering” will afford a feasible approach for regenerative medicine of the larynx and cervical trachea.

A couple of next generation artificial tracheas using collage sponge combined with several cells such as fibroblasts, adipose tissue derived stem cells, chondrocytes, and induced pluripotent stem cells and a collagen vitrigel scaffold were developed for regeneration of tracheal tissues. Outcome of these studies will be also demonstrated.

### Liver Extracellular Matrix Enhances Growth, Function and Maturation of Induced Pluripotent Stem Cell Derived Hepatocytes

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Induced pluripotent stem cells (iPSCs) represent an important cell source to both model disease and assess pharmaceutical toxicity *ex vivo*. However, one limitation is a muted functional phenotype com-

pared to endogenous cells. In this study, we investigate the effect of three-dimensional (3D) bioartificial microenvironments on maturation and biofunctionality of iPSC-derived hepatocytes (iPSC-hepatocytes) within a decellularized liver extracellular matrix (ECM scaffold). We also developed a 3D microenvironment using 3D bioprinting to create a poly-L-lactide acid scaffold infused with type I collagen (PLLA-collagen scaffold) to specifically assess the role of naturally occurring structural proteins and growth factors intrinsic to ECM scaffolds (biofunctionality) versus 3D structure alone. This bio-synthetic scaffold represents a ‘deconstructed’ 3D control with a narrowed repertoire of bioactive properties relative to ECM scaffolds yet allows for multi-dimensional cell growth. We found that iPSC-hepatocytes maintained viability, became polarized, and formed bile canaliculi-like structures on both 3D scaffolds during 14 days of culture. However, iPSC-hepatocytes grown within ECM scaffolds exhibited higher mRNA expression and functional activity of P450 isoenzymes (CYP2C9, CYP3A4, CYP1A2), which were significantly elevated compared to iPSC-hepatocytes grown in either PLLA-collagen scaffolds or standard sandwich control cultures. Moreover, the level of albumin synthesis in iPSC-hepatocytes grown within ECM scaffolds approached that of primary cryopreserved hepatocytes with lower expression of fetal-specific genes, alpha fetoprotein and CYP3A7, in iPSC-hepatocytes grown within ECM scaffolds compared to either PLLA-collagen or sandwich control environments. Taken together, these findings suggest natural liver ECM enhances biofunctional maturation of iPSC-hepatocytes when compared to a bio-synthetic multi-spatial (3D) scaffold.

### Session: Constructing a Physiologically Relevant Interface for Host Pathogen Interactions

Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM

#### Tissue Engineering and Infectious Disease - New Directions

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Tissue engineering continues to evolve as an exciting blend of science and engineering focused on generating 3D human tissues with physiological relevance. New experimental and analytical tools have evolved to support these needs, including cell sources, use of co-cultures, biomaterials with engineered structure, morphology and chemistry to meet tissue-specific needs, and bioreactor designs to establish the appropriate niche requirements. Along the way, these 3D tissues have become relevant for drug screening, toxicology and developmental biology, among other areas. In recent years, these same systems have started to provide new opportunities for the study of infectious diseases. These approaches are particularly relevant for many infectious diseases due to the limitations for human studies and the lack of animal models for the study of these diseases. The ability to exploit human tissues *in vitro* to gain new insight into infectious diseases, from lifecycles to treatments, should offer unprecedented new avenues for new understanding of these diseases, to reduce animal studies, to discover new treatment options, and to continue to establish tissue engineering as an important new tool set for the field. Some of our recent advances in these directions will be reviewed.

### *In vitro* and *in vivo* Performance of Polyethyleneimine (PEI) Brushes Integrated on Polyurethane (PU) Ureteral Stents Against Biofilm Formation and Encrustation

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Urinary tract and urologic disorders are the main causes of urinary obstruction that hinder the flow of urine. On the time of obstruction, ureteral stents are frequently used to remove the blockage. Nevertheless, urological implants are vulnerable to crystal deposition and bacteria adhesion. Because of the bacterial adhesion on the medical device, Urinary Tract Infection (UTI) is a typically encountered problem in the ureteral system. In the presence of uropathogens in the urinary system, pH of urine increases and encrustation occurs through Ca and Mg ions. Infection induces ion precipitation and encrustation induces bacterial adhesion with following biofilm formation.

Uses of PU based urological devices are very common in UTIs. In this study, surface of PU stent was modified with PEI chains as polymeric brushes and further alkylation was performed to enhance its antibacterial properties. *In vitro* studies were carried out in a dynamic bioreactor system. Results showed that, PEI brushes decreased the biofilm formation up to 2 orders of magnitude. In addition, PEI brushes along with following alkylation decreased Ca and Mg deposition by 45% and 48%, respectively. *In vivo* tests were also performed to investigate host tissue response and encrustation. *In vivo* results showed that alkylated PEI brushes decreased Ca and Mg deposition by 81% and 93.4%, respectively. Also less inflammation was triggered by PEI modified stents in the host tissue.

1. Gultekinoglu M, *et al.* Acta Biomaterialia 10.1016/j.actbio.2015.03.037, 2015.

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#### Evaluation of the Mechanisms of Action of Probiotics using a Multicellular Three-Dimensional Organotypic Model of the Human Intestinal Mucosa

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Gastrointestinal infections occur worldwide with ~700,000 hospitalizations per year in the United States (1). Preventing or treating gastroenteritis would drastically reduce hospitalizations and the burden of medical costs. Probiotics are viable bacteria that might be beneficial in preventing and treating pathogen-induced diarrhea. Despite this, probiotic health benefits, including the understanding of their potential interactions with the human host, remain poorly understood. Here, we evaluated the probiotic anti-microbial signals and intestinal epithelial responses to a pathogen using *Salmonella* infection as a model. To this end we used a multicellular three-dimensional (3-D) organotypic model of the human intestinal mucosa, which has unique characteristics with close structural and functional resemblance to the human intestinal mucosa (2). This model is composed of an intestinal epithelial cell line and primary human lymphocytes, endothelial cells and fibroblasts. We also used one of the best characterized probiotics the *Escherichia coli* strain Nissle (ECN) (3), and the wild-type pathogenic enteric bacteria *Salmonella enterica* serovar Typhi (*S. Typhi*). We found that probiotics were able to reduce pathogen-induced inflammation by controlling production of mucus, beta defensin and pro-inflammatory cytokines (e.g., IL-8 and IL-6). In summary, these results may provide surrogate indicators of both safety and efficacy of probiotics that could translate into improved diarrheal treatment as well as in the designed improved clinical trials.

#### Human Intestinal Epithelial Response to Gastrointestinal Virus Infection using Enteroids

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A limitation in translational research in the gastrointestinal tract has been the absence of models that recapitulate the diverse nature of the epithelium. Human intestinal enteroids (HIEs) contain the normal complement of intestinal epithelial cell types (stem, enterocyte, goblet, enteroendocrine, and Paneth cells). We have utilized HIE cultures as pre-clinical models to study the response of the epithelium to common viral pathogens such as human rotavirus (HRV), which kills ~500,000 children annually by causing dehydrating gastroenteritis. Studies on HRVs have been limited because they are difficult to culture in transformed cell lines and do not infect small animals. We established HIEs derived from patient small intestine tissue and showed they support HRV infection and demonstrate previously unappreciated pathophysiologic and molecular responses to infection. Undifferentiated HIEs, consisting primarily of immature enterocytes, Paneth and stem cells, were less susceptible to infection compared to fully differentiated HIEs that consist predominately of mature enterocytes confirming *in vivo* findings that the villus enterocyte is the primary target of HRV infection and replication. Enteroendocrine cells also were infected suggesting signaling that may be related to pathogenesis. Infection increased proliferation and cell death and altered cellular metabolism pathways and innate immune responses. These findings establish HIEs as new models to understand the intestinal epithelial response to gastrointestinal infections such as HRV. HIEs allow us to address new questions about human host-pathogen interactions such as innate immune responses, stem cell activity, cell-cell communication within the epithelium and to identify and test new drug therapies to prevent/treat diarrheal disease.

#### Novel *In Vitro* Urothelial Infection Model using the Self-assembly Technique

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**Objectives:** Previous *in vitro* and *in vivo* studies for investigating the development and differentiation of the urothelium have been limited, as they do not represent the normal urothelial development and differentiation process in human. Self-assembly method of matrix formation would form a biomimetic tissue without the need for exogenous materials. In this study, we aimed at the creation of an *ex vivo* urinary bladder model and investigating post-infection effects on the urothelium and changes in the cytokeratin expression.

**Methods:** Bladder stromal (BSCs), urothelial (UCs), and smooth muscle cells (SMCs) were isolated using enzymatic methods. BSCs were stimulated with ascorbate to form collagen sheets. Following, SMCs and UCs were sequentially seeded on the stacked BSC-sheets to form bladder equivalents. After maturation, constructs were analyzed by histology, mechanical tests and permeability studies. Then, the *ex vivo* bladder model was subjected to epithelial bacterial infection. Effects on urothelial proliferation and keratin expression were noted.

**Results:** BSCs formed collagen sheets that could be handled easily. UCs constituted a well-differentiated epithelial layer with biomarkers of impermeability. A well-formed basement membrane and SMCs bundles were identified. Post-infection effects included decrease in constructs' thickness, urothelial hyperplasia, increased expression of CK14 and loss of CK20 expression, reflecting skin phenotype changes.

**Conclusions:** Using the self-assembly, *in vitro* bladder model was created with many functional and biological similarities to native bladder tissue without any foreign material. The post-infection changes represent a normal urothelial response to injury, which if not reversed, may lead to squamous metaplasia of the urothelium.

#### Session: Current Bioengineering Strategies for Advanced Peripheral Nerve Injury Treatment

Date and Time: Thursday, September 10, 2015, 3:00 PM - 4:30 PM

#### Sustained Release of IL-4 Promotes Nerve Regeneration

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Peripheral nerve injury is a major cause of disability in the US, with most of the patients failing to achieve full recovery. One promising target for improving outcomes is manipulation of the cytokine milieu at the injury site. We optimized a cytokine delivery vehicle to provide controlled release of IL-4 at the site of injury for the first week post-injury and assessed effects on *in vitro* and *in vivo*. We determined the optimal concentration of IL-4 on neurite outgrowth from culture motor neurons at 30 ng/mL with neuron branching almost twice that of untreated neurons. Increasing concentration over 60 ng/mL reduced branching to below control levels ( $p < 0.01$ ). We then optimized the loading of IL-4 into a lipid microtubule (LMT) drug delivery vehicle to ~100% at 100 ng IL-4/mg LMT. At this loading dose, we characterized the release kinetics, delivering ~55% of the loaded IL-4 over the first five days. *In vivo* effects were evaluated using an 8 mm sciatic defect and embedding loaded LMT within a 0.7% agarose hydrogel. Effects of macrophage phenotype and Schwann cell recruitment were determined at 10 and 21 days. Sustained release of IL-4 provides an opportunity for nerve regeneration by promoting recruitment of alternately activated macrophages, Schwann cell migration and axon extension.

### Regeneration of Long Gap Median Nerve Defects and Functional Reinnervation of the Abductor Pollicis Brevis Muscle using Novel Tissue Engineered Nerve Guides in a Non-Human Primate Model

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**Background:** Standard treatment for peripheral nerve injuries involves excising a donor nerve and transplanting it into the defect (i.e. an autograft). Autografts have disadvantages, including neuroma formation and numbness at the donor site. Limited donor nerve availability also complicates situations where multiple injuries are sustained. Biodegradable conduits are alternatives; however, clinically available guides do not actively support regeneration through growth factor delivery. We are investigating a poly(caprolactone) (PCL) nerve guide combined with neurotrophic factor delivery in non-human primates (NHPs) to support regeneration over long gaps (>3 cm).

**Methods:** 5-cm median nerve defects were repaired with autograft, decellularized nerve allograft, PCL with empty microspheres, or PCL with glial cell line-derived neurotrophic factor (GDNF) microspheres. NHPs were trained to retrieve treats from a Klüver board. Successful retrieval percentage (a pinch between the thumb and forefinger) was recorded. Baseline and explant nerve conduction velocity (NCV) were obtained. Nerve explants were evaluated for Schwann cell and nerve fiber density.

**Results and Conclusions:** At baseline, NHPs utilized a pinch 70–80% of the time. Baseline pinch retrieval was observed at POD 100 and a trend towards increased function was observed in the GDNF and decellularized nerve groups. NCV and muscle evoked potentials were evident at 1 year in PCL/GDNF and decellularized nerve groups suggesting that treatments can regenerate across 5-cm and support reinnervation. No significant differences were observed in Schwann cell density between autograft and decellularized nerve. Future work will focus on completing this study and clinically translating an ‘off-the-shelf’ alternative for long gap nerve repair.

### Controlled Temporal and Spatial Delivery of GDNF Promotes Enhanced Nerve Regeneration in a Long Nerve Defect Model

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Treating peripheral nerve injuries with growth factors, such as glial cell line-derived neurotrophic factor (GDNF), has proven useful in supporting axon survival and regeneration. Unfortunately, developing a method that delivers the appropriate spatial and temporal release profile to promote functional recovery has proven challenging. Many release mechanisms exhibit burst release profiles that are ineffective over long regeneration periods, yet prolonged exposure to GDNF can result in axonal entrapment at the site of release. Thus, a spatially and temporally-controlled GDNF delivery system was designed using a two-phase system comprised of an affinity-based release system and conditional lentiviral GDNF over-expression from SCs. Briefly, SCs were transduced with tetracycline-inducible (Tet-On) GDNF over-expressing lentivirus prior to transplantation. Three-centimeter acellular nerve allografts (ANAs) were modified by injection of a GDNF-releasing fibrin scaffold (2 week delivery window) under the epineurium, and then used to bridge a 3 cm sciatic nerve defect. To promote axon growth past the long nerve graft, GDNF-SCs were transplanted into the distal nerve and doxycycline was administered for 4, 6, or 8 weeks to determine the optimal duration of longer GDNF expression in the distal nerve. Live imaging and histomorphometric analysis determined that 6 weeks of doxycycline treatment resulted in enhanced regeneration compared to 4 or 8 weeks. This enhanced regeneration resulted in increased gastrocnemius and tibialis anterior muscle mass for animals receiving doxycycline for 6 weeks. The results of this study demonstrate that strategies providing finely tuned spatial and temporal delivery of GDNF can improve axonal regeneration and functional muscle reinnervation.

### Towards Advanced Bioengineering Approach for Effective Nerve Regeneration

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Peripheral nerve reconstruction is critical and challenging due to lack of effective ways to create a complex, multifactorial and dynamic microenvironment required for axonal path finding. Here, we report on bioengineered nerve conduits (NCs) orchestrated with important biological functions for supporting complex requirements of axonal path finding and target reinnervation. Firstly, an efficient and tropism modified adenovirus for generating genetically modified Schwann cells (GMSCs) was identified and engineered for single or co-expression of synergistic growth factors GDNF and NGF. Resulting recombinant virus, within GMSCs, proved to be safe and efficacious both *in vitro* and *in vivo* in a nerve crush injury model. Subsequently, collagen or silk fibroin nerve conduits (NCs) possessing aligned nanotopography were fabricated using a novel technique and orchestrated with GMSCs or multiple biological functionalities. Resulting eight different bioengineered NCs varying in structural and functional features were implanted in a 10mm nerve gap model in rats. The anatomical, behavioural and electrophysiological outcomes differed significantly between the treatment groups. The virus neutralization assay indicated absence of undesired immune response against the recombinant adenovirus used for genetic modification of the Schwann cells. Importantly, bioengineered collagen NCs carrying most important biological functions, in contrast to silk fibroin NCs, showed potential to overcome the present hurdles of nerve regeneration and matched well with autograft performance. This study not only revealed the importance of new approaches for orchestrating complex biological functions for effective axonal regeneration, but also demonstrated the impact of biomaterial composition in mounting effective tissue response.

### Chitosan-based Bioartificial Nerve Guides - Developments and Findings from the EU-collaborative Project BIOHYBRID

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Previously, we have demonstrated that fine-tuned chitosan conduits, with degree of acetylation of ~5%, allow functional and structural regeneration across a 10-mm sciatic nerve gap in rats to a similar extent as autologous nerve grafts [1]. These chitosan nerve conduits (Reaxon<sup>®</sup> Nerve Guide) also allow regeneration across 15-mm gaps in 57% of the animals, while no regeneration occurred through classic silicone guides [2].

Now, hollow chitosan nerve conduits have been modified in order to further increase regeneration outcome across long nerve defects and to allow regeneration even after delayed repair (45 days after nerve transection injury). (A) Different genetically modified Schwann cells (SCs) over-expressing neurotrophic factors were seeded into a hydrogel, NVR-Gel, and introduced into the lumen of the conduits. (B) Chitosan films were longitudinally introduced into the center of the lumen of the conduits as regenerative guidance structure.

Reconstruction of 15-mm rat sciatic nerve gaps was performed as either acute intervention (in (A)+(B)) or delayed repair (in (B)). Functional recovery and histomorphometrical parameters after chitosan conduit repair were compared to repair with autologous nerve transplants.

Results from (A) demonstrate that NVR-Gel in its viscous hydrogel form represents an obstacle to regeneration *in vivo*, and only introduction of FGF-218 kDa-over-expressing SCs into NVR-Gel allowed for some regeneration. The preliminary results from (B) are more promising by demonstrating significantly increased regeneration outcome in comparison to hollow conduits in the immediate repair scenario and some support of regeneration after delayed repair.

1. Biomaterials 34, 9886, 2013.  
2. Microsurgery 2014 Dec 4. doi: 10.1002/micr.22362.

### Optimization of Hydrogels for Fast 3d Neurite Extension of Encapsulated Neurons

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We explored how hydrogels from essentially inert polymers such as alginate, polyethylene glycol (PEG) and hyaluronan (HA) could be optimized to support fast neurite outgrowth from encapsulated neurons. In particular, we investigated various methods to tune the porosities and microstructures, to reduce the stress of encapsulation, to generate stable gels at very low stiffness, and to covalently attach adhesion cues.

We found that in all cases, physical properties had an effect on neurite outgrowth dramatically more important than addition of specific adhesion cues. In particular, with optimized physical properties, we could obtain very fast neurite extension in all gels in serum-free medium without any peptide or protein addition.

Neurons from chick, rat and human, both peripheral and central, and from primary tissue or iPSC derived, could be encapsulated with high viabilities (>85% for all and >95% in the best cases). In particular, we could obtain porous non-degrading gels that support fast 3D neurite extension from encapsulated dorsal root ganglia explants while staying perfectly stable for more than a month in culture, unlike their fast degrading natural counterparts (fibrin, collagen, matrigel). We could also obtain long-term stable electrically active 3D neural networks when encapsulating cortical or stem cell derived central neurons.

This work brings important new ideas and methods to help design hydrogels with improved physical properties and that enable faster axonal regeneration.

### Session: Development of Vascularization Strategies for Tissue Engineering Constructs

**Date and Time: Wednesday, September 9, 2015, 10:30 AM - 12:00 PM**

#### Perfusion of Implanted Pre-Formed Microvascular Heart Patches

S. Riemenschneider<sup>1</sup>, D. Mattia<sup>2</sup>, J. Wendel<sup>2</sup>, L. Ye<sup>3</sup>, P. Zhang<sup>3</sup>, R. Tranquillo<sup>2</sup>;

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Aligned tissue patches with  $900 \pm 200$  human microvessels/mm<sup>2</sup> were implanted into a nude rat myocardial infarct model to achieve rapid perfusion of the implanted patch by inosculation with the host vasculature. Patch microvessels were formed by self-assembly of human blood-outgrowth endothelial cells and human pericytes entrapped in a 3D fibrin gel. These networks align via cell-induced gel compaction to achieve a high density of parallel microvessels. Microvascular patches were implanted into nude rats by suturing them onto the epicardial surface of the left ventricle after a permanent ligation of the left anterior descending artery to achieve myocardial infarction. Many pre-formed human microvessels inosculation with the host and were perfused after 6 days *in vivo*. This result is especially promising for the future survival of vascularized cardiac patches *in vivo*.

**Acknowledgment:** NIH R01 HL108670.

#### Printing Living Tissues

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The ability to pattern biomaterials in planar and three-dimensional forms is of critical importance for several applications, including drug safety screening, tissue engineering and repair. 3D printing enables one to rapidly design and fabricate soft materials in arbitrary patterns without the need for expensive tooling, dies, or lithographic masks. In this talk, our efforts to creating vascularized living tissues via 3D bioprinting will be described. I will present recent advances in the design of cell-laden inks, extracellular matrices and fugitive (vascular) inks for 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs with as well as ongoing efforts to characterize these 3D living tissues.

#### Controlling Microvascular Assembly

S. Gerecht;

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Neovascularization takes place in an intricate milieu. This unique microenvironment is situated throughout the body in diverse types of healthy and diseased tissues, yet it activates/inhibits similar mechanisms of the microvasculature. Two parameters of this microenvironment seem critical for blood vessel growth independently of its location: (i) the extracellular matrix (ECM), which provides critical support for vascular cell adhesion, proliferation, migration, and morphogenesis, and (ii) low oxygen concentrations (hypoxia), which is a critical factor promoting vascularization during embryonic development and tumor growth. Hydrogels provide a highly controlled three-dimensional (3D) environment that is structurally and biomechanically similar to native ECM and can provide a rich biochemical landscape as well as biophysical cues to influence cell behavior. In this talk I will present our recent efforts to develop hydrogel matrices that activate signaling pathways during 3D vascular assembly. Examples will include interface along stem cell differentiation and during wound healing and tissue regeneration.

### Patterned Vascular Networks Rescue Limb Ischemia via Dll4/Notch1-mediated Anastomoses

T. Mirabella<sup>1</sup>, D. Cheng<sup>1</sup>, A. Longshamp<sup>2</sup>, C. K. Ozaki<sup>2</sup>, C. Chen<sup>1</sup>;

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Over 500,000 procedures a year in the US are arterial bypasses to restore blood flow downstream an arterial atherosclerotic occlusion. Almost 40% of patients in need of bypass surgery may not have autologous vessels of the appropriate quality or length, and synthetic graft alternatives often fail because of thrombotic occlusion and poor "biointegration" within host vasculature. The printing of rigid 3D filamentous meshes of carbohydrate glass, recently developed in our lab, is a rapid and biocompatible method to construct vascular networks. The geometries generated by this approach can be: i) used as sacrificial template, ii) lined with endothelial cells, iii) blood perfused under high-pressure pulsatile flow. In the presented study we show that those engineered vessels are functional once implanted *in vivo*, meaning: a) patent, b) mural cell-covered, c) chimeric and anastomosed with the recipient vasculature. Nevertheless, they can rescue the perfusion of distal tissues when implanted in a murine model of ischemic hind limb. In detail, by providing either acellular channels or randomly organized endothelial cells, we demonstrate that the bypass function is necessary but not sufficient to establish anastomoses with the pre-existing vasculature, rather both cell source and cell alignment are required for a successful biointegration of the vascular graft. Finally, we show that the signaling pathway developed across the axis Dll4-Notch1 at the interphase between donor and recipient endothelia is fundamental for implanted vessels to "wrap and tap" the host vasculature and divert blood flow to ischemic tissues.

### 3D Printing of Engineered Vascular Beds

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A major barrier in tissue engineering is a lack of understanding of how to integrate vascular networks into 3D tissue constructs. Without perfusable vascular networks, large constructs containing living cells develop necrotic cores due to nutrient, oxygen, and waste diffusion limitations. To address this challenge, we have developed approaches to generate perfusable vascular networks that support 3D culture of assembled constructs and provide a means to study how to regulate vascular remodeling and angiogenesis. The methods are based on creating channels within constructs using 3D freeform fabrication. A cytocompatible carbohydrate glass is thermally extruded with a custom 3D printer to form an interconnected filamentous lattice, which are then used as a sacrificial element to generate 3D vasculature in many types of matrices including collagen, fibrin, agarose, alginate, and PEG-based hydrogels. We discuss how these vascular networks are able to recapitulate the physiology of *in vivo* vasculature, and how the engineered vascular networks remodel over time in response to different cues, and how these approaches can be used to engineer the architecture and performance of vasculature in implanted constructs. This approach, as well as the cellular responses observed, open new possibilities for 3D vascularized tissue culture.

### Session: Directing Cell-Driven Microenvironment Formation for Tissue Engineering *In Vitro*

**Date and Time: Wednesday, September 9, 2015, 10:30 AM - 12:00 PM**

#### Human Mesenchymal Stem Cell Differentiation Monitored by Single-Cell Gene Expression Analysis

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Typical cellular and molecular biology approaches utilize bulk-scale measurements that yield averaged gene expression values for cell populations. These approaches fail to report individual cell phenotypes and, therefore, mask heterogeneity within cell populations. Development of stem cell-based therapies will require understanding of cell homogeneity/heterogeneity.

In the case of mesenchymal stem cells, biochemical compounds (e.g., bone morphogenetic proteins) have been utilized to promote their osteogenic differentiation. Our research provided evidence that, in the absence of exogenous biochemical compounds, alternating electric current (a biophysical stimulus), induces exclusive osteodifferentiation of adult human mesenchymal stem cells (MSCs) at the population level. The present study investigated the effects of alternating electric current (AC) on MSC osteodifferentiation at the single-cell level.

MSCs were cultured on flat, indium-tin-oxide-coated glass (an electrically conductive substrate pre-coated with fibronectin), and exposed to a sinusoidal, 10  $\mu$ A, 10 Hz, alternating electric current for 6 hours daily, for 7 and 21 consecutive days. Single-cell qRT-PCR measurement of mRNAs for 45 genes (indicative of MSC osteogenic differentiation) were monitored using Fluidigm Systems. Similar heterogeneous gene expression for bone morphogenetic protein 2, bone sialoprotein, and osteonectin were observed for MSCs under control (no AC) and the AC conditions tested. In contrast, greater homogeneity in early osteodifferentiation genes (specifically, TAZ and RUNX2) was observed in cells exposed to AC at 7 and 21 days.

These results provide the first glimpse of gene expression heterogeneity in differentiating MSCs, and suggest that cell exposure to AC promotes a homogeneous response towards early osteoblastic differentiation.

### Optogenetic Skeletal Muscle Powered 3D Printed Biological Machines

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Cell-based soft robots, or "bio-bots," that can accomplish such objectives of robotics as sensing, storage and processing of signals, and a resultant response such as actuation can address many engineering challenges. Perhaps the most intuitive demonstration of a biological machine is one that can generate force and produce motion. Skeletal muscle, a natural actuator, is designed to efficiently generate force and, when coupled to an appropriate mechanical design, generate motion. We have developed a 3D Printed millimeter-scale bio-bot powered by the contraction of tissue engineered skeletal muscle. Optogenetic muscle cells seeded in a synthetic extracellular matrix apply traction forces to compact over time into a "strip" that is capable of contractility and force generation. External electrical and optical signals can drive contraction of the muscle and change the conformation of the hydrogel backbone. The resulting flexion causes the bio-bot to crawl across surfaces, demonstrating net locomotion at speeds up to 1.5 Body Lengths/minute. We have assessed the functional performance of the engineered skeletal muscle by measuring the passive and active tension forces generated during electrically and optically stimulated locomotion. FEA Modeling is used to optimize geometric and material design parameters to better understand the effect of different biochemical and mechanical cues on engineered muscle performance. This demonstration sets the stage for developing machines capable of more complex controlled actuation behaviors, advancing the goal of forward engineering integrated cellular machines which have a myriad array of applications in high-throughput drug screening, programmable tissue engineering, noninvasive drug delivery, and biomimetic machine design.

### Controlling cell Fate Through Microenvironmental Signalling for Clinical Translation and Commercialisation of Cell-based Therapies

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The driven hypothesis of scaffold-free, cell-based therapies is that replacement, repair and restoration of tissue function can be accomplished best by recruiting cells' inherent proficiency to create their own tissue-specific extracellular matrix with precision and stoichiometric efficiency still unmatched by man-made devices. Such therapeutic strategies require removal of cells from their optimal, in regards to topography, stiffness, packing density, oxygen tension and mechanical loading tissue context, and propagation thereof in far from physiological *in vitro* environment. However, bereft of their tissue context, cells perform poorly, lose their functionality, and with it, their therapeutic potential. To this end, research efforts have been directed towards reconstruction of more functional *in vitro* micro-environments by modulating substrate topography; substrate stiffness; macromolecular crowding; oxygen tension; and mechanical loading in order to provide an optimal *in vitro* niche that will closely mimic cells' natural *in vivo* conditions and thus facilitate maintenance of their therapeutic potential. Herein, the influence of topographical features, macromolecular crowding and oxygen tension will be discussed as means to control cellular function and consequently enable clinical translation and commercialization of cell-based therapies.

### Cyclic Stretch on Soft Substrates Increases Traction Force

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Substrate stiffness has been clearly demonstrated to modulate cell differentiation independent of soluble factors. Stiffness likely modulates a cell's ability to spread and generate traction forces, i.e., "inside-out" signaling. External dynamic stretch, "outside-in signaling," has also been shown to modulate cell differentiation, and we previously demonstrated that cells cultured on soft gels and cyclically stretched spread as if cultured on stiff gels, yet the effects on cell traction forces are less well defined. The purpose of this study was to determine if the traction forces of cells cultured on soft substrates subjected to long-term cyclic stretch increase to levels equivalent to cells on stiff substrates. Polyacrylamide gels (0.6 and 7.5 kPa) were attached to compliant silicone wells and were coated with fluorescent beads and monomeric collagen. Porcine valvular interstitial cells were seeded on the gels, and the wells were stretched 10% uniaxially at 1 Hz for 24 hours. On soft substrates following cessation of stretch, cell area increased significantly from 530  $\mu\text{m}^2$  to 1214  $\mu\text{m}^2$  with stretch, and traction force increased from 14 nN to 43 nN ( $p < 0.05$ ); however, these values were significantly lower than the area (2500  $\mu\text{m}^2$ ) and traction force (520 nN) observed on the static stiff gels ( $p = 0.001$ ). Thus, cyclic stretch induces spreading and generation of cytoskeletal tension, but it does not appear that 10% uniaxial stretch for one day can substitute for the inside-out signaling of a stiff matrix.

**Acknowledgments:** This work was funded in part by an AHA Predoctoral Fellowship (14PRE18310016) and the NIH (2R15HL087257-02).

### Micro-Engineered 3D ECM Array for Investigating Cell-Interaction During Stem Cell Differentiation

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Reconstructing physiologically relevant 3D extracellular-matrix (ECM) environment is important in understanding the cell-ECM interaction but it remains a challenge to perform high-throughput analysis of their interactions *in vitro*. Here, we describe a novel miniature 3D ECM array combined with quantitative imaging that permits high-throughput and sensitive evaluation of different organ-derived ECMs and their effects on stem cell fate and function.

ECM extracts derived from either decellularized pancreas, liver or heart were mixed with cells + alginate solutions and micro-patterned on

top of dried PLL-Ba2+ spots with varying ion concentration (10 mM-100 mM), which gelled and formed spatially separated 3D ECM micro-constructs. To examine the feasibility of the platform to interrogate stem cell behavior, two different human pluripotent stem cell derived populations: pancreatic progenitors and multipotential cardiovascular progenitors were examined at 2 and 6 days using LICOR IR-imager.

ECM array quantification identified distinct ECM composition differences in the three organs. The stiffness of the 3D ECM micro-constructs was specified between 3 k and 70 k Pa as evaluated by Atomic Force Microscopy. Live/Dead analysis demonstrated high cell viability ( $85 \pm 5.8\%$ ) confirming the cyto-compatibility of the platform. In-cell western analysis with LI-COR IR-Imager detected significant differences ( $n=25$ ,  $P < 0.05$ ) of the proliferation (Ki67) and differentiation (PDX and CTnT) behavior of the two tested stem cell populations.

Our methodology enables rapid and high-throughput analysis of the contribution of native-like 3D multicomponent ECMs toward stem cells fate and function. The developed 3D ECM array will allow rational design of engineered stem cell niches and support the rapidly developing field of regenerative medicine.

### Localized and Spatiotemporally Controlled RNAi Delivery from Hydrogels to Encapsulated and Surrounding Cells

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RNA interference (RNAi) is a powerful tool to regulate gene expression post-transcriptionally. Controlled delivery of siRNA and miRNA has recently garnered much attention for its potential to enhance tissue engineering strategies by modifying cell behavior. Many RNAi delivery strategies rely on incorporation into nanoparticles and liposomes, however in these approaches the genetic material is often subject to degradation by serum RNases, challenging to spatially target to a desired anatomical location and rapidly cleared from the site of interest resulting in only transient cellular effects. We have engineered macroscale hydrogels capable of localized, sustained and tunable delivery of RNAi to cells incorporated within the gels and to those in the surrounding microenvironment. In this talk, I will discuss systems in which delivery may be modulated by tailoring hydrogel degradation and affinity interactions with the RNAi. Release of bioactive RNAi via external "on-demand" stimuli, as well as the capacity to spatially control delivery of RNAi and subsequently spatially control changes in gene expression will be demonstrated. Finally, the promise of this approach to deliver RNAi relevant for tissue engineering bone *in vitro* and *in vivo* will be presented.

**Acknowledgments:** This work was supported by the National Institutes of Health (R56DE022376) and the Department of Defense Congressionally Directed Medical Research Programs (OR110196).

### Session: Elastin: Predictive Biology, Encoded Tissue Elasticity and Directed 3D Growth and Enabling Extracellular and Elastic Matrix Regenerative Repair

**Date and Time:** Friday, September 11, 2015, 9:15 AM - 10:45 AM

### Magnetically-responsive Stem Cell-derivatives Maintain Pro-elastogenic Benefits for Matrix Regenerative Therapy

G. Swaminathan, B. Sivaraman, L. Moore, M. Zborowski, A. Ramamurthi;

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Restoring degraded elastic matrix is critical for growth arrest of abdominal aortic aneurysms (AAA), local aortal expansions resulting from structural matrix breakdown by matrix-metalloproteases (MMPs). Promising towards overcoming intrinsically poor regenerative repair of elastic matrix assembly by aneurysmal smooth muscle cells (SMCs), we recently showed high elastogenicity of bone marrow mesenchymal stem cell (BM-MSC)-derived SMC-like cells (BM-SMLCs) and their pro-elastogenic and anti-proteolytic effects on cultured AAA-SMCs. To magnetically-target BM-SMLC delivery to

AAA wall, we now investigate how labeling rat BM-SMLCs with super paramagnetic iron oxide nanoparticles (SPIONs) impacts its functional benefits. Prussian blue and LIVE/DEAD staining showed SPION uptake and non-cytotoxicity of BM-SMLCs at tested doses (0.2,0.5,1.0 mg/mL). Cell tracking velocimetry showed significant magnetic mobility of labeled-BM-SMLCs (0.5 mg/mL) in an applied magnetic field. Magnetic-targeting significantly enhanced BM-SMLC uptake and retention in porcine carotids *ex vivo*. Expression levels of SMC phenotypic markers and elastin homeostatic genes & proteins by BM-SMLCs were unchanged upon SPION-labeling (0.5 mg/mL); the cells continued to lack terminal differentiation markers. Elastic matrix deposition (Fastin assay) by BM-SMLCs was also unchanged upon SPION-labeling, though proliferation was increased. In transwell-culture assays, we showed no differences between labeled and unlabeled BM-SMLCs in their paracrine stimulus to increase a) gene expression for elastic matrix assembly proteins (ELN, FBLN5, EFEMP2, LOX) by rat AAA-SMCs (RT-PCR), b) elastic matrix synthesis (Fastin assay, immunofluorescence), and c) inhibit production & activity of elastolytic MMPs2,9 (westerns, gel zymography). The results are promising towards pursuing magnetic targeting of BM-SMLCs for regenerative AAA repair.

**Acknowledgments:** NIH (HL092051, HD078820) and AHA (GRNT17080027).

#### Phenotypic Coordinates of Mesenchymal Stem Cell-derived Smooth Muscle Cells Determine their Potential for Elastic Matrix Regenerative Repair

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Abdominal aortic aneurysms (AAAs) involve chronic matrix remodeling within aortic wall. Promising towards overcoming intrinsically poor regenerative repair of elastic fibers by aneurysmal smooth muscle cells (SMCs), we showed high elastogenicity of bone marrow mesenchymal stem cell (BM-MSC)-derived SMC-like cells (BM-SMLCs) and their pro-elastogenic and anti-proteolytic effects on cultured AAA-SMCs. We now investigate if BM-SMLCs effects can be augmented by phenotypic selection. Rat BM-MSCs were differentiated on fibronectin in presence/absence of PDGF in low (2%v/v) and high (10%v/v) FBS in low/high glucose DMEM/F12 containing TGFβ1. SMLCs expressed SMC marker genes/proteins but were not terminally differentiated. Expression of elastic fiber assembly genes was higher than for healthy SMCs in all derived BM-SMLC sub-groups, with highest levels seen in 10%v/v FBS with/without PDGF. Elastic matrix deposition amounts (Fastin Assay, IF) and desmosine crosslinking of elastin (ELISA) were significantly higher in SMLCs derived under high serum conditions with/without PDGF vs. other derived groups and controls (adult rat SMCs and MSCs). Carbachol assay and whole cell patch-clamp for intracellular Ca<sup>2+</sup>/K<sup>+</sup> activity demonstrated higher contractility of SMLCs cultured in 10%v/v FBS+TGF with/without PDGF in both low/high glucose DMEM/F12. Wound closure assay showed increased migration of SMLCs derived in 2%v/v FBS+TGF with PDGF in low glucose DMEM/F12 suggesting a more synthetic phenotype. Overall, SMLCs in high serum with/without PDGF were found to be most contractile and yet exhibited highest elastogenic potential. Our results suggest that phenotype is a useful metric for selection of BM-SMLCs for elastic matrix regenerative repair applications.

**Funding support:** NIH (HL092051, HD078820) and AHA (GRNT17080027).

#### In Vitro and In Vivo Chemotactic Behavior of Silk-tropoelastin Protein Alloys

C. E. Ghezzi<sup>1</sup>, J. Moreau<sup>1</sup>, A. Weiss<sup>2</sup>, D. L. Kaplan<sup>1</sup>;

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The combination of multiple biopolymers in a new multifunctional, degradable, biomaterial system would provide versatile tools in both *in vitro* and *in vivo* environments depending on cell and tissue needs. The implementation of silk and tropoelastin in a unique biomaterial, by conjugating a mechanically robust and long-lasting protein, silk, to a highly flexible and biologically active counterpart, tropoelastin. Silk-tropoelastin protein alloys in the form of films can direct biological outcomes by controlling net charge, chemotactic properties, as well as degradation profiles. In particular, we are interested to understand how these features affect biological responses both *in vivo* and *in vitro*. Furthermore, in order to understand how autoclaving impacts the structure and responses to the two proteins, the behavior of silk-tropoelastin alloys in a subcutaneous mouse model was studied. The impact of these protein alloys on the polarization of THP-1 macrophages was also addressed. This study provides insight into the physical interactions between the two proteins to underscore their mechanisms of stabilization and chemotactic behavior, which ultimately control the alloy degradation lifetime.

**Acknowledgments:** NIH R01 EB014283.

**References:** (1) Ghezzi *et al.* Israel Journal of Chemistry 2013; (2) Hu *et al.* Advanced Functional Materials 2013.

#### Accelerated Wound Repair and Promoted Angiogenesis in a Tropoelastin-modified Dermal Regeneration Template

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Severe burn injury results in substantial skin loss and cannot be treated by autografts. The Integra Dermal Regeneration Template (IDRT) is the leading synthetic skin substitute because it allows for wound bed regeneration and wound healing. However, all substitutes suffer from slow blood vessel ingrowth and would benefit considerably from enhanced vascularization to nurture tissue repair. It is shown here that by incorporating the human elastic protein tropoelastin into a dermal regeneration template (TDRT) we can promote angiogenesis in wound healing. In small and large animal models comprising mice and pigs, the hybrid TDRT biomaterial and IDRT show similar contraction to autografts and decrease wound contraction compared to open wounds. In mice, TDRT accelerates early stage angiogenesis by 2 weeks, as evidenced by increased angiogenesis fluorescent radiant efficiency in live animal imaging and the expression of endothelial cell adhesion marker CD146. In the pig, a full thickness wound repair model confirms increased numbers of blood vessels in the regenerating areas of the dermis closest to the hypodermis and immediately below the epidermis at 2 weeks post-surgery. It is concluded that including tropoelastin in a dermal regeneration template has the potential to promote wound repair through enhanced vascularization.

#### Stem Cell Secretions Rescue the Pelvic Organ Prolapse Phenotype in Lysyl Oxidase Like-1 (Loxl1) Knockout (KO) Mice

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Pelvic organ prolapse (POP) is an elastolytic disorder resulting in herniation of pelvic organs. As in women, lysyl oxidase-like 1 (Loxl1) knockout (KO) mice develop POP with increasing age and parity, and have disorganized elastin composition. Stem-cell based therapies have potential and can work by paracrine secretions of growth factors & cytokines collected from cell culture media. We hypothesized that concentrated conditioned media (CCM) from mesenchymal stem cells (MSC) of wild type (WT) mice (+/+CCM) would be more beneficial than CCM from Loxl1 KO mice MSC (-/-CCM) in preventing POP development in Loxl1 KO mice. CCM was prepared



by removing MSCs from culture and concentrating the media containing MSC secretions 50×. Concentrated control media (CM) was produced similarly but without conditioning by cells. Lox11 KO female mice (n=77) received 300 µl intraperitoneal injections of either +/+CCM, -/-CCM, or CM within 48 h after first & second deliveries. POP was assessed weekly for 20 weeks after the first delivery. Kaplan-Meier survival analysis compared time-to-prolapse between groups.  $p < 0.05$  shows significant difference. Lox11 KO mice treated with -/-CCM showed a significant reduction of time to prolapse ( $p = 0.04$ ) compared to CM treated mice. In contrast, time to prolapse in Lox11 KO mice treated with +/+CCM was not significantly different compared to CM treated mice ( $p = 0.14$ ). IP injection of -/-CCM significantly decreased development of POP in Lox11 KO mice, indicating that the mechanism of CCM treatment is not by replacing the missing LOXL1 and suggesting CCM as a potential non-invasive cell-based therapy to prevent or delay POP.

### Engineering a Highly Elastic Surgical Sealant

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Approximately 114 million surgical and procedure-based wounds occur annually worldwide, including 36 million from surgeries in the US. Post-operative reconnection of tissues is crucial for restoring adequate function and structure. Sutures, wires, and staples are widely used for this purpose. Despite their common use in the clinic, these methods exhibit limitations when being applied in fragile and elastic tissues, especially if the sealing is intended to inhibit fluid or air leakage against high pressure, as e.g. in vascular and lung surgeries. Additionally, the application of these methods can be demanding in minimally invasive procedures with restricted access. To address these limitations, various types of surgical materials have been used for sealing, reconnecting tissues, or attaching devices to tissues. However, existing surgical sealant materials often feature limited adhesion strength, are toxic, lack appropriate mechanics, and do not function in wet environments. We aim to overcome these limitations by engineering a highly elastic, biocompatible and biodegradable sealant through photocrosslinking of a human protein. This light-activated elastic sealant proves to be suitable to seal air and body fluid leakages after surgical procedures. We envision that this sealant will be easily commercialized as it is fabricated based on a recombinant human protein, and its degradation and physical properties are controllable. Our *in vitro* and *in vivo* data suggest that this material is superior to the existing products in the market and may generate a paradigm-shifting surgical sealant that may not require additionally supporting sutures due to its superior mechanical and adhesive properties.

### Exploiting Elastin's Mutability: Regulated Design of Elastin-Like and Silk-Elastin-Like Protein Polymer Libraries in Simulation and Experiment

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Elastin is a key mammalian extracellular matrix protein found in the human body, conferring resilience, compliance and elasticity to the lungs, blood vessels and skin. Soluble elastin-like peptides can be engineered into a range of physical forms, from hydrogels and scaffolds to fibers and artificial arteries, finding numerous applica-

tions in medicine and engineering as "smart polymers". Importantly, elastin is a great candidate as a platform material for novel biomaterial design as it exhibits a highly tunable response spectrum, with reversible phase transition capabilities at a temperature of 40–60°C. Here, we designed a library of elastin-like protein material models, using classical Molecular Dynamics and Replica Exchange Molecular Dynamics methods, to study the effect of sequence and environmental triggers on elastin's structural transition, exposing molecular mechanisms controlling these transitions that have been poorly understood until now. This library is a valuable standalone resource for recombinant protein design and synthesis and may elucidate the design of composite elastin-based materials. As an example of such a system, we study silk-elastin-like protein polymers (SELPs). Silk is uniquely strong, exhibiting strength values that surpass those of engineering materials such as Kevlar and steel. Thus, SELPs, which have repeating silk and elastin blocks, combine the distinctive properties of the composing parts to achieve strong and extensible, mutable biomaterials. The design of highly specific materials that can react controllably to triggers opens up avenues for applications in drug delivery and smart material design.

### Elastin-PLGA-Perlecan Peptide Nanofiber Scaffolds for Salivary Cell Attachment and Growth

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Biomimetic scaffolds for tissue engineering of salivary glands hold great promise for treatment of Xerostomia, or dry mouth, a condition caused by head and neck radiation therapy and Sjögren's syndrome. Our previous work demonstrated that electrospun poly(lactic-co-glycolic acid) (PLGA) nanofiber scaffolds promote an *in vivo*-like salivary epithelial cell tissue organization. To enhance the extracellular matrix (ECM) biomimetic character of the scaffold, we fabricated electrospun scaffolds composed of PLGA and elastin followed by functionalization with perlecan domain IV peptide. Elastin, a structural protein of the ECM responsible for tissue elasticity and cell attachment, is present at high levels in adult glands. Characterization of the scaffolds showed an increase in the fiber diameter of the blend scaffolds over PLGA nanofiber scaffolds and the appearance of elastin-specific ribbon and coil-like structures, as shown by scanning electron microscopy. Contact angle measurements showed increased hydrophilicity of elastin-PLGA scaffolds over PLGA alone. Cell viability studies suggested improved cell attachment and viability of both NIH/3T3 fibroblasts and submandibular immortalized mouse salivary epithelial cells (SIMS) on elastin-PLGA scaffolds as compared to PLGA nanofiber scaffolds. To promote apical polarity of the epithelium, perlecan domain IV peptide was conjugated to elastin-PLGA scaffolds. Perlecan is a component of the salivary gland basement membrane, and perlecan domain IV peptide was reported to promote salivary epithelial apical polarity. Cell viability studies showed increased attachment and growth of NIH/3T3 and SIMS cells on elastin-PLGA-perlecan nanofiber scaffolds relative to the other scaffolds. In conclusion, nanofiber-based, biomimetic scaffolds show promise for salivary gland tissue engineering.

### Session: Emerging Organ Models and Organ Printing for Regenerative Medicine

Date and Time: Thursday, September 10, 2015, 3:00 PM - 4:30 PM

#### Assembly of Stem Cell-derived Human Tissues for High Throughput Screening Applications

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The need for human, organotypic culture models coupled with the requirements of contemporary drug discovery and toxin screening

(i.e. reproducibility, high throughput, transferability of data, clear mechanisms of action) frame an opportunity for a paradigm shift. The next generation of high throughput cell-based assay formats will require a broadly applicable set of tools for human tissue assembly and wholistic analysis. Toward that end, we have recently focused on: i) generating iPS-derived cells that properly represent the diverse phenotypic characteristics of developing or mature human somatic cells; ii) assembling organotypic cell culture systems that are robust and reproducible; iii) translating organotypic cell culture models to microscale systems for high throughput screening; and iv) combining genomic analyses with bioinformatics to gain insights into organotypic model assembly and the pathways influenced by drugs and toxins. This talk will emphasize recent studies in which we have achieved biologically driven assembly of organotypic vascular and neural tissues. These tissues mimic critical aspects of human tissues, and can be used for predictive neurodevelopmental toxicity, and for discovery of vascular disrupting compounds.

#### Scaffold-free Spheroid Assembly to Generate Organoids

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*In vitro* human models hold great promise as more relevant platforms in drug screening than traditional approaches based on animal tests<sup>1</sup>. Therefore, the ability to generate tissue organoids representing basic cellular structures, such as the hepatic acinus in the liver and the nephron in the kidney, is of benefit for drug screening as well as diverse tissue engineering applications. Despite intense research on generating scaffold free platforms, a number of challenges still constrain the engineering of organ models for practical application including: 1) the fidelity of organization of cells and their surrounding microenvironment with microscale resolution in the engineered tissue functional units; 2) sufficient vascularization inside tissue functional units for minimizing necrosis and loss of function; 3) other complex 3-D mimicry aspects. Here, we demonstrate a novel bottom-up approach for scaffold-free spheroid assembly using Faraday waves in a fluidic environment. This technique can generate repetitive and symmetric cellular constructs with high cell-packing density and sustained cell viability. Standing waves established at the air-liquid interface by hydrodynamic instability are used to pack cells without scaffold into 3D architecture at the bottom of the assembly chamber in less than 15 seconds. Using this technique, we demonstrate assembly of fibroblast, human umbilical vein cell spheroids into homogenous/heterogeneous organoids. In addition, we generated long-term viable hepatic constructs and evaluated these constructs specifically for formation of bile canaliculi, hepatic gap junctions and extracellular matrix. We envision this scaffold-free spheroid assembly will find broad applications in engineering tissue models for drug screening and personalized medicine.

#### A Microphysiological 3D Model of the Renal Proximal Tubule to Evaluate Regenerative Therapies

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The human kidney maintains solute, water and acid-base balance through reabsorption, excretes metabolic waste, and produces hormones. The majority of reabsorption occurs in the proximal convoluted tubule (PCT) through epithelial and endothelial cell monolayers. Many diseases including diabetes and hypertension impair PCT function, leading to chronic kidney disease and poten-

tially complete kidney failure. Current renal replacement therapies are dialysis, which does not replace full renal function, or kidney transplantation, which suffers from a lack of donor organs and from infectious complications. Thus, there is an unmet medical need for repairing or rebuilding kidneys which will provide full kidney function to a patient. However, a tissue framework to quantifiably replicate native kidney function *in vitro* is lacking. Here, we mimic the PCT physiological barrier architecture in a microfluidic device, which directly quantifies renal-specific reabsorptive function. The microfluidic device comprises a topographically-patterned membrane that separates a microchannel representing the tubule filtrate from a microvascular microchannel. We co-cultured human primary renal proximal tubule epithelial cells (hRPTEC) and microvascular endothelial cells (hMVEC) to confluence in their respective channels of the microfluidic device. Co-culturing hRPTEC in presence of hMVEC enhanced tissue structure, extended viability, and increased proliferation rates. hRPTEC expressed transport proteins and barrier characteristics of PCT, demonstrated glucose uptake, and dynamically altered transport in response to a sodium transport inhibitor drug. Such devices will enable evaluation and optimization of kidney regenerative therapies, reconstructing human physiology at the cellular and organ level, and provide a scaffold strategy to eventually build or rebuild the human kidney.

#### Microphysiological Platforms for Modeling of Cardiac Disease

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Engineering of human tissues is becoming increasingly successful in recapitulating the environmental milieu of development, regeneration, and disease in many different organs, including the heart. In recent years, robust protocols have been developed for the staged molecular differentiation of human iPS cells into phenotypically stable, but immature cardiomyocytes and endothelial cells. These cells, with the addition of fibroblasts, can be induced to assemble into functional tissue units through the use of "biomimetic" culture systems, which provide the native tissue matrix and biophysical signals leading to tissue maturation. Here we discuss the engineering of millimeter-sized functional human cardiac organoids, and their utility for physiological studies and disease modeling. Specifically, human iPS-derived cardiomyocytes, endothelial cells and fibroblasts were encapsulated in hydrogel and subjected to electromechanical conditioning, using a microfluidic platform capable of on-line measurements of force, contractility and conductive function. Over 4 weeks of cultivation, the cardiac tissues matured, as evidenced by the changes in gene expression, accumulation of cardiac-specific proteins, ultrastructural features representative of mature myocardium, synchronous contractions, force generation, and responses to drugs. Remarkably, engineered tissues exhibited a positive force-frequency relationship, a hallmark of a maturing heart muscle, and consistent with physiological calcium handling. The same methods were then used for the study of inflammation and hypertrophy, two important disease models. Finally, patient-derived iPSCs were used for the modeling of Timothy Syndrome, an inherited cardiomyopathy.

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#### Acoustic Printing of Multilayer Neuronal Networks

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Generation of multilayered neural constructs is motivated by the need to better understand inter cellular interactions and build three-dimensional (3D) microenvironments for disease models<sup>1</sup>. A large body of research is focused on how neuronal and supporting cell types connect and work together to build neuronal networks and

perform computations, and to better understand neurodevelopmental, neurodegenerative and psychiatric disorders. Engineering of neural tissue from human cells will facilitate broad applications in 3D neuroscience ranging from understanding neurons mapping and wiring to drug testing. Current strategies mostly utilize 2D monolayer platforms to reveal the neuron cell interactions, which poorly represent the native spatial organization and functionality. Alternative 3D bioprinting approaches are implement harsh manipulation of fragile neurons and fail to spatially organize the cells<sup>2</sup>. Here, we developed an acoustic bioprinting platform allowing fabrication of 3D multilayer human neural progenitor cells (NPC) in a simple and rapid way. NPCs were driven to nodes of acoustic standing waves by acoustic radiation force<sup>3</sup> and levitated to form multilayers in less than 10 seconds within a fibrin hydrogel. Levitated NPCs are directly differentiated into neurons in 3D microenvironment and formed interlayer neuronal connections. We validate the developed acoustic bioprinting technology that holds great potential to be utilized in neural tissue engineering applications. We also show the differentiation of human embryonic stem cell derived neural progenitor cells in 3D microenvironment. Generating multilayered interconnected neuronal networks can be used for generation of *in-vitro* neuronal tissue models as an alternative for animal studies and brain slices.

### Session: Enabling Tissue Engineering Technologies for Vascular Therapies

**Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM**

#### Nanobiomaterials for Stem Cell-Based Vascular Therapies

**H. Kong:**

Chemical & Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL.

Human bodies are highly vascularized to transport oxygen, nutrients, and hormones to and from cells residing in tissues and organs. Vascular disease resulting from occlusion, leakage, or rupture is the world's leading cause of death. Extensive efforts were made to treat these vascular diseases by recreating new functional microvascular networks using stem cells that can sustainably secrete endogenous proangiogenic factors and also engraft new blood vessels via differentiation. However, there are still needs to improve therapeutic efficacy of cells. These cells are often integrated with various engineering technologies to further improve their performance. To contribute these efforts, we have been developing various implantable nanobiomaterial systems that can elevate the quality of vascular repair and regeneration by integrating material chemistry, 3D printing, and self-assembly. In this talk, I will introduce a few nanobiomaterials assembled as a cell carrier, including (1) nanomaterials devised to deliver stem cells to inflamed blood vessels, and (2) a "Living" microvascular stamp to control the organization of blood vessel during regeneration, and (3) a self-folding hydrogel to modulate vascular drug release with its shape change.

#### Fabrication of Vascular Smooth Muscle Cell Sheets using an Enzymatic Sacrificial Hydrogel System

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Engineered blood vessels must replicate the cellular and structural organization of native tissue in order to be fully functional. Both scaffold- and cell-based approaches have been described, but each suffers important disadvantages. Seeding cells onto polymers or decellularized tissue scaffolds has the potential to achieve the proper structural organization, but generally results in insufficient oxygen delivery and poor survival of the seeded cells. Cell-based approaches using direct injection of cells or pellets can overcome the cell-survival problem, but at the cost of improper localization/geometrical organization. Here, we present a novel sacrificial-

hydrogel system for engineering tissue sheets that allows for both organizational control and improved survival of the component cells. We show the sacrificial hydrogels, tyramine-conjugated carboxymethylcellulose or tyramine-conjugated alginate can easily be enzymatically degraded with cellulase or alginate lyase, allowing intact cell sheets to be harvested without harm to either the cells or their basement membrane. We also show the surfaces of these substrates can be patterned using a gelatin mold made from PDMS (poly-dimethyl-siloxane) mold cured on a photolithographically-treated silicon wafer, and that vascular smooth muscle cells grown into sheets on this patterned substrate show cellular and cytoskeletal (F-actin) orientations consistent with the original pattern. More importantly for tissue engineering purposes, this pattern was retained once cell sheets were transferred to another substrate and when VSMC sheets were stacked to mimic smooth muscle structure in native blood vessels. We conclude this strategy should be applicable for the creation of complex tissues via the use of more intricate topographical patterns.

#### Vascularization of Type I Collagen Extracellular Matrix by Recombinant Bacteriophages Displaying Vascular Endothelial Growth Factor

**J. Yoon<sup>1</sup>, N. Korkmaz<sup>2</sup>, H. Park<sup>3</sup>, S. Han<sup>1</sup>, K. Hwang<sup>4</sup>, J. Shin<sup>5</sup>, S. Cho<sup>6</sup>, C. Nam<sup>7</sup>, S. Chung<sup>1</sup>;**

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Angiogenesis, which is of great importance for implanted tissues in regenerative medicine, has inspired the development of new vascularizing scaffolds conjugated with angiogenic factors. Direct delivery of stimulus-functionalized extracellular matrix has therefore attracted clinical interest for tissue implantation, wound healing, and replacement of damaged tissues. Here, we present a growth factor-integrated natural extracellular matrix (ECM) of type I collagen that induces angiogenesis without requiring any complex or toxic chemical procedures. The developed matrix adapts type I collagen functionalized with recombinant bacteriophages that display vascular endothelial growth factor (VEGF). The functionalized matrix enabled spatial delivery of VEGF into a desired region, and successfully induced *in vitro* and *in vivo* angiogenic responses within the matrix. These findings offer the promise of accelerating recovery after tissue graft or preventing parenchyma necrosis through induction of vascularization.

#### Engineering Vascular Tissue with Biomimetic Scaffolds and Patient-specific Induced Pluripotent Stem Cells

**J. Hu<sup>1</sup>, Y. Wang<sup>2</sup>, J. Jiao<sup>2</sup>, K. Forde<sup>3</sup>, B. Yang<sup>2</sup>, Y. E. Chen<sup>2</sup>, P. X. Ma<sup>1</sup>;**

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Vascular tissue engineering is a promising approach to regenerating replacement vascular tissue for patients. However, obtaining a sufficient number of functional vascular smooth muscle cells (VSMCs) in a clinical setting to construct a patient-specific tissue-engineered blood vessel remains a major challenge. Additionally, it is critical to develop a biomimetic scaffold that accommodates these cells and retains their functional phenotype to regenerate vascular tissue. Previously, we established patient-specific induced pluripotent stem (iPS) cells through viral

transfection of patient vascular fibroblasts. In this study, integration-free iPS cells were established from patient peripheral blood mononuclear cells through nonviral transfection with episomal vectors. The cells were characterized with pluripotency marker expression and potential to differentiate into all three germ layer cells. A highly efficient directed lineage specification method was then developed to induce these established iPS cells into mesoderm-originated VSMCs. The derived VSMC exhibited a functional phenotype characterized by contracting in response to carbachol treatment, up-regulating specific collagen genes under transforming growth factor  $\beta$ 1 treatment, and up-regulating matrix metalloproteinase genes under cytokine stimulation. Subcutaneous implantation of these functional VSMCs seeded on macroporous nanofibrous biomimetic scaffolds with an optimized pore size exhibited formation of functional vascular tissue with robust collagenous matrix deposition inside the scaffolds and the maintenance of differentiated VSMC phenotype. In summary, our study establishes an efficient platform to construct patient cell-derived vascular tissue through combining advanced iPS cell and scaffolding technologies, which enables the development of patient-specific vascular therapies.

#### Network Formation by Microvascular Endothelial Cells within Modular Fibrin Microtissues

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Appropriate cell source and delivery are key enabling technologies for engineered revascularization strategies. Endothelial cells are highly immunogenic, hence there is an important need for autologous sources. Microvascular endothelial cells (MVEC) present an attractive cell source since they can be isolated from autologous tissue samples. Delivery of cells in defined biomaterial microenvironments has the potential to enhance survival, engraftment, and function of transplanted cells. In the present study, we developed modular microtissues designed to direct cell function and allow minimally invasive cell delivery. Microtissues consisted of fibrin microbeads ( $210 \pm 35 \mu\text{m}$  in diameter) formed using a simple water-in-oil emulsification process, with MVEC and fibroblasts (Fb) embedded directly in the matrix. The MVEC:Fb ratio was set at either 1:1 or 1:3 and microbeads were further embedded in a surrounding fibrin gel to assess the extent of vessel network formation. By day 7 there was a significant increase in total number of endothelial networks in microbeads with the higher Fb fraction, however, no significant difference was observed by day 14. Both conditions exhibited high cell viability ( $94 \pm 2\%$ ) and total cell proliferation was comparable between the treatments over the two-week culture period. Vessels formed by embedded MVEC were robust, exhibiting relatively large diameters compared to human umbilical vein endothelial cells (HUVEC) and hierarchical networks resembling capillary beds. These results suggest that MVEC-laden fibrin microbeads have potential for delivery of cells for therapeutic vascularization. The microtissue format allows minimally invasive delivery which, in combination with autologous MVEC, offer a promising therapeutic vascularization strategy.

#### Engineering *In Vitro* Differentiation Microenvironments to Study Heart Valve Calcification

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Valvular calcification is one of the most common cardiac conditions affecting the elderly population. The reasons behind the calcification process and the cellular mechanisms involved are not well understood. Valve interstitial cells (VICs) are predominant cell type of cardiac valves with high heterogeneity in cell phenotype. We hypothesized that through engineering *in vitro* microenvironment, one VIC subtype can activate and differentiate into osteoblast-like

cells. Thus, the activation process and the isolated subgroup cells can be developed into a facile technique to study the cause of heart valve calcification, the effect of aging on the calcification process and the effect of new drugs on calcification. In this study, primary aortic VICs have been isolated by sequential dissection of porcine aortic valves. The digested VICs were cultured on a specially treated substrate which effectively separated the VICs into distinct subgroups with different morphologies. The subgroup with cell cluster morphology stained strongly for Alizarin Red, von Kossa and E-cadherin. The subgroup prevalence increased with passage number under both osteogenic media and basal media, with a more considerable increase under osteogenic media. Clonal experiments confirmed the observation. PCA analysis via synchrotron-sourced microFTIR demonstrated that the cell cluster subgroup had a different spectral signature in comparison to the other subgroup; e.g. in monolayered cells, with increasing passage number expression difference in lipid and phosphate regions concomitantly increased. A preliminary drug assay using atorvastatin in the culture system revealed that the cell cluster's size was reduced and the shape assumed a less dense morphology.

#### Session: Engineering an Artificial Ovary: A New Tool for Fertility Preservation in Cancer Patients

Date and Time: Friday, September 11, 2015, 12:45 PM - 2:00 PM

#### Engineering Artificial Ovarian Tissue using Multi-Arm Poly(ethylene glycol) Hydrogel

J. Kim<sup>1</sup>, A. S. Perez<sup>2</sup>, O. M. Nelson<sup>2</sup>, H. Zhou<sup>2</sup>, A. Shikanov<sup>1</sup>;

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Autotransplantation of ovarian tissue is emerging as a possible solution for restoration of fertility in cancer patients who have suffered from premature ovarian failure and early menopause caused by radiation and chemotherapy. However, the quality of ovarian tissue in terms of follicle count can vary depending on a patient's age, and there is also potential risk for re-introducing malignant cells when implanting an ovarian graft. We investigated poly(ethylene glycol) (PEG) modified with vinyl-sulfone groups that crosslink by Michael-type addition as supportive matrix for immature follicle development *in vivo*. To investigate the combined effect of mechano-biological properties of the PEG hydrogel on follicle survival and growth, the enzymatically isolated from day 6 mice ovaries follicles were encapsulated in the PEG gels modified with RGD and protease sensitive peptide, LGPA and then, these gels were transplanted into the ovarian bursa of ovariectomized mice. An increase in the number of secondary follicles was observed in the graft at 14 days after transplantation and antral follicles by day 30. Elevated follicle-stimulating hormone levels declined to physiological levels by 30 days post-transplantation, showing the graft's capability of restoring endocrine function. Also, cell infiltration from the surrounding ovarian tissue was observed at 14 days after transplantation without causing inflammatory responses. Controlled proteolytic degradation allowed the graft remodeling, which can improve follicle survival and neovascularization and the graft longevity. These results show how using synthetic and tunable PEG hydrogels has the potential to restore both endocrine and reproductive function.

#### Evaluation of a Rigid Fibrin Matrix (F50/T50) for the Survival of Human Isolated Follicles after 2 and 7 Days of Xenografting

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**Objective:** Fibrin is a promising scaffold for the artificial ovary. Recently, a matrix with low fibrinogen and thrombin concentrations (12.5 mg/ml and 1 UI/ml respectively) tested for encapsulation of

murine follicles showed ~30% follicle recovery rate 1 week after autotransplantation. Results for human follicles in this matrix are however deceiving. Human ovarian cortex being more rigid than the mouse ovary, human follicles may require a stiffer matrix for their survival. The study was designed to test this hypothesis.

**Methods:** Fresh human ovarian tissue was enzymatically digested and follicles isolated under stereomicroscope. Around 50 follicles were encapsulated in a more rigid fibrin matrix (50 mg/ml fibrinogen and 50 U/ml thrombin) and grafted to ovarian bursa of 9 nude mice. Mice were euthanized after 10 minutes (control), 2 days and 7 days, and recovered grafts analyzed at histology (every 10  $\mu$ m) for the presence of follicles. Only healthy looking follicles with an oocyte were counted.

**Results:** All grafts (3 for each time period) were recovered. Just 48% of the follicles initially embedded in the matrix were found in the control group (10 min). At day 2 and day 7, the follicle recovery rate was 28% and 23.4% respectively.

**Discussion:** A non-negligible proportion of follicles are lost during clot formation and grafting. However, >50% of remaining follicles survive after 2 and 7 days. This is the first time isolated human follicles are shown to survive in fibrin *in vivo* and confirms the need for a rigid matrix. These results are very encouraging for the construction of the artificial ovary.

### 3D Printed Scaffold Architecture Influences Ovarian Follicle Function

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Approximately 86,000 women and girls under 45 years old will be newly diagnosed with cancer this year. Cancer survivors are more likely to be infertile or to have difficulty getting pregnant, due to iatrogenic effects of treatment. Autotransplantation of cryopreserved ovarian tissue, harvested prior to treatment, has resulted in live human births, but carries the risk of reintroducing cancer cells. To minimize this risk, we bioengineered an artificial ovary consisting of ovarian follicles seeded within a 3D printed scaffold modeled from the ovarian extracellular matrix composition and architecture. Gelatin scaffolds were printed in multiple microporous patterns, which resulted in different pore geometries, or 3D printed follicle niches (3DP-FN), for follicles to reside and grow. Seeded follicles settled in the 3DP-FNs, and somatic cells along the follicle periphery adhered to the scaffold. We discovered that follicle survival was dependent on the architecture of 3DP-FNs. An essential feature of ideal 3DP-FNs is promotion of two or more follicle adhesion points within the niche. These architectures outperformed those that supported only one follicle adhesion point where oocyte survival was significantly decreased. In the optimized 3DP-FNs, follicles not only survived but also were functional. Follicles secreted estradiol, matured in response to LH, and oocytes resumed meiosis. Transplant studies were also performed where mouse ovaries were replaced with 3DP ovaries to evaluate their potential for *in vivo* maturation and ovulation. These are important steps toward creating a safer artificial ovary that will provide fertility to young women who have survived cancer.

### In Vitro Maturation of Primordial Follicles-Study of the Biomaterial for the Task

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The options for women fertility preservation are limited, especially for young women facing chemo and/or radiotherapy. Gaining

knowledge and the technology for guiding primordial follicles through the folliculogenesis path will dramatically improve the chances of preserving fertility. While such protocols for growing primary and secondary follicles, *in vitro*, were developed, the efforts for growing isolated primordial follicles from human were not as successful. Herein, we investigated the applicability of the macroporous alginate scaffold decorated with two cell adhesion peptides - RGD and YIGSR- as a platform for *in vitro* maturation of primordial follicles. Primordial follicles ( $13.67 \pm 2.16 \mu$ m) and ovarian cells extracted from the cortex of prepubertal porcine ovaries were cultivated within either: 1) Pristine (non-modified), 2) RGD-, 3) YIGSR-, or 4) RGD/YIGSR-attached alginate scaffolds. By scanning electron microscopy, the different macroporous scaffolds had a similar mean pore size of  $64 \pm 26 \mu$ m. The Young's modulus of the peptide-attached scaffolds was twice the value of the pristine alginate. Follicular maturation and follicle size distribution, evaluated overtime under a light microscope, showed an increase in follicle size in all scaffold groups; a finding that was correlated to anti-Müllerian hormone (AMH) secretion levels. At day 28 of cultivation, developing follicles, > 120  $\mu$ m in diameter, were more frequent in the RGD/YIGSR attached scaffolds compared to the pristine scaffolds. In summary, our study shows that given the right environment primordial follicles can be developed through the folliculogenesis path.

### The Effect of Substrate Elasticity on the Function of Ovarian Follicles

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In the ovary, follicular cells are exposed to a large array of stimuli that participate in determining their phenotype and function. This *in vivo* microenvironment is lost when ovarian follicular cells are cultured in standard cell culture, making it challenging to maintain their cellular function *in vitro*. We examined the effect of the substrate elasticity on ovarian follicular cell phenotype and function, including hormone production and oocyte maturation. In this study, rat ovarian follicular cells were placed in type I collagen hydrogels with different substrate elasticity that was varied from 0.87 to 64.05 kPa by controlling the concentrations of collagen. The results showed that extended survival, follicle formation and growth, hormone production, and oocyte maturation were achieved by altering the substrate elasticity. Our findings point to the importance of the substrate elasticity on the ovarian follicular cell function.

### Session: Engineering Biointerfaces to Control Cellular Behavior

**Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM**

### Engineering Synergistic Microenvironment for Growth Factor Presentation

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Most cells assemble rich protein matrices via an integrin-dependent mechanism that incorporates e.g. fibronectin (FN) molecules into matrix fibrils. The process involves integrin binding and activation of cell contractility to extend FN and expose cryptic domains that promote protein-protein interactions. We have shown that this process can occur by simple adsorption of individual protein molecules onto particular surface chemistries - in absence of cells. FN - material interactions would induce exposure of self-assembly sites to drive FN assembly, a process that we have named material-driven fibronectin fibrillogenesis.<sup>1</sup> This FN matrix assembled at the material interface involves conformational changes of FN upon adsorption and enhanced FN-FN contacts on the material surface.

The resulting material-driven FN matrix assembled at the material interface consists of a protein network with enhanced biological activity: it supports cell adhesion, matrix remodelling, and trigger cell differentiation. Moreover, it provides a robust platform to engineer advanced microenvironments in combination with growth factors to tune stem cell differentiation and promote tissue repair. We have engineered these systems as robust and translational platforms to present BMP-2 and VEGF and promote osteogenesis and vascularisation in pre-clinical models.

#### Particle Shape as a Biointerface Parameter to Control the Behavior of Immune Cells

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Interactions between biomaterial surfaces and cells are critical for many phenomena in regenerative medicine and immunoengineering. These interactions include ligand-receptor binding, extracellular signaling, and intracellular particle uptake. We investigated particle shape and found that it can be a key parameter in these interactions. Utilizing an automated multidimensional film-stretching device, we fabricated anisotropic particles in a range of shapes including prolate and oblate ellipsoids of varying aspect ratio on both the micro- and nanometer length scales. The strain rate during film stretching does not affect the final particle shape and different polymers including poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone), and blended PLGA/poly(beta-amino ester) can be similarly stretched into anisotropic shapes. Biomolecules can be encapsulated within these particles during stretching and the anisotropic particles resist cellular uptake by macrophages compared to spherical particles. When immunostimulatory biomimetic proteins are conjugated to the surface of biodegradable PLGA microparticles, these particles can function as artificial antigen presenting cells (aAPCs) and stimulate T-cell proliferation *in vitro* and *in vivo*. Anisotropic ellipsoidal microparticle-based aAPCs demonstrate superior T-cell proliferation of up to 20-fold compared to analogous spherical PLGA aAPCs. The ellipsoidal aAPC surface as compared to the spherical aAPC surface, as a biointerface, increases the number of particle-T-cell conjugates formed and the area of contact between these surfaces. PLGA nanoparticle aAPCs show similar trends with anisotropic ellipsoidal aAPCs superior at stimulating T-cells as compared to spherical aAPCs. Ellipsoidal nanoscale aAPCs had extended circulation time compared to spherical nanoscale aAPCs and led to greater antigen specific CD8 + T-cell expansion *in vivo*.

#### The Effect of Substrate Stiffness and Surface Topography on the Fate of Human Mesenchymal Stem Cell Chondrogenic Differentiation and Cartilage Phenotype Formation

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*In vivo*, cells reside in a complex microenvironment, where they interact and respond to multiple signals including that between cells and their matrix, cell-cell interactions, and spatio-temporal exposure to bioactive factors. Stem cells differentiation is affected by biochemical cues of the extracellular matrix, as well as physical cues in the form of matrix mechanical properties and topography. In this study, the combine effect of substratum topography and mechanical stiffness in directing bone marrow mesenchymal stem cells (MSC) chondrogenesis was investigated. Three polyesters, polycaprolactone (PCL), polylactide (PLA) and polyglactide (PGA); of varying mechanical stiffness were thermally imprinted to create nano-grill or pillar patterns of the same dimension. The cell morphology adopted on the nano-topographic surfaces were accounted by F-actin distribution, and correlated to the cell proliferation and chondrogenic differentiation outcomes.

Results show that chondrogenic differentiation was highly sensitive to both the substratum stiffness and topographical cues, affecting cell morphology and aggregation, and influenced the phenotype of the derived cartilage. Hyaline cartilage with middle/deep zone cartilage characteristic was derived on softer nano-pillar surface, and fibro-superficial zone cartilage on softer nano-grill surface. Nano-pillar of stiff material induced a mixed hyaline/fibro/hypertrophic cartilage. Our data demonstrate that cellular morphology can be manipulated with the combined cues of nano-topography and material stiffness, and employed to regulate the differentiation of MSCs towards appropriate cartilage phenotype. Such knowledge will be crucial in designing scaffolds for stem cell-based cartilage tissue engineering, especially for the generation of zonally-defined cartilage.

#### Engineering Stem Cell-Material Interaction

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Material surface is an important and versatile avenue for manipulation of cell-material interaction which eventually leads to modulation of cellular behavior. In our laboratory, we work on surface patterning to modulate cellular morphology with the hypothesis that cellular morphology has an intimate relationship with the cellular behavior. In this project, we study the differentiation of human mesenchymal stem cells into the myogenic lineage using macroscopic and microscopic manipulation of the interface. At the macroscopic level, we used contact transfer printing to print lanes of fibronectin to coerce cells to elongate and grow in the direction. The cells adopted not only elongated macroscopic shapes but the focal adhesions (FA) were also elongated and were at the apical ends of the cells. These cells exhibited upregulation of cardiomyogenic markers both at the mRNA and functional protein levels. As the FAs of the patterned and non-patterned(control) cells were significantly different, next, we took to patterning these FAs (microscopic level), as we strongly believe these FAs would be responsible for the mechanotransduction cascades. We designed different patterns to simulate elongated, dense and random FAs. We found that the elongated FAs, regardless of cell shape, is the determining factor for the differentiation. As we investigate further using dStorm, we observed a very interesting clustering and spatial distribution of the integrins in the patterned cells which are entirely different from the control. We are now in the process of investigating how this natural clustering and spatial distribution could trigger differentiation.

#### Spatial Regulation of Valve Interstitial Cell Phenotype within Three-Dimensional Micropatterned Hydrogels

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The mature heart valve leaflets are made up of highly organized and aligned extracellular matrix (ECM) and valve interstitial cells (VIC) with quiescent fibroblastic phenotype. Calcific aortic valve disease (CAVD) is associated with disruption of aligned valve ECM structure, maladaptive ECM remodeling, and activated VIC. However, very little is known about how the aligned topography of the 3D microenvironment affects VIC phenotype under physiological and pathological conditions. The goal of these experiments was to characterize the effect of cell alignment to 3D micropatterns on human aortic VIC (HAVIC) phenotypes, as well as the ability of conjugated basic fibroblastic growth factor (bFGF) to maintain fibroblastic HAVIC phenotypes in the presence of these micropatterns in normal and pro-osteogenic conditions. We generated micropatterned hybrid hydrogels consisting of methacrylated hyaluronic acid (Me-HA) and methacrylated gelatin (Me-Gel) using custom photomask guided local photocrosslinking. HAVIC isolated from both healthy and calcified valve leaflets were encapsulated and micropatterned with or without conjugated bFGF in both normal and osteogenic media. There was no difference in cell viability and circularity

between micropatterned and nonpatterned HAVIC, but aligned micropatterned promoted VIC alignment. HAVIC from calcified valves showed greater myofibroblastic and osteogenic phenotype than healthy HAVIC. Aligned HAVIC from either healthy or calcified valve showed greater ALP, Runx2 and osteocalcin expression within 3D micropatterned hydrogels compared with nonaligned cells. Immobilized bFGF upregulated vimentin expression and promoted the fibroblastic differentiation of HAVIC. These results suggest that aligned HAVIC are more likely to undergo osteogenic differentiation, but fibroblastic differentiation is encouraged through bFGF.

### Session: Engineering the Soft-Hard Tissue Interface; EU-NZ Consortium SkelGEN

**Date and Time: Friday, September 11, 2015, 12:45 PM - 2:00 PM**

#### Bovine Lactoferrin Promotes Bone Healing in a Rat Critical-Sized Calvarial Defect Model

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**Introduction:** Lactoferrin is a multifunctional glycoprotein with therapeutic potential in bone tissue engineering. *In vitro*, lactoferrin enhances osteoblast proliferation and differentiation, is anti-apoptotic and inhibits osteoclastogenesis. Furthermore, lactoferrin is antimicrobial and modulates the immune response. The aim of this study was to assess the effect of lactoferrin on bone regeneration *in vivo*.

**Methods:** Five-millimetre critical-sized defects were created over the right parietal bone in 60 Sprague-Dawley rats. These were randomized into 3 groups: group 1 = empty defects; group 2 = defects filled with collagen gels (3 mg/mL); and group 3 = defects filled with collagen gels containing bovine lactoferrin (10 µg/gel). These gels induce rapid release of lactoferrin within 6 hrs of seeding, *in vitro*. The rats were sacrificed at 4 or 12 weeks, the calvaria excised and imaged by micro-CT (Skyscan 1172) with a 12 µm voxel size. Cylindrical VOI were created. Bone regeneration, tissue mineral density (TMD) and porosity were measured.

**Results:** The percentage of bone regeneration (compared to intact, contralateral parietal bone) for groups 1, 2 and 3 were 42.7 ± 4.2%, 35.9 ± 5.9% and 62.6 ± 2.5%, respectively, at 4 weeks (p = 0.0019); and 41.3 ± 5.2%, 45.8 ± 4.8% and 74.6 ± 4.3%, respectively, at 12 weeks (p < 0.0001). There was no difference in TMD between groups, while porosity was lower in group 2 compared to group 3 at 4 weeks (p < 0.05).

**Conclusions:** This study demonstrated that a burst release of low dose lactoferrin significantly increased bone regeneration in a rat calvarial defect model. The profound effect of lactoferrin on bone regeneration has huge therapeutic potential.

**Acknowledgments:** Partial funding by EU FP7-'SkelGEN' under grant agreement n° [318553]

#### Calcium Phosphates-based Biomaterials with Sr- and Zn-Dopants for Osteochondral Tissue Engineering

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The repair and regeneration of osteochondral (OC) defects has been increasing owing to the high number of trauma related injuries or osteoarthritis. Although current clinical options are effective for the treatment of OC defects, advanced therapeutic options that simultaneously preserve the native tissue structure and address a proper regeneration of bone and cartilage defects are therefore fundamental, namely in respect to neurovascular regeneration in large defects. The main purpose for OC tissue engineering is to recreate a biomimetic and monolithic scaffold consisting of a cartilaginous layer and an

underlying osseous layer, for regeneration of cartilage and bone, respectively, involving different combinations of materials, morphologies and properties in both parts of the scaffolds[1]. This study aims to develop scaffolds composed of biopolymers (silk fibroin) and calcium phosphates incorporating different ions (e.g. Sr, Zn), for the simultaneous regeneration of cartilage and bone. These scaffolds present great bioresorbability and osteointegration, and high mechanical strength[2]. In particular, Sr and Zn plays vital roles in the formation, growth, and repair of bone, thus it can promote osteogenesis and angiogenesis[3]. Besides, these elements can lend controlled degradation and increase the mechanical strength of the new materials. Porous composite scaffolds with distinct cartilage and bone sides were produced through solvent casting and particulate-leaching technique, followed by freeze-drying. The scaffolds presented macroporosity highly interconnected and microporosity with sizes around 500 µm, and 1–10 µm, respectively. Current studies are on-going to evaluate the scaffolds *in vitro* degradation and ions release profiles, and mechanical properties.

#### The Effect of Oxygen Inhibition in Biofabrication of Photocrosslinked Hydrogels

K. S. Lim, G. C. Brown, N. V. Mekhileri, B. S. Schon, G. J. Hooper, T. B. Woodfield;

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Biofabrication of cell-laden hydrogels (or bioinks) by combining 3D-bioprinting techniques with photo-initiated radical crosslinking offers the ability to develop engineered constructs with high shape fidelity, as well as supporting cell growth, and are hence widely explored [1]. Often this crosslinking process is performed in the presence of oxygen to maintain cell survival and viability. However, oxygen is known to quench the radicals causing incomplete crosslinking, which in turn leads to physical deformation of the scaffold [1]. Therefore, the aim of this study was to minimise the effect of oxygen inhibition in photo-curable hydrogel constructs. 3D printed hydrogel constructs (20 × 20 × 3 mm) consisting of 10 wt% gelatin-methacrylate + 0.8 wt% collagen-I + 0.05 wt% Iracure2959 were fabricated using a BioScaffolder (Syseng, Germany), then irradiated with UV light (330–450 nm) in the presence of oxygen for 15 minutes. UV light intensity was varied from 3–50 mW/cm<sup>2</sup>. Fiber diameter within the constructs was measured before and after equilibrium swelling. Hydrogel constructs irradiated with UV light intensity commonly adopted in literature (3 mW/cm<sup>2</sup>) [1], had poor structural integrity, with constructs degrading completely in PBS within 15 mins. Increasing light intensity to 30 mW/cm<sup>2</sup> allowed successful fabrication of mechanically stable constructs, but had significant reduction in fiber thickness (~70%) after equilibrium swelling. Further increase in light intensity to 50 mW/cm<sup>2</sup> successfully reduced the fiber deformation to 30%, yielding constructs with high fidelity and discrete porosity throughout. Current work is investigating biofabrication of cell-laden hydrogels and evaluating cellular behavior (survival, proliferation and extracellular matrix formation) for osteochondral tissue repair.

**References:** 1. Malda *et al.* Advanced Materials.2013,25(36).

**Acknowledgments:** SkelGEN(318553).

#### Bridging the Gap - Harnessing Skeletal Cells, Biomimetic Materials and Environmental Niches for Bone Regeneration

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Medical advances have led to a welcome increase in world population demographics. However, increased aging populations pose new challenges and emphasize the need for innovative approaches to augment and repair tissue lost through trauma or disease. Key in addressing these issues are the development of relevant *in vitro*, *ex vivo* and *in vivo* models to determine factors involved in the maintenance

and repair of skeletal tissue and development of a functional strategy for repararised tissues for bone is critical in any reparative approach.

We have protocols for the isolation, expansion and translational application of skeletal stem cell populations with cues from developmental biology informed by *in vitro* and *ex vivo* models as well as, nanotopography and nanoscale architecture and biomimetic niche development informing our skeletal tissue engineering approaches. Furthermore, we have developed *ex vivo* approaches to skeletal tissue formation evaluation and analysis and, central to clinical application, large animal *in vivo* translational studies to examine the efficacy of skeletal stem and cell populations in innovative scaffold compositions for orthopaedics. The talk will also highlight current clinical translational studies to examine the efficacy of skeletal populations for orthopaedic application.

Advances in our understanding of skeletal stem cells and their role in bone development and repair, offer the potential to open new frontiers across the hard tissue interface and offer exciting opportunities to improve the quality of life of many.

#### Development of Bone-Like Tissue Graft *in Vitro* using Multilayer Cell Sheet Technology

M. Kiamehr<sup>1</sup>, T. Yoshida<sup>2</sup>, H. Takahashi<sup>2</sup>, K. Washio<sup>2</sup>, T. Iwata<sup>2</sup>, M. Yamato<sup>2</sup>, T. Okano<sup>2</sup>, X. Yang<sup>1</sup>;

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The use of stem/osteoprogenitor cells, scaffolds and growth factors has been the gold standard in bone tissue engineering. However, many disadvantages have limited their efficacy in clinical therapy. This study aims to use multi-layered cell sheet (MLCS) technology and human dental pulp stem cells (DPSCs) to regenerate bone-like tissue grafts *in vitro*.

Human DPSCs were cultured on temperature-responsive culture dishes and harvested as an intact single cell sheet by temperature reduction and MLCSs were assembled from monolayer cell sheets. Then, the MLCSs were continuously cultured in osteogenic medium for two weeks to allow more extracellular matrix production and mineralisation. The formation of mineralised 3D MLCSs, a bone-like tissue constructs, was confirmed by histological, immunohistochemical assessment and imaging analysis.

After two weeks of osteogenic culture, hematoxylin/eosin staining and SEM confirmed the formation of MLCSs 3D structure *in vitro*. Live/dead fluorescent labelling and confocal microscope showed that the majority of hDPSCs stayed viable within the construct. Sirius Red staining and birefringence microscope results indicated the enhanced collagen matrix deposition between the cell layers. Formation of the bone-like tissue within the MLCSs was confirmed by positive staining for human specific type I collagen and Von Kossa.

In conclusion, this study indicated that the additional two weeks culture of HDPSCs MLCS in osteogenic medium is essential for enhancing type I collagen production and extracellular matrix mineralisation to form bone-like tissues as 'bone grafts' for clinical bone augmentation.

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#### Session: Engineering Tissue Interfaces

**Date and Time: Thursday, September 10, 2015, 3:00 PM - 4:30 PM**

#### Scaffold Design Strategies for Integrative Tendon Repair

H. H. Lu;

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Musculoskeletal motion is facilitated by synchronized interactions between multiple tissue types and the seamless integration of bone with soft tissues such as tendons, ligaments or cartilage. Many of these soft tissues transit into bone through a multi-region fibrocartilaginous interface, which serves to minimize the formation of stress concentrations while enabling load transfer between soft and

hard tissues. Given its functional significance, re-establishment of the soft tissue-to-bone interface is thus critical for promoting the integrative repair of biological and synthetic soft tissue grafts. To address the challenge of biological graft fixation, our approach centers on interface tissue engineering, guided by the working hypothesis that this multi-tissue transition may be regenerated by controlled culture of interface-relevant cell populations on a stratified scaffold pre-designed with a biomimetic gradient of structural and functional properties. Focusing on the rotator cuff tendon-to-bone insertion site, and inspired by current understandings of the native interface structure-function relationship, the evaluation of biomimetic, nanofiber-based stratified scaffolds for tendon-to-bone interface regeneration in small and large animal models will be presented. Through these optimization studies, we seek to elucidate key interface scaffold design criteria which can also be relevant for the regeneration of other soft tissue-to-bone interfaces.

#### An Aligned Cardiac Construct from Fibrin-Adhered Microvessel and Cardiomyocyte Patches

R. T. Tranquillo, J. A. Schaefer;

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Cardiovascular disease is the leading cause of death worldwide, and many of these deaths result from myocardial infarctions. Because there is a severe shortage of donor hearts to replace these damaged tissues, it is desirable to engineer replacement cardiac tissue. Unfortunately, current engineered cardiac tissues are limited in size by the diffusion of nutrients due to a lack of vascularization. Our previous studies have demonstrated the ability to generate microvessel-dense patches using human blood outgrowth endothelial cells (BOECs) and human brain pericyte support cells (PCs), which were able to inosculate with host vasculature and become perfused in a rat infarct model (unpublished). We have also demonstrated the beneficial effect of implanting aligned cardiomyocyte patches from neonatal rat CM and iPS-CM. These patches were formed by cell entrapment in mechanically-constrained fibrin gels. Towards the generation of a vascularized heart patch, we have adhered an aligned microvessel patch to an aligned iPS-CM patch by incubating each in a fibrinogen solution, adding a thrombin solution to one surface of the cardiomyocyte patch, and placing the microvessel patch on top. This results in a mechanically stable bi-layered patch. *In vitro* characterization revealed that these bi-layered patches generate up to 8x more force compared to control iPS-CM patches and maintained the microvessels, with pericytes from the microvessel layer migrated through the interface into the iPS-CM layer within one week.

#### Tissue Engineering the Meniscus to Bone Interface

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<sup>1</sup>Cornell University, Ithaca, NY, <sup>2</sup>Biomedical Engineering, Cornell University, Ithaca, NY.

The complex structure of the meniscus to bone interface, or entheses, is challenging to reproduce *in vitro*. Previous studies have attempted to recreate the entheses of tendon or ligament with limited success, with no attempts to tissue engineer the meniscal entheses. The goal of this study was to establish an experimental test platform for meniscal entheses and determine the effect of clamping on the organization of collagen fibers at the soft tissue to bone interface.

Fibrochondrocytes were homogeneously mixed with a 20 mg/mL collagen gel at 25 million cells/mL and then injection molded into tygon tubing preloaded with decellularized bone plugs at each end. Samples were clamped or unclamped at the bone plugs and cultured for 0-, 2-, and 4-weeks. After culture, constructs were analyzed for contraction, mechanical properties, and fiber organization.

Clamping reduced collagen contraction in construct length, width, and area. Tensile strength was greater in clamped samples and increased with time, however, samples failed primarily at the interface after 4 weeks. Collagen fibers were axially aligned in the collagenous



region and continued to penetrate into the bone plugs at the interface, showing integration of the collagen into bone.

This study showed that collagen and bone can be integrated into a test platform for enthesis tissue engineering. Furthermore, collagen alignment in the axial direction was increased by clamping. Our test platform is amenable to targeted experiments investigating mineralization gradients, collagen fiber alignment, cell population phenotype, and media conditioning with experimental impact on enthesis studies for meniscus, tendon, and ligament.

### **Injury and Repair of Tendons and their Insertion to Bone**

**J. Temenoff;**

Biomedical Engineering, Georgia Tech/Emory University, Atlanta, GA.

Tendon overuse injuries are a significant source of pain and disability world-wide. These injuries represent a complex pathology arising from multiple intrinsic factors as well as external (mechanical) factors and, therefore, the exact etiology remains unknown. Our laboratory has been studying the progression of this disease using a rodent model of rotator cuff tendon overuse. Recent histological results suggest that more tissue damage occurs near the tendon insertion to bone, and that collagen-cleaving protease (cathepsin) activity is also higher in this region, thus providing a potential therapeutic target. In response, we have developed injectable carriers for cells and therapeutic proteins that may prevent further degeneration or promote tissue healing after overuse injuries. This presentation will summarize both the structure and unique function of the tendon insertion site, as well as potential interventions that would address injury to this area.

**Acknowledgments:** NIH R01 AR063692.

### **Spatially Organized Differentiation of High-Density Mesenchymal Stem Cell Constructs for Osteochondral Tissues Engineering**

**E. Alsberg, L. D. Solorio;**

Biomedical Engineering, Case Western Reserve University, Cleveland, OH.

Bone marrow-derived mesenchymal stem cells (hMSC), which are capable of giving rise to both bone and cartilage during development, can generate all the cells and tissues of the osteochondral interface. This talk will focus on a scaffold-free hMSC system used to engineer biphasic osteochondral constructs incorporated with two different types of bioactive microparticles enabling spatially organized cell differentiation. Using incorporated bioactive microparticles to drive the formation of complex, stem cell-derived osteochondral tissues could enable earlier *in vivo* implantation, and potentially improve the rate and quality of osteochondral defect repair.

**Acknowledgments:** This work was supported by the National Institutes of Health (R01AR063194; T32AR007505) and the AO Foundation.

### **Tissue Engineering the Tendon Enthesis: Lessons from Development**

**A. Schwartz, S. Thomopoulos;**

Orthopaedic Surgery, Washington University, Saint Louis, MO.

The attachment of dissimilar materials is a major challenge because of the high levels of localized stress that develop at such interfaces. An effective biologic solution to this problem can be seen at the attachment of tendon to bone. The enthesis, a transitional tissue that exists between healthy tendon and bone, is not recreated during healing, so surgical reattachment of these two dissimilar biologic materials often fails. To define the design criteria and roadmap for tissue engineering this attachment system, we must first understand the mechanisms by which the healthy attachment transfers load between tendon and bone, and then define how cells build a functional

attachment during development. Our work has examined the biophysical and biochemical events that drive enthesis formation and mineralization. Muscle loading is necessary to drive mineralization and growth of the enthesis; paralysis of the rotator cuff muscles at birth led to severe defects in enthesis development in mice. Furthermore, we have identified a unique population of hedgehog-positive cells that are necessary for defining and mineralizing the enthesis. These cells are active in regenerative enthesis healing in young mice but not in scar-mediated enthesis healing of adult mice. Understanding how these cells build a functional attachment between tendon and bone provides a roadmap for development of tissue engineered replacements. To apply these lessons from development, we created nanofiber scaffolds that mimicked the structure and composition of the natural enthesis, and studied cell responses to those cues. These cell-based biomaterials are currently being optimized for *in vivo* use.

### **Session: Evolution of Regenerative Medicine to Restore Urogenital Tract Function**

**Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM**

#### **Nine Year Follow Up on Tissue Engineered Buccal Mucosa used for Urethroplasty - What have we Learnt?**

**S. MacNeil<sup>1</sup>, A. Mangera<sup>2</sup>, A. J. Bullock<sup>1</sup>, J. Patterson<sup>3</sup>, N. Osman<sup>1</sup>, C. Chapple<sup>4</sup>;**

<sup>1</sup>Dept of Materials Science & Engineering, University of Sheffield, Sheffield, UNITED KINGDOM, <sup>2</sup>Department of Reconstructive Urology, Royal Hallamshire Hospital, Sheffield, UNITED KINGDOM, <sup>3</sup>Department of Reconstructive Urology, Royal Hallamshire Hospital, Sheffield, UNITED KINGDOM, <sup>4</sup>Dept of Reconstructive Urology, Royal Hallamshire Hospital, Sheffield, UNITED KINGDOM.

When there is extensive disease of the urethra then the use of autologous buccal mucosa has proved successful. However it's not always possible to obtain enough tissue to replace all of the urethra and harvesting extensive buccal mucosa can cause morbidity at the site of excision. Accordingly our laboratory was the first to explore the use of tissue engineered buccal mucosa as a graft material for tissue substitution. We treated 5 patients with complex long anterior urethral structures and reported a 3 year follow up in 2008 and more recently a 9 year follow up in 2014.

Our aim is to reflect on what has gone well and to highlight for discussion what remains a challenge for the future. Production of autologous tissue engineered buccal mucosa was straightforward, the material handled well surgically and initial neovascularisation was excellent. Where problems occurred (in 2 out of the 5 patients) they occurred at 8 and 9 months respectively with recurrent fibrosis. In one patient this required replacement of the TEBM with autologous tissue. In the other patient this was partial. Of the four patients with surviving TEBM at 9 years, the urethra appeared normal on examination and all can void urine.

In conclusion TEBM has been moderately successful for this group of patients but the major challenge is in the recurrent fibrosis occurring many months post successful take of the grafts. Our results argue for long term follow up of patients and investigations of the nature of this fibrosis.

#### **Restoration of Penile Erectile Function using Stem Cells in a Rat Model of Neurovascular Erectile Dysfunction**

**H. Shi<sup>1</sup>, W. Li<sup>1</sup>, Y. Zhang<sup>1</sup>, C. Clouse<sup>1</sup>, S. Sink<sup>1</sup>, S. George<sup>1</sup>, T. Lue<sup>2</sup>, J. Yoo<sup>2</sup>, A. Atala<sup>1</sup>, J. Jackson<sup>1</sup>;**

<sup>1</sup>Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston Salem, NC, <sup>2</sup>University of California San Francisco, Wake Forest School of Medicine, San Francisco, CA.

Erectile dysfunction due to traumatic pelvic injury is difficult to treat. One treatment method that is gaining increased attention is stem cell therapy. The goal of this study was to determine whether

stem cells injected into the penis could improve and restore erectile function in a rat model of neurovascular erectile dysfunction (NVED). Three different cell types were used in this study; human endothelial cells (ECs), adipose derived stem cells (ASCs) and amniotic fluid derived stem cells (AFSCs). Each cell type was characterized using flow cytometry assay using cell specific markers; ECs expressed CD31 and CD34, while human AFSCs and ADSCs expressed CD73, CD90, CD105, CD146 but did not express CD31 and C34. A nude rat model of NVED was established by crush injury of the bilateral cavernous nerves and ligation of the bilateral internal pudendal bundle. Animals were divided into five groups (G): G1 was treated with ECs, G2 with ASCs, G3 with AFSCs, G4 with normal saline and G5 was an age match group. Approximately 2.5 million cells in 0.2 ml serum free medium per animal were injected intracavernously into penile tissue. At 12 weeks post-injection erectile function analysis showed that the ratio of intracavernous pressure and mean artery pressure was increased in the cell therapy groups. Among the cell treated groups, the endothelial cell injection group showed the most improvement in intracavernous pressure. These results show that cell-based therapy may be an effective treatment modality for patients with trauma induced ED.

### Cell-based Therapies for Urogenital Tract Disorders

**J. J. Yoo;**

Wake Forest Institute for Regenerative Medicine,  
Winston-Salem, NC.

The genitourinary system is constantly exposed to different adverse conditions, which can lead to structural and functional damage. Over the past decade, the field of tissue engineering and regenerative medicine has made a significant progress in developing cell-based therapies for genitourinary tissue applications. One approach to augment tissue function is through cell therapy. A wide variety of cell sources, ranging from somatic to stem cells, have been investigated. Another method commonly employed is tissue engineering to restore damaged tissues. This approach uses cells and biomaterials to build tissue constructs that results in functional tissues when introduced *in vivo*.

The genitourinary tract system is one of the more studied areas in the field, as many technologies have already been translated into patients. It is becoming increasingly clear that the field has the potential to treat various urological disorders that are known to be a challenge with conventional treatment methods. Clinical experiences with regenerative therapies for genitourinary disorders, such as tissue engineered bladder, urethra and vagina, have demonstrated that successfully developed technologies can profoundly affect and improve the lives of patients who do not have many options. The progress of tissue regeneration in the genitourinary system will be discussed.

### Tissue Engineering a Cell-based Therapy for Pelvic Organ Prolapse (POP) using Autologous Human Endometrial MSC and Novel Scaffolds

**C. E. Gargett**<sup>1,2</sup>, S. Edwards<sup>3</sup>, A. Rosamilia<sup>2</sup>, J. Werkmeister<sup>3,2</sup>;

<sup>1</sup>The Ritchie Centre, MIMR-PHI Institute of Medical Research, Clayton, AUSTRALIA, <sup>2</sup>Obstetrics and Gynaecology, Monash University, Clayton, AUSTRALIA, <sup>3</sup>Manufacturing Flagship, CSIRO, Clayton, AUSTRALIA.

POP is the herniation of the pelvic organs into the vagina due to childbirth injury, causing incontinence and sexual dysfunction. 19% of women are treated for POP by surgery with or without polypropylene mesh. Surgical failure and complication rates approach 30%. We propose to use autologous MSC, purified from the endometrium (eMSC) delivered in a composite mesh scaffold as a new approach for treating POP. eMSC are isolated from biopsies obtained without anaesthesia from pre-menopausal and estrogen-treated post-menopausal women by magnetic bead (W5C5/SUSD2+) cell sorting. We are developing protocols for eMSC culture expansion under cGMP conditions.

We fabricated a polyamide and gelatin (PA+G) composite scaffold for delivering human DiO-labelled eMSC into a fascial defect in a nude rat model. We showed that eMSC promoted early neo-

vascularisation and a rapid influx of M1 macrophages which changed to a M2 wound healing phenotype. Longer term, fewer macrophages and minimal fibrosis surrounded the PA filaments compared to non-cell-seeded scaffolds. The eMSC/PA+G tissue complexes were less stiff and more distensible compared with PA+G alone, indicating improved biocompatibility.

We are developing an autologous, large animal, pre-clinical model of vaginal surgery using sheep. We have developed an objective clinical measurement that shows a subset of parous ewes have similar vaginal tissue laxity as women, suggesting subclinical POP. Pilot data showed vaginally implanted lentiviral-mCherry-labelled-autologous eMSC on PA+G constructs survived 21 days. Our tissue engineering approach using autologous eMSC delivered on polyamide/gelatin scaffolds may be an alternative approach for treating POP with potential to improve surgical outcomes.

### Off-the Shelf Collagen-fibrin based Scaffolds for Bladder Augmentation

**H. M. Larsson**<sup>1,2</sup>, E. Vardar<sup>2</sup>, K. Pinnagoda<sup>1</sup>, E. Balet<sup>2</sup>, J. A. Hubbell<sup>2,3</sup>, P. Frey<sup>2</sup>;

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In the management of congenital and acquired bladder pathologies augmentation of the bladder is commonly performed using vascularized digestive tract patches. This can be linked to severe complications, such as metabolic disturbances and even cancer. Therefore tissue engineering and laminated collagen-fibrin hybrid grafts is a promising alternative. We compared the mechanical, functional and cellular behavior of different collagen-fibrin based scaffolds used for augmentation in 5 groups of 4 nude rats each. In the first acellular scaffolds were applied. In the second human smooth muscle cells (hSMC) were added within the scaffold. In the third group hSMC's have been cultured within the scaffold for 4 weeks, followed by decellularization and DNA removal. In the fourth a low dose of IGF-1 was covalently bound to fibrin and in the fifth group a high dose of IGF-1 was used. After a transverse hemi-cystectomy the bladder was augmented with the respective collagen-fibrin based patches. Four weeks post hemi-cystectomy all animals showed the same bladder volumes than before surgery, voiding capacity was normal and host cells had populated all the grafts. The cellular, the decellularized and the high dose IGF-1 scaffolds showed faster regeneration as compared to the acellular and low dose IGF-1 scaffolds. Apart from the cellular scaffold all the remaining can be manufactured as an off-the-shelf products and may become beneficial in clinical use.

### Session: Fibrin and Fibroin – What “O” Difference

**Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM**

#### Silk: From Textiles to Tissue Regeneration

**A. Motta, Sr., C. Migliari, Sr.;**

Industrial Engineering, University of Trento, Trento, ITALY.

Silks, and in particular silks from silkworms cocoons, have been used for many years as suture filaments, more recently acquiring novel attention for other applications in medicine and namely in tissue engineering. The “silks success” should be attributed to the material versatile properties that can be tuned according to the requirements via tailored processing and post-treatment methods that, in turn, affect molecular structure, supramolecular conformation and final properties.

By adjusting biopolymer architecture and chemical composition, the formation of the extra cellular matrix (ECM) can be triggered such as to guide cells to the generation of functional tissues.

Crystallinity and morphologies at different scale level can induce different blood responses in terms of platelet adhesion and activation, modulate the interaction with the inflammatory system, and control the stem cell fate.

Materials source, advanced processing strategies and selective chemical modifications can control chemistry and thus silk's function.

Silk fibroin can be used as polymer model to debate the relationship between material structure, physical and biological properties.

The lecture will explore the history, uses and potential future of silk fibroin as elective materials for tissue engineering applications, starting from basic "primitive" bidimensional substrates till the design and fabrication of microniches populated 3D structures able to guide the cell behavior and the regenerative process.

### Generation of Highly Aligned Skeletal Muscle-Like Tissue through Strain-Induced Patterning of Fibrin

P. Heher<sup>1,2</sup>, B. Maleiner<sup>3</sup>, J. Pruessler<sup>1</sup>, A. Teuschl<sup>3</sup>, J. Kollmitzer<sup>3</sup>, S. Wolbank<sup>1,2</sup>, H. Redl<sup>1,2</sup>, D. Ruenzler<sup>3</sup>, C. Fuchs<sup>3</sup>;

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The natural hydrogel fibrin is considered a suitable scaffold material for skeletal muscle engineering since its mechanical properties can be adjusted to match those of native skeletal muscle tissue. In addition, fibrin responds to uniaxial mechanical stimulation with fibril alignment along the axis of strain, allowing for guided cellular patterning. To exploit this feature in a biomimetic skeletal muscle engineering approach, we have developed a novel bioreactor system (MagneTissue) for the rapid generation of highly aligned skeletal muscle-like tissue constructs. With this system, murine myoblasts embedded in ring-shaped fibrin scaffolds are subjected to static mechanical strain via magnetic force transmission, leading to cellular alignment concomitant with the patterning of the scaffold material into highly organized fibrin fibrils. Within 9 days, a parallel array of myotubes with a more mature phenotype in terms of sarcomeric patterning, width and length is obtained using a daily stimulation protocol of 10% static strain for 6 hours and 3% for 18 hours. Moreover, static mechanical stimulation leads to enhanced myogenic determination/differentiation on the gene expression level, demonstrated by the upregulation of MyoD and Myogenin as well as the contractile structural marker TnnT1. The MagneTissue bioreactor system provides a versatile platform for engineering tissues whose functionality requires parallel cellular patterning, such as skeletal muscle - with the advantage that strain protocols can be individually adjusted. In future work, this will allow implementing mechanical stimulation with different strain regimes in the maturation process of tissue engineered skeletal muscle constructs and elucidating the role of mechanotransduction in myogenesis.

### Fibrin or Fibroin: Effect of Hydrogel Formulation on Behavior of Neonatal Cardiomyocytes Cultured within a Bioreactor

W. L. Stoppel<sup>1</sup>, K. Y. Morgan<sup>2</sup>, B. P. Partlow<sup>1</sup>, D. L. Kaplan<sup>1</sup>, L. D. Black, III<sup>1,3</sup>;

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Though extensive studies have investigated the development of engineered myocardium, current methods result in constructs with contractile stresses that are at least one order of magnitude smaller than that of cardiac tissue, necessitating continued improvements in biomaterial design and development. We present a comparison between neonatal cardiomyocyte behavior in fibrin gels or silk fibroin-cardiac extracellular matrix (ECM) hydrogels. The maturation and function of cultured cardiomyocytes was investigated using a dual electromechanical bioreactor that allows for tight control over the timing of the two modes of stimulation. Fibrin constructs cultured under delayed electromechanical stimulation (isovolumic contraction) had the highest twitch forces and an increase in contractility

(Troponin I) and calcium handling (SERCA2a) proteins, resulting from upregulation of physiological hypertrophic pathways (Akt).<sup>1</sup> When electrical and mechanical stimulation were completely offset (non-physiological conditions), a reduction in twitch force generation was measured, indicating that the timing of dual electromechanical stimulation is important in generating optimal construct function.<sup>2</sup> On-going work aims to compare these fibrin hydrogel results to cardiomyocyte response in silk fibroin-ECM elastomeric hydrogels formed via horseradish peroxidase-H<sub>2</sub>O<sub>2</sub> crosslinking.<sup>3</sup> These highly tunable silk hydrogels present a new class of silk-based materials which support the growth and maturation of cells from neonatal isolations as well as compaction and remodeling by isolated cardiac fibroblasts. Ultimately, we aim to develop a new injectable platform for cardiovascular applications.

1. Morgan KY *et al.*, *Tissue engineering. Part A* **2014**, 20(11–12).
2. Morgan KY *et al.*, *Organogenesis* **2014**, 10(3).
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### Silk Fibroin for Peripheral Nerve Regeneration: A Novel Preparation Method Improved Mechanical Characteristics and Supports Regeneration in Rat Sciatic Nerves

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Over the last decade, silk fibroin has been emergently used in tissue engineering. In this study, we describe a novel procedure to produce silk fibroin nerve graft conduits (SF-NGCs): a braided tubular structure of raw *Bombyx mori* silk was subsequently processed with the ternary solvent CaCl<sub>2</sub>/H<sub>2</sub>O/ethanol, formic acid and methanol to improve its mechanical and topographical characteristics.

The combination of the treatments resulted in a fusion of the outer single silk fibers to a closed layer. In contrast to the outer wall, the inner lumen still represented the braided structure of single fibers. Mechanical stability, elasticity and kink characteristics were evaluated and the modifications drastically improved these properties of the SF-NGC. An advanced cell migration assay with NIH/3T3-fibroblasts revealed the impermeability of the SF-NGC for possible invading and scar-forming cells. However, cytocompatibility assays with primary Schwann cells showed its suitability to serve as a substratum for Büngner-band forming Schwann cells.

*In vivo*, the SF-NGC was implanted to bridge a rat sciatic nerve defect and evaluated histologically (immunofluorescence, axon count) and functionally (electrophysiology, Catwalk analysis). No signs of elicited inflammatory reactions could be detected in the *in vivo* setting and we could demonstrate morphological and functional re-innervation of the distal targets after 12 weeks.

The novel SF-NGC presented here shows promising results for the treatment of peripheral nerve injuries. The novel modification of braided structures to adapt its mechanical and topographical characteristics may support the translation of SF-based scaffolds into the clinical setting, not restricted to the nerve field.

### Optimizing Peptide Incorporation Efficiency and Characterizing Changes in the Physical Properties of Fibrin Gels

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**Objectives:** Recently, the Wu lab has developed a new strategy for light actuated drug delivery-on-demand that uses the photo-thermal response of biocompatible chromophores to trigger release from thermally-responsive delivery vehicles. However, there is a

lack of biocompatible thermally-responsive biomaterials. Fibrin is a traditionally non-stimuli-sensitive biomaterial with a long history of use in FDA approved products, and has previously been functionalized with exogenous peptides by enzymatic incorporation to alter its bioactivity<sup>1</sup>. This same approach can be used to functionalize fibrin with thermally-responsive elements. The goal of this study is to optimize the enzymatic incorporation efficiency and characterize changes in the resulting fibrin gel.

**Methodology:** Four peptides from various Factor XIIIa substrates have been identified and included in the fibrin polymerization. Incorporation efficiency is measured using fluorescently tagged peptides and changes in the fibrin mechanical properties are measured by a rheometer and the swelling ratio.

**Results:** Each peptide exhibits unique physical properties (i.e. different net charges and hydrophobic amino acid content), and the effect each peptide has on the mass swelling ratio of fibrin has currently been studied. Specifically, peptides with a net charge have an increased swelling ratio over control gels, and the swelling ratio decreases when the hydrophobic content of the peptide is above 30%. Ongoing studies are measuring the degree of peptide incorporation and changes in the fibrin gel's viscoelastic properties. The findings from this study will aid future studies aiming to not only modify a biomaterial's stimuli-responsiveness, but also its mechanical properties, degradation kinetics and physicochemical properties.

### Fibrin or Fibroin - not only the "o" makes the Difference

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By its nature, fibrin is the resulting polymerized network formed during blood clotting by the action of the protease thrombin on fibrinogen monomers. Since the 1970s commercially prepared fibrin sealants consisting of human plasma-derived concentrated fibrinogen and thrombin have been used in clinics. On the base of its main physiological functions sealing and hemostasis fibrin was mainly used as tissue glue for clinical applications such as hemostasis, wound closure or fixation of grafts. Due to its additional properties such as improved wound healing and its complete *in-vivo* resorption fibrin has also been used as a potent biomaterial. Here, we describe the use of fibrin as a hydrogel to "train" cells in bioreactors, to deliver cells, or growth factors to the site of injury or as a gene activated matrix for non-viral gene therapy.

In contrast to the human origin of fibrin silk fibroin (SF) is produced by all spiders and many insects. The mainly used SF is derived from the cultivated *Bombyx mori* silkworm. Numerous studies in the last years have demonstrated the safety of this material. After degumming it can be used in its native fibrous structure or can be dissolved and post-processed in multiple ways. A new degumming procedure as well as partially dissolving/recrystallization techniques will be described. SF shows remarkable mechanical, degradation and biocompatibility properties, favoring its use to generate highly loaded grafts, especially in the musculoskeletal field. In this regard, evaluation results of a SF ligament graft in a large animal study will be presented.

### Session: Functional Bone Tissue Engineering – From Bone Development to Regeneration/Translation

**Date and Time: Thursday, September 10, 2015, 10:30 AM - 12:00 PM**

#### Stem Cell Transplantation into Epiphysis of Piglet Perthes' Model Promoted Restoration of Femoral Head Sphericity

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The deformation of the femoral head in Legg-Calve-Perthes' disease is thought to occur during the remodeling that follows the ischemic necrosis of an immature femoral head. We aim to assess whether autologous bone marrow mesenchymal stem cells (MSCs) transplanted into the operated femoral head after revascularization can favorably alter the history of femoral head deformation. In a surgically induced avascular necrosis piglet model, the femoral heads were injected with hydrogel loaded with  $5 \times 10^7$  osteogenically induced MSCs ( $n=9$ ) and hydrogel in the absent of MSCs were used as control ( $n=2$ ). Animals were then sacrificed at 3 to 5 weeks. On gross inspection 7 of 9 experimental femoral heads were not morphologically collapsed comparing with 100% collapsing in controls. Of these 7, 4 had maintained epiphyseal height (range 9 to 12 mm) within 1 mm of the height of the contralateral femoral head (range 10 to 13 mm). The epiphyseal quotients of the operated femoral heads of these four animals were also maintained (range 0.37 to 0.47) compared to the contralateral heads (range 0.41 to 0.45). Histological analysis of these 4 heads showed extensive new bone formation including osteoblastic and osteoclastic activities associated with and independent of vascular invasion, suggesting that the restoration of the femoral head height and quotient was due to increased bone formation. We have shown that the transplantation of induced MSCs into the femoral heads in this piglet model restored femoral head shape, demonstrating a tantalizing potential for stem cell therapy in the treatment of Perthes'.

#### Transplantation of Culture-expanded Bone Marrow Cells and Platelet Rich Plasma during Distraction Osteogenesis of the Long Bones

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**Objectives:** We have developed a cell therapy using culture-expanded bone marrow cells (BMC) and platelet rich plasma (PRP) during distraction osteogenesis of the long bones. We introduce this technique and evaluate the efficacy of this treatment.

**Methods:** Approximately 40 ml of bone marrow aspirates were collected from the iliac crest during bone lengthening procedure and mononuclear cells were cultured in the differentiation medium for osteoblastic differentiation for 3 weeks. Third-passage BMC were used for transplantation. PRP was prepared from approximately 200 ml of venous blood and concentrated after centrifugation. Cultured BMC were dissolved in PRP, and the mixture was injected into the distraction gap with thrombin and calcium to form the gel within the injected site. We have performed BMC and PRP therapy for more than 130 bones since 2002. We evaluate clinical outcome between the bones with and without BMC and PRP in patients who have achondroplasia or hypochondroplasia.

**Results:** There were no transplantation-related complications. The BMC group consisted of 74 bones in 26 patients while the control group consisted of 44 legs in 14 patients. There were no significant differences in the age of surgery, the length gained, and the percentage of lengthening between the two groups. Healing index (total treatment period/extent of lengthening, days/cm), however, was significantly smaller in the BMC group than in the control group. The rate of complication was also lower in the BMC group.

**Significance:** Transplantation of BMC and PRP shortened the treatment period by accelerating new bone regenerates during bone lengthening.

#### Pre-vascularized Bone Implant Composed of Cell-seeded Naturally Derived Collagen Matrix and Synthetic $\beta$ -TCP

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Bone regeneration is a major challenge regarding defects that exceed a critical size. The current standard of autologous bone transplantation faces problems as donor site morbidity or limited availability of transplantable bone. Methods of tissue engineering present an alternative to established procedures by *in vitro* assembly of bone implants that can be equipped with vascular structures for enhanced regeneration potential.

The present studies' bone implants consist of synthetic  $\beta$ -TCP granules with osteoconductive properties and a naturally derived collagen matrix (BioVaSc) that provides vascular structures. The BioVaSc is a de-cellularized porcine jejunum that can be reseeded with endothelial cells in order to re-establish the function of its inherent vessels. After re-endothelialization in a bioreactor system, the tubular-shaped BioVaSc was filled with  $\beta$ -TCP granules which were seeded with osteoblasts or mesenchymal progenitor cells. The combination of BioVaSc and  $\beta$ -TCP granules results in a construct that presents characteristics that favor fast bone formation by introducing cells with osteogenic potential on a material with osteoconductive properties as well as a matrix that provides nutritional support for the resident cells via its vascular structures that can be connected to a host's circulatory system. The regenerative potential of the implant was assessed in a tibia defects as well as in defects in the mandible in sheep.

This study describes the *in vitro* construction of cell-seeded bone substitutes with pre-vascularization via bioreactor technology. Additionally, it gives an insight into the regenerative potential of the bone substitute and illustrates the feasibility of the anastomosis of such an implant.

#### **Clinical Research on Safety and Efficacy of Autologous MSC Integrated with Porous Ceramics for Bone Defect Repair after Benign Tumor Removal**

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The safety and the efficacy of the bone regeneration technique using autologous mesenchymal stem cells with highly porous ceramics scaffold have not been revealed yet.

We carried out a prospective clinical research of the cell-ceramics composite technique for the treatment of bone defect after bone tumor removal. Briefly, bone marrow aspirate was harvested and the adherent cells were cultured and expanded with medium containing autologous serum. Then, cells were integrated with porous hydroxyapatite ceramics artificial bone and further cultured in medium that induces osteoblastic differentiation.

Eleven patients (7 males, 4 females; median 43 years old) with benign bone tumors in extremities were registered. Nine of them completed the protocol treatment and 48 week follow-up. All the cell-ceramics composite products met the quality control criteria. Two severe adverse events were reported. However, only one, local recurrence of the original tumor, might have a possible causal relation to the treatment. Primary endpoint for efficacy was bone defect repair evaluated by a 4-grade scoring system based on the findings in plain radiographs. The score improved with time in all cases except in the case with local recurrence.

In conclusion, our study suggests that the autologous mesenchymal stem cells integrated with ceramic scaffold is a safe and effective method for the repair of bone defect after the removal of bone tumors. However, for the superiority in efficacy to conventional artificial bone, and for the possible causal relation with the

enhancement of tumor relapse, there is a need for careful evaluation in the future.

#### **Basic and Clinical Research on Custom-made Artificial Bone and Tissue-engineered Cartilage in Oral and Maxillofacial Region**

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Recently, there have been remarkable advances in regenerative medicine, and almost all the disorders of the oral and maxillofacial region could be research targets of regenerative medicine. Meanwhile, treatment in this region has been well established using biomaterials, prostheses and microsurgery. Therefore, to surpass such a conventional approach as an alternative, regenerative medicine should take an approach to be less invasive and/or more effective. In this report, we present our basic and clinical research on bone and cartilage regenerative medicine in the oral and maxillofacial region.

Regarding bone regenerative medicine, we have tried to develop artificial bone that would maximize bone formation at the transplanted site, but would be subsequently replaced by autologous bone. We have made custom-made artificial bone (CT-Bone) using  $\alpha$ -TCP particles and an ink-jet printer, and have conducted clinical research and trials on 30 patients.

To develop tissue-engineered cartilage with proper 3D morphological form and mechanical strength, we have optimized the culture medium of chondrocytes and the scaffold. Following a preclinical study confirming efficacy and safety, we have conducted clinical research in 3 patients with nasal deformity associated with cleft lip and palate, and are now starting multicenter clinical research.

#### **How Natural Bone Formation can Inform Functional Bone Tissue Engineering - Biomimetics.**

X. Yang;

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To engineer functional bone tissues for the restoration of bone lost is still a big clinical challenge. The choice of ideal scaffolds, suitable cells, proper inductive factors and mechanical simulation/microenvironment or the best combination of these basic elements are still not fully optimized. The efficacy of current approaches on enhancing healing of fracture non-union and critical bone defect cannot yet meet the clinical need. Thus, it is the time to look the 'past, present' and inform the 'future' of functional bone tissue engineering. This talk will cover the topics from fundamental bone development, fracture healing, bone remodelling to current progress on bone tissue engineering both *in vitro* and *in vivo*. It aims to emphasize the importance of 'biomimetics' for functional bone tissue engineering and add more educational value to the congress. The talk will end up with some questions for open discussion with research students and junior scientists, such as 1) what we can learn from natural bone formation and fracture healing? 2) What the current challenges are regarding to cell sources, scaffolds, delivery methods? 3) What is the bottle neck for clinical translation; 4) What do you think about the future directions?

#### **Session: Green Technologies for the Preparation of Enhanced Biomaterials for Tissue Engineering and Regenerative Medicine**

**Date and Time: Wednesday, September 9, 2015, 2:15 PM - 3:30 PM**

#### **What Can CO<sub>2</sub> Do For You? Carbon Dioxide for Polymer Processing in Tissue Engineering**

M. S. Detamore<sup>1</sup>, M. Bhamidipati<sup>2</sup>, C. J. Berkland<sup>1</sup>, A. M. Scurto<sup>1</sup>;

<sup>1</sup>Chemical & Petroleum Engineering, University of Kansas, Lawrence, KS, <sup>2</sup>Biomedical Engineering, Rutgers University, Piscataway, NJ.

Widespread advances have made the use of CO<sub>2</sub>, ubiquitous in our tissue engineering laboratories, a tremendous asset for the tissue engineering community. In our group, we have leveraged CO<sub>2</sub> for sintering microsphere-based scaffolds. In contrast to CO<sub>2</sub> foaming, which typically relies on supercritical CO<sub>2</sub> at very high pressures, by using pressures an order of magnitude lower we have created scaffolds of complex geometries in the presence of cells in a single step by sintering microspheres with CO<sub>2</sub>. CO<sub>2</sub> has a number of advantages for polymer processing in tissue engineering, including its ease of use, low cost, and the opportunity to circumvent the use of organic solvents. We have established a precedent for various operating parameters to achieve desired levels of sintering with different polymers, and quantified the effect of CO<sub>2</sub> on release from the microspheres. For our colleagues in drug delivery and tissue engineering, we invite and encourage you to leverage CO<sub>2</sub> as a new tool to enhance your own unique capabilities.

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#### The Intersection Between Chemical and Biomedical Engineering: Green Technologies Towards the Development of Enhanced Biomaterials

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Despite the advances on biomaterials development and polymer processing technologies, this remains still one of the major scientific challenges that tissue engineering and regenerative medicine (TERM) faces to go from benchtop to bedside. Ideal scaffolds should be biocompatible, biodegradable and promote cellular interactions and tissue development and possess proper mechanical and physical properties. The preparation of 3D matrices must result, hereafter in structures with adequate porosity, interconnectivity, pore size distribution and compression properties which make them suitable for the tissue to be engineered. A wide range of biomaterials has been proposed for biomedical applications, from metals to ceramics and polymers. Due to their versatility, polymers are the straightforward choice. These must comply with different requirements such as hydrophilicity, biocompatibility, degradation rate, cytotoxicity, among others. The use of natural based polymers in tissue engineering and regenerative medicine applications has long been proposed, precisely due to their chemical/biological versatility. Nonetheless, its processing using supercritical fluids only recently has started to received more attention from researchers. Supercritical fluids appear as an interesting alternative to the conventional methods for processing biopolymers as they do not require the use of large amounts of organic solvents and the processes can be conducted at mild temperatures. Different processing methods based on the use of supercritical carbon dioxide have been proposed for the creation of novel architectures able to fulfill the particular needs of each tissue to be regenerated and these will be unleashed in this presentation.

#### Design of Biomaterials for Musculoskeletal Tissue regeneration

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Hybrid biomaterials fabricated from ceramics, polymers and biopolymer are deemed to be the material of choice due to their tunable physicochemical properties. In our studies, we prepared hybrids from natural and synthetic polymers with bioactive glass by formation of covalent bond. It was demonstrated that the presence of chemical

bonds between the polymers and bioactive glass eradicates the issue of phase-separation and enhances the uniform distribution of components in the structure of these materials. Assessment of *in vitro* bioactivity of these samples showed that a homogenous apatite layer was formed on the surface of hybrid scaffolds. Furthermore, it was shown that the presence of chemical bond resulted in uniform degradation of different components in hybrids. The fabricated materials could be considered as viable candidates for bone regeneration. We also synthesized thermo-responsive hydrogels with the capacity to chemically bond with primary amine groups of proteins. These hydrogels were injectable and their gelation time was favourable for clinical applications. The unique feature of these hydrogels include their biocompatibility, tunable mechanical properties and adhesiveness that make them suitable for broad range of musculoskeletal tissue repair. This work will provide an insight about these highly bioactive materials and their *in vivo* applications for bone and cartilage tissue regeneration.

#### Nanocomposite Prepared of Whiskers of Biological Chitin and Enzymatic Poly-ε-(Caprolactone)

A. U. Espadín, Sr.<sup>1</sup>, L. Tamay de Dios<sup>2</sup>, E. Ruvalcaba<sup>2</sup>, M. Gimeno<sup>3</sup>, A. Tecante<sup>3</sup>, H. Vazquez<sup>1</sup>, C. Velasquillo<sup>2</sup>, K. Shirai<sup>1</sup>;

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Cationic polysaccharides in the nanoscale improve the mechanical properties, biocompatibility and biodegradability in polymer matrix composites. In this regard, chitin is obtained herein by fermentation of shrimp wastes by lactic acid bacteria (LAB), from which chitin whiskers (CW) were prepared in an acid medium at 4 °C and 90 °C. The size and morphology of the particles were determined by TEM and Zetasizer-nano, Powder X-ray diffraction (PXRD) and <sup>1</sup>H NMR to achieve Nanowhiskers (Nw) of desired size (67 nm). The biodegradable polyester PCL matrix was enzymatically (lipase B from *Candida antarctica*) synthesized in compressed 1,1,1,2-tetrafluoroethane (65°C, 25 bar) media and characterized by SEC (Mn 20774 g/mol-1), thermal analysis and X-ray (DSC and TGA). Several nanocomposites (NCs) films were prepared with PCL containing chitin-Nw. NCs were characterized by elemental analysis, confocal microscopy, as an indication of the distribution of Nw, FTIR and powder PXRD. The films were also characterized by thermal analysis, mechanical properties, swelling and erosion. The results indicate that the best material is achieved with the lowest amount of Nw, which were used for the *in vitro* culture of osteoblasts, presenting the highest cell viability without toxicity.

#### Processing of Materials for Regenerative Medicine using Supercritical Fluid Technology

C. A. García-González, L. A. Díaz-Gómez, A. Concheiro, C. Alvarez-Lorenzo;

Departamento Farmacia y Tecnología Farmacéutica, University of Santiago de Compostela, Santiago de Compostela, SPAIN.

Novel processes are being sought for the production of advanced materials acting as the future "platinum standard" in regenerative medicine. The objective is to overcome the current implant concerns regarding supply availability limitations as well as clinical complications with infection and rejection episodes. Technologies based on the use of supercritical CO<sub>2</sub> as a green-solvent are being endeavoured for the massive production of solvent-free scaffolds using mild operating conditions compatible with the loading of thermally sensitive compounds, such as growth factors and other bioactive agents.<sup>1</sup> The technological platform based on the use of supercritical CO<sub>2</sub> for regenerative medicine is being tackled by three main approaches: processing medium of synthetic scaffolds, treatment and purification of tissues and post-treatment of medical devices. In this work, recent advances in the state-of-the-art on the manufacturing of grafts using supercritical fluid technology are presented. Namely, strategies for the

incorporation of bioactive agents (drugs, bioactive glasses, growth factors) in scaffolds are highlighted with results from our research group showing high loading efficiencies and retained activity after supercritical processing.

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Disclosures: The authors declare that there are no conflicts of interest.

### Session: Human on a Chip for Tissue Engineering and Screening

**Date and Time: Friday, September 11, 2015, 9:15 AM - 10:45 AM**

#### 2d and 3d Brain on a Chip for Cns Disease Model

**S. Lee;**

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Understanding the mechanisms of CNS functions is important to discover the causes and treatment method of disease and the *in vitro* CNS model mimicking the *in vivo* neuronal environment as possible is highly required. Recent progress in microfluidics allows the development of such CNS models. Here, we have developed several 2D and 3D brain model in and on the microplatform. For the creation of 3D brain model, we have cultured cells on the arrayed concave microwell. To mimic the *in vivo* environment, we have employed osmotic pump which enables ultra-slow flow continuously. By the osmotic pump, chemical gradient could be applied to the neurons and the effect of chemical cue's concentration could be observed in single microfluidic platform. Neuron and glial cells from the fetus of rat were cultured and they are networked 2- and 3- dimensionally in the microplatform. Alzheimer's disease (A-disease) was chosen as CNS disease model, and we analyzed the effect of amyloid- $\beta$  (A- $\beta$ ) which is known to be one of main cause of A-disease to the 2D and 3D neurons. The effect of flow was also investigated. As result, networked 2D neurons were affected to the gradient of A- $\beta$  and flow, and networked neurons were severely damaged. Similarly, networked 3D neuro spheroids were affected by the A- $\beta$  and flow. We expect the proposed system can be used as *in vitro* screening tool to analyze the effect of diverse physical and chemical cues of CNS disease, and will contribute to develop treatment method of CNS diseases.

#### A Microfluidic Model of the Blood Brain Barrier

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Considerable interest has focused on the generation of an *in vitro* model for the blood brain barrier (BBB) due to its critical importance in regulating the passage of drugs and other factors into the brain from the microvasculature. Complicating factors include the need to capture the three-dimensional microenvironment, the importance of multiple cell types, and differences in the media compositions that support these different types of cells. Previous attempts have drawn upon existing culture methods such as the well-known transwell systems. We have recently adapted our microfluidic platform assay [1] to incorporate primary rat neurons and astrocytes cultured beside human brain endothelial cells (hCMEC/D3) in a single system that mimics the physical arrangement of these cells in the brain. Two different media

compositions are used to maximize survival of the neurons and to maintain low permeability of the endothelial monolayer. Measurements of endothelial monolayer permeability of a 70 kDa dextran after 2 weeks of co-culture show values < 10–6 cm/s, approaching those *in vivo*. Neuronal function was assessed by calcium staining.

#### Reference

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#### Smoking Lung-on-a-chip: A Tissue-engineered Microphysiological Model of Cigarette Smoke-induced Airway Disease

**M. J. Mondrinos, W. Byun, C. Blundell, D. Huh;**

Bioengineering, University of Pennsylvania, Philadelphia, PA.

Cigarette smoking is a primary cause of chronic medical conditions and life-threatening malignancies in the lung. Biological underpinnings of these diseases, however, remain poorly understood due to a lack of surrogate models for mechanistic investigation of pathological responses to cigarette smoke in a physiological environment. Here we introduce a biomimetic microengineering approach to emulate and probe smoking-induced disease processes in a tissue-engineered microenvironment that recapitulates the complexity of human airways. Specifically, we developed an airway-on-a-chip model that integrates human airway epithelial cells, basal stromal tissue and airway lumen macrophages with programmable microfluidic delivery of cigarette smoke to study deleterious effects of smoke exposure on the airway epithelium. Single smoke exposures induced acute injury of human bronchial epithelial cells and small airway epithelial cells, leading to significant loss of epithelial integrity and barrier function. This injurious response was accompanied by increased stress in the endoplasmic reticulum, as manifested by robust activation of the unfolded protein response (UPR). Using patient-derived cells, we also created a microengineered chronic disease model that recapitulated constitutive UPR activation typically observed in chronic obstructive pulmonary disease (COPD). Our microphysiological system provides unprecedented capabilities to interrogate key biological processes involved in smoking-induced lung diseases and may contribute to identification and screening of new therapeutic targets.

#### 3d Tissue Fabrication Based on Cellular Building Blocks

**S. Takeuchi;**

IIS, University of Tokyo, Tokyo, JAPAN.

Large-scale 3D tissue architectures that mimic microscopic tissue structures *in vivo* are very important for not only in tissue engineering but also drug development without animal experiments. In this presentation, I will talk about several MEMS/Microfluidic-based approaches for the rapid construction of 3D tissue. We demonstrated a bottom-up tissue construction method using different types of cellular modules that serve as building blocks for thick and dense 3D tissues (eg. cell beads and cell fibers).

To prepare the cellular beads, we used an axisymmetric flow focusing device (AFFD) that allows us to encapsulate cells within monodisperse collagen beads; the cells can also be seeded on the surface of the collagen beads. Moreover, by stacking these capsules in a 3D chamber and incubating them, we successfully constructed a complicated and millimeter-sized 3D tissue structure. We believe that various 3D shapes can be possible by changing the shape of the mold, and this method is useful to create more complex structures with multiple types of cells.

As the cell fibers, a cell-encapsulating core-shell hydrogel fiber was produced in a microfluidic device. When with myocytes, endothelial cells, and nerve cells, each fiber showed the contractile motion, the tube formation and the synaptic connections, respectively. A method of manipulating these cell fibers into desired three-dimensional configurations (such as reeling, weaving and folding) enables us to build spatially ordered and bio-functional fiber-based 3D tissue constructs.

### 3d Reconstitution of Brain Neural Tissue

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The brain is the most complex organ with 3D structures of neuron, glia and brain vasculature. Neuron processes and transmits information through electrical signals, having electrically insulating myelin around its axon. Production of the myelin sheath is called myelination, which helps proper functioning of neurons with its 3D winding structure. 3D axonal myelination was successfully induced *in vitro*, in our extracellular matrix (ECM) incorporating microfluidic device, utilizing multipotency of neural stem cells (NSCs). NSCs and neurons were co-cultured inside the device for three weeks under precisely optimized co-culture condition.

The developed microfluidic device has precisely controlled ECM microenvironment, which regulates myelination by differentiated oligodendrocytes from NSCs on axons 3 dimensionally dispersed in ECM. The 3D *in vitro* myelination model can be a tool for understanding brain diseases and drug development.

### A Microphysiological Heart-on-a-Chip using Electroconductive Myocardial Matrices

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Drug-induced cardiac arrhythmias account for roughly a third of safety-related drug recalls, indicating an inability for current screening platforms to predict compound action on human myocardial tissue adequately. Consequentially, the production of a human cardiac tissue mimic that is capable of producing predictive data in preclinical pharmacological studies remains an important target for bioengineers. This project focuses on the development of a microfluidic model of the human myocardium that recapitulates the form and function of native cardiac tissue. The model was generated by adapting a microfluidic platform to support the development and maturation of engineered cardiac tissue derived from human embryonic stem cells (hESCs). A cardiac reporter cell line for monitoring Ca<sup>2+</sup>-dynamics (derived from GCaMP3-expressing hESCs) was used to facilitate real-time assessment of the engineered tissue's electrophysiology. Cardiomyocytes were mixed with decellularized myocardial extracellular matrix-derived hydrogels containing electroconductive reduced graphene oxide. The cell-matrix solution was injected into the lumen of the microfluidic device and maintained under constant flow rates for 7 days. Measurements of cardiac conduction velocity, action potential anisotropy, and beat frequency/rhythmicity were then made in real time by analyzing GCaMP3 expression. Functional measurements were supported by post hoc assessment of sarcomere striation, actin cytoskeleton alignment, connexin43 expression, and construct cell density. Comparison of output metrics from baseline and drug-treated constructs demonstrate that this system can be effectively employed for screening the arrhythmogenic potential of novel compounds. The collected results provide important information and standards concerning the production of mature myocardial micro-tissues in a microfluidic system.

**Session: Hydrogels for Printing Tissue Constructs**

**Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM**

### Transparency Improvement for Artificial Cornea bases on 3d Printing Technique

H. Kim, J. Jung, D. Cho

POSTECH, Pohang, KOREA, REPUBLIC OF

In cornea regeneration, the arrangement and the concentration of collagen fibrils are important factors which can control the transparency of the cornea. So far, some groups control the arrangement of collagen and regulate the topography of keratocytes in 2-dimension (2D) to obtain the transparency of cornea [1, 2]. However, 3D artificial cornea stacked by 2D sheets has low transparency due to disarrangement of collagen fibrils. In this study, we developed advanced techniques to improve the transparency of the artificial cornea. The artificial cornea was fabricated with bio-ink called corneal derived extracellular matrices (Co-dECM) by 3D printing technology. Cell arrangement with less than 100 μm of interspace was realized by integrated composite tissue/organ building system (ICBS) developed in-house. Beforehand, we determined the concentration of Co-dECM and the cell density in Co-dECM for improving transparency of the artificial cornea through *in-vitro* test. We confirmed that cell viability of keratocytes-laden Co-dECM was over 80%, and collagen fibrils were aligned around keratocytes. In comparison with control group composed of Co-dECM and keratocytes, the 3D printed artificial cornea has consistent arrangement of collagen fibrils in same direction and uniform distribution of keratocytes. Also, it caused less deformation after cell culture periods, because of the uniform distribution of keratocytes generating mechanical tension on collagen fibrils. Overall, we can fabricate the artificial cornea which has high transparency through this advanced technology, and it can be applied to corneal regeneration.

1. Torbet J, *et al.*, Biomaterials 28, 29, 2007.

2. Kilic C, *et al.*, Biomaterials Science 2, 3, 2014.

### Design of a Sugar-responsive Hydrogel as a Sacrificial Template to Fabricate Collagen Scaffolds with a Vessel-like Structure

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Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, JAPAN.

The objective of this study is to fabricate collagen scaffolds with a vessel-like structure. A sugar-responsive hydrogel was designed as a sacrificial template that can be removed by the sugar-responsive water-solubilization without any cytotoxicity, to create a vessel-like structure in collagen scaffolds. In this study, m-aminophenylboronic acid (APBA) of a sugar-responsive moiety was introduced into gelatin, while sugar-responsive hydrogels were fabricated from the APBA-introduced gelatin. The resulting hydrogel was dried up and cut into a rod with a diameter of 300 μm and a length of 1 cm. Mouse vascular endothelial cells (MS-1) were pre-seeded onto the surface of the rod and the resulting MS-1-attached rod was embedded into type I collagen gels. The embedded rod was water-solubilized by adding sorbitol of a sugar, whereas no water-solubilization of the rod was observed in the absence of sorbitol. It is likely that the sorbitol binding allows the boronic acid group to process negative charges, resulting in disrupted hydrophobic interaction between the APBA groups. This interaction disruption would lead to the water-solubilization of rod and the subsequent creation of a channel in the collagen gel. The pre-seeded MS-1 were found on the channel surface, probably due to transferring the attached MS-1 to the channel surface simultaneously with the rod removal. As a result, a vessel-like structure was introduced into the collagen gel. The present hydrogel system of sugar-responsive water-solubilization is a promising sacrificial template to create cell scaffolds with a vessel-like structure *in vitro*.

### Design of Self-assembling Bio-inks for Cell-based 3d Printing

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Materials Science and Engineering, Stanford University, Stanford, CA.

Despite the rise of 3D printing of thermoplastics both in industry and the general public, a key limitation preventing the widespread use of cell-based additive patterning is the lack of cell-compatible bio-inks that have the required properties for printing. Current challenges of many commonly used bio-inks include difficulty



maintaining a homogeneous cell suspension, preventing long-term cell exposure to photo-activators, avoiding cell damage during extrusion, customizing the printed matrix properties to facilitate cell-matrix interactions, and printing within a bath to prevent cell dehydration while preserving high print resolution. In response, we synthesized a new family of tunable biomaterials specifically designed for cell-based additive patterning. These hydrogel-based bio-inks are produced from a blend of recombinantly engineered self-assembling peptide sequence and peptide-modified alginate polysaccharide. This design is advantageous due to the ability of the bio-ink to undergo two-stages of crosslinking: (i) weak, peptide-based, self-assembly to homogeneously disperse cells within the ink cartridge and to mechanically shield the cells from damaging forces during extrusion and (ii) electrostatic crosslinking of alginate upon printing within a bath to rapidly (within seconds) stabilize the construct and to tailor the final mechanical properties for optimal cell-matrix interactions. The resulting gel modulus was tuned to span 400 Pa to 2000 Pa. The use of engineered proteins provides control of ligand presentation for cellular attachment and signaling. Encapsulated human adipose-derived stem cells demonstrated excellent viability and spreading and experienced significant protection from damage during extrusion. Furthermore, the rapid gelation kinetics resulted in a homogeneous cell dispersion throughout the extruded hydrogel.

### Decellularized Extracellular Matrix based Bioinks for 3D Printing Technology

D. Cho<sup>1</sup>, J. Jang<sup>1</sup>, J. Park<sup>1</sup>, J. Lee<sup>1</sup>, J. Shim<sup>2</sup>, S. Kum<sup>3</sup>, J. Rhie<sup>3</sup>;

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Research at IMS Laboratory focuses on tissue engineering and regenerative medicine based on 3D cell printing technology which mainly utilizes bioinks composed of decellularized extracellular matrix (dECM) to fabricate complex three-dimensional (3D) tissue constructs. The ability to pattern and print the components of a tissue (cells and matrix materials) in 3D structure is a fascinating prospect of 3D cell printing technology. However, majority of the matrix materials used so far for 3D cell printing are unable to realize the complexity of natural extracellular matrix. And also, they cannot fully reconstitute the intrinsic cellular morphologies and functions.

Recently, we have successfully developed printable dECM based bioinks for tissue-specific regeneration. We developed a 3D cell printing method for printing of cell laden construct with novel dECM based hydrogels, which are capable of providing an optimized microenvironment conducive to the growth of 3D structured tissue. And here, we show the versatility and flexibility of developed 3D cell printing process using tissue-specific dECM based hydrogels derived from various tissues. These dECM based hydrogels were capable of providing crucial cues for cells engraftment, survival, and long-term function. Moreover, we achieved high cell viability and functionality of the cell printed dECM structures. Throughout this research, we ultimately aim to regenerate tissue/organ by constructing and integrating the pre-tissues using 3D cellprinting technology with dECM based hydrogels.

### 3D Bioprinting of Physical Hydrogels Based on Peptide Oligosaccharide Interaction

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Non-covalent biopolymer that possess simple components with basic biochemical properties similar to the extracellular matrix are considered highly promising for various biomedical applications, ranging from drug

delivery to 3D cell culture. The combination of biomaterials with additive manufacturing (3D bioprinting), is driving major innovations in regenerative medicine, drug discovery and toxicology using printed structures as tissue models. In this study, we have realized 3D bioprinting of vascular-like structures by using an in-house built microextrusion printer and a novel physical hydrogel system developed in our lab. We designed and screened minimal peptide motifs whose conjugates with PEG (peptide-PEG conjugate) interact with sulfated oligosaccharides (like heparin) to form non-covalent hydrogels. Namely, the peptides contain repeats of a basic amino acid and alanine ((RA)<sub>n</sub> and (KA)<sub>n</sub> motifs). Hydrogel was formed by mixing KA5-PEG conjugate, human fibroblasts and dextran sulfate in 250 µl syringe. Hydrogel was formed after 5 minutes and ready to print after one hour. Using the in-house built microextrusion printer, constant filaments could be formed and printed into 3D structures containing channels. These stable channels can be flushed with liquid. Cell survival in printed hydrogel is 96.4%, cells proliferate and spread after printing. Additionally, the hydrogel does not swell or shrink so structures stay as they are. Therewith, this hydrogel is an ideal biomaterial for 3D bioprinting. It could be used for more complex applications as printing organ-like structures to avoid animal experiments in drug screening and regenerative medicine.

### Bioprinting Vascular Networks for Engineered Tissue Constructs

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A key challenge in engineering tissue constructs is integrating adequate vascular networks to prevent necrosis. A microcirculation supply is not only necessary for the exchange of nutrients and waste, but it is often coupled with many functions of the organs. To address this challenge, we use a direct ink write system to print complex geometries of high cell density 3D tissues that can self-assemble and remodel into lumenized microvascular networks. Initial viability testing immediately after printing the cell-laden constructs showed viability of greater than 90%. Using high cell density fibrinogen based bioinks, we 3D printed geometries that were maintained in culture for 2 weeks. During this time the metabolically active tissue constructs consisting of co-printed human fibroblasts and human endothelial cells, remodeled their surrounding scaffold and self-assembled into lumenized vascular networks with diameters ranging from 15–25 µm. Additionally, we have demonstrated the ability to coaxially print perfusable tubes using custom made coaxial needles. Tubes printed with stromal cells in the walls had high viability immediately after printing. Printed tubes were also seeded with human cerebral microvascular brain endothelial cells (HCMEC) that attached well and proliferated in the tubes forming a perfusable endothelialized vascular channel. Future efforts include integrating tissues of interest (i.e. blood brain barrier) with our vascularized printed tissue platform to create organ-specific microvascular networks.

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### Session: Immunomodulation in Bone Tissue Engineering

Date and Time: Wednesday, September 9, 2015,  
1:00 PM - 2:15 PM

#### Impact of CCR2-Mediated Immunomodulation in the Outcome of Bone Repair Process

C. C. Bigueti<sup>1</sup>, A. E. Vieira<sup>1</sup>, P. Colavite<sup>1</sup>, F. Cavalla<sup>1</sup>, C. Francisconi<sup>1</sup>, A. F. Trombone<sup>1</sup>, C. Sfeir<sup>2</sup>, S. R. Little<sup>2</sup>, G. P. Garlet<sup>1</sup>;

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CCR2 receptor is important to macrophage recruitment during inflammatory response, but its role in bone repair process remains

unknown. The purpose of this study was to evaluate the role of CCR2+ cells in the alveolar bone repair in mice, and its potential for therapeutic immunoengineering. C57BL/6(WT) and CCR2KO mice strains were compared by microscopic (histomorphometry, birefringence and immunohistochemistry), microtomographic and molecular (PCRArray) methods during bone repair process (0, 7, 14 and 21 days) post-extraction of the right upper incisor. Our results demonstrated that CCR2+ cells are present in the inflammatory infiltrate at repair in WT mice, as well F4/80+ and CCR5+ cells. CCR2 association with F4/80+ monocytic cells migration was confirmed by the significantly reduced F4/80+ and CCR5+ cell number in CCR2KO mice. CCR2 absence resulted in an increase of inflammatory infiltrate, collagen fibers, osteoblasts and osteoclasts counts and decrease of blood vessels in CCR2KO mice compared to WT mice. The molecular analysis demonstrated that growth factor TGF $\beta$ 1, bone markers RUNX2 and CTSK, immunological markers TNF- $\alpha$ , CCR1 and CCR5, and MSCs markers (CD106, OCT-4, NANOG, CD146, CXCL12 and CD105) were decreased in CCR2KO mice, RANK, RANKL, DMP1, IL-6 and CXCR1 mRNA levels were increased. The remaining F4/80+ dependent immunomodulation in the absence of CCR2 is supposed to be driven by CCR5+ cells, since F4/80+ are double positive for CCR2/CCR5. CCR2/CCR5 mediated F4/80+ cells migration play an active role in alveolar bone repair, unraveling the potential for immunoengineering host response by interfering with such receptors with therapeutic aims.

### Osteoimmunomodulation in the Development of Bone Substitutes

Y. Xiao;

Queensland University of Technology, Brisbane, AUSTRALIA.

The important role of immune cells in regulating bone formation and remodelling has been recognized recently. This knowledge has a significant impact on the paradigm shift in the development of bone substitutes from an inert to an osteoimmunomodulatory material, highlighting the important role of immune cells. The current study has systematically investigated the interactions between bone cells and immune cells, with an emphasis on how immune cells affect bone dynamics in relation to the development of bone substitutes. The importance of osteoimmunomodulation in the assessment and development of advanced bone biomaterials has been demonstrated by the surface coating, materials composition, and the release of ions in the local environment. The phenotypic change of macrophages and cytokines produced significantly directed the differentiation and recruitment of mesenchymal stem cells. BMP 2/4 and NF- $\kappa$ B pathways played a regulatory role in the biomaterials induced osteogenesis. The information generated from the study will help to develop new biomaterials with osteoimmunomodulatory property for bone regeneration.

### The Immunoregulatory Property of Bone Marrow Mesenchymal Stem Cells during Osteogenic Differentiation

Y. Zhou, R. Huang, Y. Xiao;

Queensland University of Technology, Brisbane, AUSTRALIA.

The bone remodeling process requires a precise regulatory network among bone marrow mesenchymal stem cells (BM-MSCs), macrophages and bone cells such as osteoclasts and osteoblasts. The coupling between osteoclasts and osteoblasts has been intensively studied. Recent studies have shown that macrophages can cause the activity changes of osteoclasts and osteoblasts. However, it remains elusive whether MSCs are associated with the activity changes of macrophages during osteogenesis, leaving a certain gap unfilled in the field of bone remodeling. Our study aims to investigate the immunoregulatory properties of MSCs during osteogenic differentiation through the analysis of migration and immune responses of macrophages. Our data has demonstrated that the conditioned media (CM) collected from BM-MSCs at the early stage of osteogenic differentiation significantly inhibited the expression of IL-1 $\beta$  and IL-6 in RAW264.7 cells. However, the conditioned media collected from osteogenically differentiated BM-MSCs (OCM) could enhance

the production of IL-1 $\beta$  and IL-6. Meanwhile, increased cell migration of RAW 264.7 was observed when cultured with OCM. The results show that there is an immunoregulatory shift during osteogenic differentiation of BM-MSCs which reflects the involvement of immune responses in bone repair and regeneration

### Engineered Three Dimensional Mesenchymal Stem Cell Constructs to Enhance Immunomodulatory Activity

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Gladstone Institute of Cardiovascular Disease, San Francisco, CA.

The paracrine activity of mesenchymal stem/stromal cells (MSCs) to suppress innate and adaptive immune responses offers a potent cell therapy for modulating inflammation and promoting tissue regeneration. While the immunological mechanisms of MSCs are still actively under investigation, inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$  play a critical role in stimulating MSC immunomodulatory activity. Additionally, three-dimensional aggregation of MSCs alone has been demonstrated to further enhance the paracrine activity of MSCs. Therefore, we hypothesized that biomaterial-based presentation of cytokines within three-dimensional MSC constructs may provide a means of locally concentrating and sustaining presentation of immunomodulatory stimuli to potentiate the MSC paracrine response after transplantation *in vivo*. The secretome of MSCs cultured as spheroids was analyzed using cytokine antibody arrays. MSC spheroids exhibited a unique cytokine expression pattern compared to MSCs cultured as adherent monolayers and secretion of 39 cytokines was increased at least 2-fold or greater in spheroid cultures. To further enhance MSC immunomodulation, heparin microparticles loaded with IFN- $\gamma$  or TNF- $\alpha$  were incorporated within MSC spheroids. Incorporation of TNF- $\alpha$  loaded microparticles increased secretion of the immunomodulatory factors prostaglandin E2 (PGE2) and interleukin-6 (IL-6). IFN- $\gamma$  loaded microparticles induced sustained expression of the immunomodulatory factor indoleamine 2,3-dioxygenase (IDO) over at least 7 days of culture. In contrast, pre-stimulation of MSC spheroids with IFN- $\gamma$  only transiently upregulated IDO for up to 2 days. These results demonstrate that biomaterial-based stimulation within 3D MSC aggregates can promote an enhanced and sustained expression of immunomodulatory factors and controlled means of regulating MSC paracrine activity after transplantation.

### Adipose Tissue-derived Stem Cells do not Elicit Alloreactivity after Chondrogenic and Osteogenic Differentiation

T. C. Santos<sup>1</sup>, D. B. Rodrigues<sup>1</sup>, M. T. Cerqueira<sup>1</sup>, R. P. Pirraco<sup>1</sup>, A. G. Castro<sup>2</sup>, R. L. Reis<sup>1</sup>, A. P. Alexandra<sup>1</sup>;

<sup>1</sup>3B's Research Group, Headquarters European Institute of Excellence on TERM, Guimaraes, PORTUGAL, <sup>2</sup>Institute of Life and Health Sciences, Health Sciences School, University of Minho, Braga, PORTUGAL.

Mesenchymal stem cells lack or express low levels of major histocompatibility complex (MHC) class II and other immunoreactive costimulatory molecules rendering them "immunoincompetent". We have shown that human adipose tissue-derived stem cells (hASCs) implanted subcutaneously in mice did not elicit adverse immune responses. In this study we hypothesized that undifferentiated hASCs, and derived osteoblasts and chondrocytes, are able to evade xenogeneic immune system by failing activating murine bone marrow-derived macrophages (mBMM $\phi$ s) and dendritic cells (DCs).

Murine BMM $\phi$ s or DCs were plated in direct contact with undifferentiated and osteo- or chondro-differentiated hASCs for 4h, 10h and 24h. The cytokine profile was evaluated by qRT-PCR and the surface markers detected by flow cytometry. The direct interaction of both cell types was observed by time lapse microscopy. Results showed that mBMM $\phi$ s and DCs did not depict an activated profile after contacting tissue culture polystyrene. This profile was maintained along the experiment in direct contact with undifferentiated, osteo- or chondro-differentiated hASCs. This was confirmed by the expression of IL-1, IL-4, IL-10 and TNF- $\alpha$  and

surface markers (CD206<sup>++</sup>, CD336<sup>++</sup>, MHC II<sup>+</sup> and CD86<sup>++</sup>) detection. These data suggest the potential of hASCs in a xenogenic tissue engineering and regenerative medicine approach for research routine procedures, as well as for host immune system modulation in autoimmune diseases.

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I-Santos, TC *et al.*, Tissue Eng A, 19(7-8): 83, 2013.

### Session: Immunoprotection and Tissue Engineering Strategies for Type 1 Diabetes

**Date and Time: Thursday, September 10, 2015, 10:30 AM - 12:00 PM**

#### Bioengineering of a Microfabricated Porous Bioartificial Endocrine Pancreas for Extrahepatic Islet Transplantation

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Intrahepatic clinical islet transplantation (CIT) is affected by mechanical stress, high drug or toxin loads and an immediate blood mediated inflammatory reaction causing a 60% loss of the transplanted islets. The use of a tailored islet specific macroporous bioengineered scaffold for extrahepatic islet transplantation can help overcome the aforementioned disadvantages.

By combining advanced microfabrication and high-resolution laser drilling we manufactured thin-film microwells arrays of non-degradable poly(ethyleneglycol)terephthalate-poly(butylene)terephthalate. We used femtosecond pulsed laser drilling to create a regular pattern of pores ( $\varnothing$  40  $\mu$ m) suitable for vascular ingrowth.

We transplanted a marginal mass of 300 IEQ seeded in scaffolds into the epididymal fat pad of diabetic BALBc mice and compared blood glucose levels over time with free islets in the same location. We performed intraperitoneal glucose tolerance tests (IPGTT) at day 14 and 28 to test glucose responsiveness and systematically quantified vascular ingrowth and changes in  $\beta$ - and  $\alpha$ -cell composition using immunofluorescent microscopy.

We found that in the scaffold group 6 of 8 mice recovered from type 1 diabetes, becoming normoglycemic within 2 days after transplantation, in contrast to the non-scaffold group (2 of 8). IPGTT revealed that the glucose responsiveness of the scaffold group was similar to the positive kidney control group at 28 days. Extensive histological evaluation revealed that revascularization of islets occurs from the outside-in via the pores, and that the islet cellular composition followed this trend in regard to  $\beta$ -cell density showing an anisotropic vascular, and beta cell distribution, with the highest density on the outside declining inwards.

#### Human Islet Transplantation, Current Status and Challenges

**J. Oberholzer;**

Surgery, University of Illinois at Chicago, Chicago, IL.

Type 1 diabetes (T1DM) is a chronic autoimmune disease that results in the permanent destruction of insulin secreting beta cells present in the islets of Langerhans. In T1DM patients, the destruction of pancreatic beta cells results in insulinopenia, which in turn results acutely in hyperglycemia and ketoacidosis. Use of exogenous insulin can correct hyperglycemia to some degree, but does reliably prevent

chronic diabetic complications. Such complications include retinopathy, nephropathy and cardiovascular disease. Near normal glucose control may only be achieved by a cell-based therapy. Islet transplantation is currently applied as an experimental cell-based therapy for worst case, brittle T1DM patients. It involves the isolation of viable islets from a deceased organ donor, the purifying, culturing and infusing of these cells into a T1DM patient. This treatment not only provides insulin independence for most T1DM patients but also delivers long-term graft function and symptomatic benefit in terms of avoidance of severe hypoglycemia. At present this treatment is nearing biological licensure in order for it to become the standard of care for brittle T1DM patients. However, research is still needed to improve this procedure further, with the ultimate goal of providing long-term insulin independence without immunosuppressive medication. Such challenges to this goal include, but are not solely limited to, the inadequate supply of insulin producing cells, the inability to fully determine islet function prior to and following transplantation and the resultant immunorejection of donor islets post transplantation. The future development of islet transplantation hinges on the ability to overcome these obstacles.

#### Delivery of Supplemental Oxygen Improves Oxygenation of Tissue-Engineered Grafts *in Vitro* and *in Vivo* as measured with Fluorine-19 Magnetic Resonance Spectroscopy

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Pancreatic islet transplantation is a promising treatment for diabetes, but favorable long-term outcomes are inconsistent. Macroencapsulation and immunoisolation of islets within a tissue-engineered graft (TEG) could offer many advantages to current techniques. Oxygenation, however, remains a critical limitation for the development of large-scale implanted TEGs. This study demonstrates successful delivery of supplemental oxygen (DSO) to implanted TEGs, and non-invasive measurement of oxygen partial pressures ( $pO_2$ ) using fluorine-19 magnetic resonance spectroscopy (<sup>19</sup>F-MRS) *in vitro* and *in vivo*.

TEGs were constructed by injecting an acellular emulsion containing perfluoro-15-crown-5-ether, or the same emulsion with porcine islets into TheraCyte<sup>TM</sup> immunoisolation devices. Grafts were maintained using a bioreactor (N=5), or implanted into a dorsal subcutaneous pocket of Lewis rats while oxygenation was monitored with (N=13) and without (N=12) DSO. Graft  $pO_2$  was measured continuously during *in vitro* studies, and one day following implantation for *in vivo* studies using <sup>19</sup>F-MRS relaxation methods.

TEG  $pO_2$  measurements demonstrated efficient oxygen delivery *in vitro*, achieving an average of  $205 \pm 23$  and  $471 \pm 38$  mmHg internal  $pO_2$  during 300 and 760 mmHg DSO, respectively, despite a controlled hypoxic external environment (30 mmHg). Implanted acellular TEGs and TEGs containing islets without DSO were severely hypoxic ( $33 \pm 10$  and  $16 \pm 5$  mmHg respectively), while  $pO_2$  was significantly increased in grafts receiving 760 mmHg DSO for both conditions ( $557 \pm 18$  and  $370 \pm 49$  respectively). Implanted acellular TEGs returned to hypoxic  $pO_2$  levels ( $22 \pm 2$  mmHg) following cessation of DSO. DSO can alleviate hypoxia for implanted macro-encapsulated TEGs, and <sup>19</sup>F-MRS is a promising non-invasive method for assessment of implanted TEG oxygenation.

#### Long term Glycemic Control using Polymer Encapsulated, Human Stem-Cell Derived $\beta$ -cells in Immune Competent Rodents.

**O. Veisoh,** R. Langer, D. Anderson;

Massachusetts Institute of Technology, Cambridge, MA.

The transplantation of glucose-responsive, insulin-producing cells offers the potential for restoring glycemic control in diabetic patients. Pancreas transplantation and the infusion of cadaveric islets are currently implemented clinically, but their utility is limited by both the adverse effects of lifetime immunosuppression and the limited supply

of donor tissue. Recently, glucose responsive mature  $\beta$ -cells derived from human embryonic stem cells, called SC- $\beta$ , have been developed as an essentially unlimited human cell source for replacement therapy. The immunoisolation of insulin-producing cells with porous biomaterials to provide an immune-barrier is one strategy to overcome the need for immunosuppression. However, clinical implementation has been challenging due to host immune responses to implant materials. Here, we report the first long term glycemic correction of a diabetic, immune-competent animal model with human SC- $\beta$  cells. SC- $\beta$  cells were encapsulated with novel, alginate-derivatives capable of mitigating foreign body responses *in vivo*. Our results show glycemic correction in streptozotocin-treated (STZ) C57BL/6J mice for over 170 days with encapsulated human SC- $\beta$  cells implanted in the intraperitoneal (IP) space without any immunosuppression. Human c-peptide and *in vivo* glucose responsiveness demonstrate therapeutically-relevant glycemic control. Retrieved implants revealed viable insulin-producing cells even after 170 days in immune-competent mice.

#### Microwell Textured Porous Flat Membranes for Immunoprotection and Macro-encapsulation of Pancreatic Islets

K. Skrzypek<sup>1</sup>, M. Groot Nibbelink<sup>2</sup>, A. van Apeldoorn<sup>2</sup>, D. Stamatialis<sup>1</sup>

<sup>1</sup>Biomaterials Science and Technology, University of Twente, Enschede, NETHERLANDS, <sup>2</sup>Developmental BioEngineering, University of Twente, Enschede, NETHERLANDS.

**Objectives:** Clinical islet transplantation (CIT) is accompanied by an immunosuppressive drug protocol to avoid rejection of allogeneic donor cells. The lifelong intake of these drugs increase the risks of infections, and certain types of cancer, and limit the treatment of type 1 diabetes with CIT to patients suffering from severe glycemia unawareness and kidney failure. We are developing a macro-encapsulation device based on thin membranes, able to shield donor islets from the host immune cells without compromising their glucose responsiveness and survival.

**Methodology:** Polyether sulfone (PES) based microwell textured flat membranes were fabricated and used for encapsulation of mouse MIN6 insulinoma cell aggregates mimicking islets of Langerhans. Hydraulic permeance and transport of glucose and insulin were determined. Insulin secretion of MIN6 cells was evaluated by a glucose stimulated insulin secretion test.

**Results:** MIN6 aggregates (150  $\mu$ m) were successfully encapsulated inside microwell textured flat membranes of approximately 150  $\mu$ m thickness. The dimensions of the microwell were designed based on the size of the islets. The membrane porosity was optimized for efficient glucose and insulin transport enabling a proper endocrine response at 1 and 7 days of culture.

**Conclusions:** Transport measurements indicate that glucose and insulin are able to diffuse through the porous PES membrane. The MIN6 cell aggregates confined to the microwell structures were able to respond to alternating glucose concentrations, suggesting microwell textured PES membranes are suitable for encapsulation and immunoprotection of islets.

**Acknowledgments:** This research is funded by JDRF, grant number: 17-2013-303.

#### High Islet Density within a Tissue Engineered Device has a Negative Impact on Viability and Function *In Vivo*

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Islet transplantation is emerging as a promising treatment option for patients with Type 1 Diabetes. However, there are limitations including human islet availability and the need for lifelong immu-

nosuppression. The use of immunoisolating devices and alternative cell sources (such as porcine or human derived stem cell islets) offer potential to overcome these limitations. However, the use of these devices (at least in theory) is impractical due to the size needed to effectively oxygenate the cells within them so as to support their viability and function in humans.

In this study, we utilize the TheraCyte immunoisolating device to test the effect of islet density on islet viability and function post-transplant. Devices were loaded with 500, 2000, 4000, or 8000 human IE/cm<sup>2</sup> and then transplanted subcutaneously using a nude rat model. The devices were explanted after 7 days and assessed for islet viability by histology, oxygen consumption rate (normalized to DNA, OCR/DNA), and function by insulin secretion rate measurements. Histological examination confirmed minimal islets (tissue) remained viable within the high density devices (containing 2000 IE/cm<sup>2</sup> or more). Any remaining tissue stained positive for cleaved caspase-3 (apoptotic marker) that was co-localized with insulin. Insulin secretion and OCR/DNA were substantially decreased (~10–20% relative viability as a percent of 500 IE) from explanted devices containing more than 500 IE/cm<sup>2</sup>. These results suggest innovative approaches are needed to supply the devices with oxygen to achieve high cell density loading without compromising cell viability and function.

**Acknowledgments:** Project supported by JDRF: 5-2013-141 and NIH SBIR P1 R43 DK100999.

#### Engineered *In Situ* Oxygen Generator Reduces Pro-Inflammatory Activation of Pancreatic Islets under Hypoxia

M. M. Coronel, C. Stabler;

Biomedical Engineering, University of Florida, Gainesville, FL.

Clinical Islet transplantation has the potential to restore glycemic control on type 1 diabetic patients; however, long-term success has been hindered by many factors, including poor oxygenation. Hypoxic beta cells experience stress, which elevates production of abnormal proteins and pro-inflammatory cytokines that can increase their vulnerability to immune-mediated destruction. Thus, beta cell therapies can benefit from engineered oxygen delivery platforms that provide optimal oxygenation. We have developed an *in situ* oxygen generating biomaterial capable of generating oxygen tensions comparable to normoxic culture conditions for extended time periods. In this study, we sought to investigate the effects of our oxygen generator on the pro-inflammatory activation of *in vitro* cultured pancreatic islets exposed to hypoxia. For this purpose, pancreatic islets were co-cultured with or without our oxygen generator at two oxygen tensions: 0.01 mM oxygen, hypoxic conditions; or 0.2 mM, normoxic conditions. Exposure of islets to hypoxia resulted in upregulated mRNA expression of iNOS and CCL2 inflammatory factors in control group; however, when the oxygen generating material was present, iNOS and CCL2 levels were comparable to normoxic controls. Exposure of islets to hypoxia induced the release of known pro-inflammatory cytokines IL-6, IL-8, and MIP1- $\alpha$ , whereby induction of these pro-inflammatory markers could be damped by the introduction of our oxygen generator. Herein, we have illustrated the ability of our oxygen generator to not only enhance viability, but to also mitigate the pro-inflammatory activation of beta cells under hypoxia. This can be highly beneficial in protecting the islet graft from the local immune reaction.

#### Session: *In Vitro* Model Microsystems for Tissue Engineering

Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM

#### Development and Application of an Osteochondral Microphysiological System

R. S. Tuan;

Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Degenerative joint diseases, such as osteoarthritis, represent a major cause of physical disability. Discovery and development of disease modifying osteoarthritis drugs (DMOADs) has been hampered by the lack of experimental models that accurately represent disease pathogenesis and tissue pathology. This presentation will report on the development and characterization of a recently developed 3-dimensional osteochondral microphysiological system (OC-MPS), constructed using adult human stem cells and photocrosslinked polymeric scaffolds. The OC-MPS exhibits physiological response to pro-inflammatory cytokines, including interleukin-1 beta, as well as hormonal influences. The OC-MPC is being optimized in terms of its capability to respond to mechanical load and interaction with other MPS's. The improved throughput, human cell composition, uniformity, and experimentally accessibility of the OC-MPC support its potential utility for the study of osteoarthritis pathogenesis, and its application as a platform for the screening of candidate DMOADs. (Funding support: NIH U18 TR000532)

### Organ-Specific ECM Constructs by Self-Assembly for the Study of Stromal Induction of Epithelial Maturation

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Surgery, Laval University, Quebec, QC, CANADA.

**Objectives:** Synthetic extracellular matrices have many shortcomings and do not replicate normal extracellular matrix (ECM) biology. The self-assembly method for ECM formation using stromal cells, closely mimics natural ECM. In this study, we studied the impact of organ-specific stromal cells on the maturation of genitourinary epithelium and compared it to non-specific stromal cells.

**Methods:** Epithelial and stromal cells were individually isolated from human skin, bladder and vaginal biopsies. Stromal cells were cultured with ascorbic acid to form collagen sheets that were superimposed to form a single construct, followed by seeding of epithelial cells. Four types of equivalents were formed (dermal stroma/urothelium, bladder stroma/urothelium, dermal stroma/vaginal epithelium, vaginal stroma/vaginal epithelium). All four equivalents were cultured at the air-liquid interface to allow maturation and further evaluation. The analyses of all equivalents included histological and functional assessments for epithelial maturation and differentiation.

**Results:** Different stromal sheets were formed and could be handled easily. Regarding urothelium, a well-stratified epithelium was formed on both stromas; however, it was more differentiated when placed on bladder stroma than on dermal fibroblasts as shown by expression of CK20 and lack of expression of CK14. As for vaginal epithelium, it was better stratified and differentiated when it was grown on vaginal stroma than dermal stroma. Mucin and glyco-gen production were seen only with vaginal stroma.

**Conclusions:** Organ-specific stromal cells and ECM induce more efficiently their corresponding epithelium maturation. Self-assembled Organ-specific ECM biomimetics are novel models for studying mesenchymal-epithelial interactions involved in tissue development, repair and regeneration and epithelial differentiation.

### Substrate Stiffness Effect on the Epigenetic Conversion of Fibroblasts into Insulin-producing Cells

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Diabetes mellitus is a diffused metabolic disorder, with severe secondary complications. Islet cell transplantation represents an alternative to long-term exogenous insulin administration. We recently developed a method to convert fibroblasts into insulin-secreting cells (EpiCC), using the DNA methyltransferase inhibitor, 5-aza-cytidine (5-aza-CR), followed by a three-step pancreatic induction protocol. Here we investigate the effect of substrate stiffness on the maturation of EpiCC. Murine skin fibroblasts were plated either on standard plastic dish (group A) or on polyacrilammide based gel (PAA) of

low stiffness (1 kPa) (group B). After 18-hour exposure to 1  $\mu$ M 5-aza-CR, cells were subjected to pancreatic induction for 10 days. At the end of pancreatic induction all EpiCC modified their typical fibroblast elongated shape and acquired an epithelioid morphology. However, while A cells remained monolayer, B cells formed three-dimensional spherical structures, reminiscent of *in vitro* cultured pancreatic islets. The latter showed a significant increase of pancreatic hormone-positivity ( $82.83 \pm 6.8\%$  vs.  $26.86 \pm 5.8\%$ ) and became mono-hormonal ( $65.33 \pm 2.5\%$ ) with only  $17.5 \pm 0.98\%$  persisting in a poly-hormonal mode. In contrast, 100% of A cells remained poly-hormonal. Glucose triggered insulin release was significantly higher in B EpiCC ( $262.57 \pm 0.79$  mU/ $\mu$ gDNA) than in A ( $171.22 \pm 0.9$  mU/ $\mu$ gDNA). Overall, these data demonstrate that a soft substrate is able to improve pancreatic differentiation and promotes the acquisition of a mono-hormonal mode, which is associated with a mature pancreatic phenotype.

### Novel *In Vitro* Exercise Model of Engineered Human Skeletal Muscle

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Exercise is critical to maintenance of muscle function and mass and is an effective intervention against multiple chronic diseases including obesity, type II diabetes, and sarcopenia. *In vitro* models of exercised human muscle have the potential for discovery and testing of new therapeutic targets for metabolic and muscle wasting disorders. Recently, starting from primary myogenic cells we engineered the first electrically and chemically responsive, contractile human skeletal muscle tissues ("myobundles") (Madden *et al.*, 2015). Here, we present a novel *in vitro* platform to evaluate acute and long-term effects of exercise on myobundle metabolism and function. We evaluated a range of stimulation frequencies, 1–40 Hz, during chronic (1 week) stimulation of myobundles and found that all frequencies led to significant (3–5 fold) increase in myobundle contractile force amplitude compared to unexercised control. The increase in force generation was at least in part attributable to myofiber hypertrophy (25% increase in cross-sectional area), lengthening, and improved sarcomeric organization. Furthermore, a genetically encoded calcium sensor, GCaMP6, was used to non-destructively assess calcium transients of exercised and control myobundles. Significant increase in calcium transient amplitude during twitch and tetanus contractions was found in exercised versus control myobundles. Of all the applied stimulation regimes, high frequency stimulation shortened the contraction relaxation time (20–25%). This novel *in vitro* model of working human engineered muscle will be applied to study exercise-related effects on muscle metabolism and regeneration towards the discovery of new therapeutics against muscle loss and metabolic dysfunction.

### The Impact of Heterotypic Cell Communication Between MSCs and Mature Cells

E. A. Otte<sup>1,2</sup>, A. R. Pinto<sup>3</sup>, N. A. Rosenthal<sup>3</sup>, J. J. Cooper-White<sup>1,2</sup>;

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An important and under-examined aspect of the microenvironment driving cell fate is the presence of different cell types. There are well known relationships between functional cells (such as cardiomyocytes) and supporting cells (such as cardiac fibroblasts) but the nature, extent and specific effects of communication between cell types is not well defined, particularly with respect to differentiation. Presented here is a novel microfluidic device that allows the impact of cell-cell contact and paracrine signalling on cell behaviour to be examined. Standard photolithographic techniques were used to create the simple and highly reproducible device from PDMS and glass. As the device is designed to run under perfusion, cultures can be maintained for extended periods and easily assayed for end-point analysis using

standard stains or antibodies to cell surface antigens. The device has been applied to co-cultures of mesenchymal stem cells (MSCs) with a number of differentiated cell types including endothelial cells and osteoblasts. Results show the importance of heterotypic communication in MSC differentiation as well as preliminary experiments characterising the connections MSCs make to facilitate this communication. MSCs are able to form junctions with other cells that are permeable to proteins that have impacts on MSC behaviour. Heterotypic interactions impact both MSCs and mature cells in co-culture, with improved outcomes from a tissue engineering perspective. Stem cell behaviour is strongly impacted by interactions with differentiated cells and understanding the mechanism of these interactions could lead to improved practices in tissue engineering.

### Generation of Stably Expandable Human Neural Progenitor Cells for Innervating Multiple Tissue Models

D. M. Cairns, D. Kaplan;

Biomedical Engineering, Tufts University, Medford, MA.

Many tissue engineered models have been developed for a variety of highly innervated tissue types such as the skin, muscle and intestine. Despite the recognized importance of innervation within these tissues, most *in vitro* systems do not include a functional neuronal component. Major reasons for this include the lack of a stable source of human neurons, the relative difficulty in culturing and differentiating stem cells, and the inability to maintain a neuronal phenotype within complex tissues. This study describes an optimized protocol for generating stable human neural progenitors using primary cells such as fibroblasts or adipocytes. These human induced neural progenitors (hiNPs) can be passaged and expanded indefinitely as colonies on feeder layers. Once removed from feeders, these cells exclusively and efficiently differentiate into functional neurons in under one week. hiNPs can be differentiated and subsequently incorporated into relevant tissue models, or alternatively can be seeded as neural progenitors and allowed to differentiate within the engineered tissue. Preliminary studies have shown that hiNPs can successfully innervate 2D skeletal muscle as well as 3D skin equivalent models. Furthermore, because hiNPs are generated using somatic cells as starting material, this technique could be expanded to harvest primary cells from specific patients for the future study of disease models related to innervation.

### Session: Integrated Cell Systems for Biological Machinery and Regenerative Therapies

Date and Time: Thursday, September 10, 2015,  
10:30 AM - 12:00 PM

### Modular Inductive High-Density Cell Culture Systems for Engineering Complex Tissues

E. Alsberg;

Biomedical Engineering, Case Western Reserve University, Cleveland, OH.

High-density cultures of cells can mimic immature condensates present during many developmental processes. Presenting specific soluble signals, such as growth factors, exogenously in tissue culture media can regulate cell behavior in these cultures and promote new tissue formation. However, shortcomings of this approach include transport issues, limited spatial control over signal presentation, and required repeated dosing in the media. We have engineered technology that overcomes these challenges by incorporating polymer microspheres containing bioactive signals within the high-density cell cultures, which permits localized spatial and temporal control over the presentation of regulatory signals to the cells. In this talk, I will present our research using this strategy to engineer a variety of tissues, including bone, cartilage and trachea. The capacity to deliver a diversity of signals, including growth factors and plasmid DNA, for driving new tissue formation will be demonstrated. In addition, this technology will be shown to have value in engineering a wide range of tissue shapes, including spheres, sheets, rings and tubes. Finally, the

utility of providing cell-instructive bioactive factors in a controlled manner in assembly of modular tissue units for engineering complex constructs comprised of multiple tissue types will be explored.

**Acknowledgments:** This work was supported by the National Institutes of Health (R01AR063194), the Department of Defense Congressionally Directed Medical Research Programs (OR110196), the AO Foundation, and a New Scholar in Aging grant from the Ellison Medical Foundation.

### Microvascular Networks for 3D Integrated Biological Systems

J. Whisler<sup>1</sup>, M. B. Chen<sup>1</sup>, R. D. Kamm<sup>2</sup>;

<sup>1</sup>Mechanical Engineering, MIT, Cambridge, MA, <sup>2</sup>Biological Engineering, MIT, Cambridge, MA.

Tissue engineered constructs or organ models often require some form of vascularization in order to maintain viability of the cells or to allow for the exchange of secreted factors. Several approaches have been developed over the years including the casting of networks within a hydrogel matrix that can subsequently be lined with vascular cells, and the growth of networks from cells seeded either on the side of the gel by angiogenesis, or from cells uniformly suspended in gel by a process more akin to vasculogenesis. Our previous work has followed the second path in producing networks within microfluidic platforms that can be perfused within several days of seeding. These networks can be grown in various matrices and either with endothelial cells in co-culture with other cell types or in isolation. To date, the best results have been obtained by co-culture with normal lung fibroblasts in separate chambers, using a fibrin-based extracellular matrix. Recently, these systems have been scaled up to mm-sized regions and the fibroblasts have been co-seeded with the endothelial cells, leading to vascularized and perfusable networks with potential applications *in vitro* organ-on-chip systems. Support from the National Science Foundation (CBET-0939511) is gratefully acknowledged.

1. Whisler JA, Chen MB, Kamm RD. Control of perfusable microvascular network morphology using a multiculture microfluidic system. *Tissue engineering Part C, Methods* 20, 543, 2013.

### 3D Printed Neuromuscular Biological Machines

C. Cvetkovic<sup>1</sup>, M. H. Rich<sup>2</sup>, H. Kong<sup>2</sup>, R. Bashir<sup>1</sup>;

<sup>1</sup>Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, <sup>2</sup>Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL.

Combining cells and tissues with soft robotics can enable the fabrication of biological machines with the ability to sense and process signals, as well as produce force, in response to external signaling. The demand of these machines to respond to stimuli and exhibit controlled movement merit the use of skeletal muscle, which does not demonstrate significant spontaneous contractility, as a contractile power source. We report the development of a skeletal muscle powered machine ('bio-bot') capable of biomimetic and directional locomotion, as well as a method for attachment of motor neuron-containing embryoid bodies (EBs) for neuromuscular junction (NMJ) formation. This demonstration aids in the realization of forward-engineered integrated cellular machines and systems, which can have myriad applications in drug screening and programmable tissue engineering. The 3D printed 'bio-bots' were powered by the actuation of a mammalian skeletal muscle strip in response to electrical stimulation, which was harnessed to trigger contraction of cells and net locomotion of the bio-bot with a velocity of over 1.5 body lengths/minute. This engineered hydrogel-muscle platform is ideal for introducing different cell types and biomaterials. We demonstrate preliminary methods for integrating motor neuron-containing embryoid bodies onto the bio-bots, an important first step leading to innervation of the muscle. Integration of motor neurons was achieved by differentiating mouse embryonic stem cells into motor neurons. The neuron systems were then co-cultured with muscle strips differentiated in parallel. Resulting NMJs can lead to more complex functional outputs as well as specific means of control over muscle contraction and bio-bot locomotion.

### Nitric Oxide Signalling Enhances Cardiomyocyte Reprogramming of Adipose Derived Stem Cells

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Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, AUSTRALIA.

Reprogramming of adult stem cells may provide a source of cardiomyocytes for cardiac repair. Previous studies have shown that three defined transcription factors, GATA4, MEF2C, and TBX5 (GMT) can directly reprogram fibroblasts into cardiomyocyte-like cells (CLCs). Here, we demonstrate that manipulation of the nitric oxide (NO) signalling pathway can enhance GMT driven reprogramming of adipose derived stem cells (ASCs) to CLCs. NO is an important signalling molecule which can promote stem cell differentiation and endothelial nitric oxide synthase (ENOS) is one of the three enzymes responsible for producing NO, and is located in a membrane caveolae in a tight conformation with the caveolin 1 (CAV-1) protein. This ENOS-CAV-1 interaction inhibits NO production. To enhance NO production we modified the CAV-1-ENOS interaction to be non inhibitory by co-expressing a mutated caveolin protein (CAVF92A) with ENOS. Co-expression of ENOS and CAVF92A increased the production of NO ( $3.08 \pm 0.1 \mu\text{M}$ ) significantly ( $p < 0.05$ ) compared to ENOS alone ( $1.08 \pm 0.08 \mu\text{M}$ ). There was no significant difference between cells co-transfected ENOS and CAV-1(wt).

The effect of NO on reprogramming process was monitored by GFP expression using a cardiac specific alpha-MHC promoter. Reprogramming was further modified by culturing transduced cells within a fibrin hydrogel. Our data shows that cells co-expressing ENOS and CAVF92A significantly enhanced GMT driven reprogramming compared to reprogramming without NO which was evident with alpha MHC-GFP expression appearing after 3 days and increasing numbers of fluorescent cells up to 4 weeks demonstrating a cardiomyocyte like morphology combined with alpha actinin immunostaining.

### Cell Fibers for Cell Therapies

S. Takeuchi;

IIS, UTokyo, Tokyo, JAPAN.

Fiber based assembly is broadly attractive in various industrial fields including clothes, optical communications, and minimal invasive operations with endoscopes. Here, I propose versatile fiber-shaped cellular building units, named "cell fiber". The cell fiber was produced using a double coaxial laminar flow microfluidic device. Using the device, to treat diabetes mellitus, we prepared a pancreatic islet cell fiber using primary rat dissociated islet cells; in this case, we used an alginate-agarose interpenetrating network (IPN) hydrogel as the shell of the fiber. Using a microcatheter, the primary islet cell fiber was injected into the subrenal capsular space of a diabetic mouse. As a result, we found that the implanted fibers normalized blood glucose concentrations. In contrast, simple injection of a number of dispersed primary islet cells equivalent to the cells in the fibers failed to normalize blood glucose concentrations. Moreover, we found that the removal of the fiber caused the immediate reappearance of hyperglycaemia in all of the mice. Thus, our cell fiber could be useful for applications in medical transplantation using regenerated glucose-responsive insulin-secreting cells and could potentially be used as a removable graft when the cell fiber is covered with a stable shell *in vivo*.

### Development of Vascularized iPSC Derived 3D-Human Tissue Constructs for Regenerative Medicine

M. Matsusaki, M. Akashi;

Department of Applied Chemistry, Osaka University, Osaka, JAPAN.

*In vitro* development of highly-organized three dimensional (3D)-engineered tissues consist of multiple types of cells and ECM, which possess a similar structure and function as natural tissues, is a key challenge for tissue engineering and pharma-

ceutical assay. Especially modulation of 3D-cell-cell interaction inside the 3D-artificial tissues is one of the significant issues. We have developed a simple and unique bottom-up approach, "hierarchical cell manipulation", using nanometer-sized Layer-by-Layer films consisting of fibronectin and gelatin (FN-G) as a nano-extracellular matrix (nano-ECM). The FN-G nanofilms were prepared directly on the cell surface, and we discovered that at least 6 nm thick FN-G films acted as a stable adhesive surface for adhesion of the second cell layer. We have also developed a rapid bottom-up approach, "cell-accumulation technique", by a single cell coating using FN-G nanofilms, because the fabrication of two-layers (2L) was limitation through the above technique due to the time required for stable cell adhesion. This rapid approach easily provided more than twenty-layered 3D-tissues after only one day of incubation. Moreover, fully and homogeneously vascularized tissues of 1 cm width and over 100  $\mu\text{m}$  height were obtained by a sandwich culture of the endothelial cells. The both manipulations will be promising to achieve one of the dreams of biomedical field, *in vitro* creation of artificial 3D-tissue models for regenerative medicine. We are now performing construction of vascularized 3D-induced pluripotent stem cell (iPSC) derived cardiac myoblast tissues as an implantable cardiac tissue for regenerative medicine and a 3D-cardiac tissue model for pharmaceutical assessments.

### Session: Lessons Learned from Regenerative Medicine Therapies in Veterinary Patients (Part 2)

Date and Time: Wednesday, September 9, 2015, 4:00 PM - 5:30 PM

#### Stifle Injuries Treated With Regenerative Therapy with or without Arthroscopic Surgery Fare Better Than with Arthroscopic Surgery Alone: A Study of 98 Horses

R. Rich, E. Carpenter, M. A. Vidal;

CAVE CREEK EQUINE SURICAL CENTER, Phoenix, AZ.

Stifle injuries are common in performance horses, usually demand long recovery times and may prematurely end the career of an equine athlete. To-date there are no reports describing the combinatorial use of adipose tissue-derived stromal vascular fraction (SVF) and interleukin-1 receptor antagonist protein (IRAP) for equine stifle injuries. Medical records of 98 horses (189 stifle injuries) were evaluated. These horses were treated with regenerative medicine (SVF and IRAP) (group 1, n=21 horses) or arthroscopy and regenerative medicine (group 2, n=38 horses) or arthroscopy (group 3, n=39 horses). All stifle injuries were diagnosed through lameness examination, diagnostic anesthesia and radiographs, and some via ultrasound, nuclear scintigraphy, and MRI. All horses completed a standardized, 6-month rehabilitation program. Horses that returned to full work (RFW) for >1 year, without recurrent lameness were considered a success. In group 1, 2, and 3, 66.7%, 68.4% and 59% of horses, respectively, RFW at prior or higher level of performance for >1 year. Approximately 28% of horses RFW at a lower level and 5% of horses in groups 1 and 2 and 13% of horses in group 3 were retired. Pre-operative use of corticosteroids and lameness score negatively affected return to prior level of performance. Lameness and chondromalacia reduced the chance of healing within 270 days. Treatment with regenerative therapy and arthroscopy or just regenerative therapy improved the chance of healing in <270 days compared to arthroscopy alone.

### Equine Bone Marrow and Adipose Tissue Mesenchymal Stem/Stromal Cells Putatively Derive from Perivascular Stem Cells

C. L. Esteves<sup>1</sup>, T. Sheldrake<sup>1</sup>, L. Sawson<sup>1</sup>, T. Menghini<sup>1</sup>, N. Khan<sup>2</sup>, B. Peault<sup>2</sup>, F. X. Donadeu<sup>1</sup>;

<sup>1</sup>The Roslin Institute, The University of Edinburgh, UNITED KINGDOM, <sup>2</sup>Scottish Centre for Regenerative Medicine, The University of Edinburgh, UNITED KINGDOM.

Mesenchymal Stem/Stromal Cells (MSCs) derived from bone marrow (BM-MSC) and adipose tissue (AT-MSC) have been used in

equine regenerative medicine for more than a decade now and, although efforts have been made to characterize equine MSCs the true origin of these cells has not been elucidated. Recently, Perivascular Stem Cells (PVSCs) were identified as a native source of MSCs in multiple human tissues, and it was shown that they may provide a superior source for regenerative therapy. The present study aimed at determining 1) if equine MSCs express PVSC markers and 2) whether the expression of these markers is maintained during culture. Equine adipose samples were analysed by Immunohistochemistry (IHC) and this showed that both MSC (CD73, CD44, CD29) and PVSC (CD146, NG2) markers were present and had a perivascular location. MSCs were obtained by culturing equine BM and AT stromal vascular fraction. Flow cytometry and qPCR analyses showed that the expression of MSC (CD105, CD73, CD90, CD44, CD29) and PVSC (CD146, NG2 and PDGFR $\beta$ ) markers was indeed maintained in the cultured MSCs, with no differences being observed between BM- and AT-MSCs. Importantly, dual-antibody IHC and flow cytometry indicated that

PVSC (CD146, NG2,  $\alpha$ SMA) and MSC (CD105, CD44, CD29) markers co-localised in MSCs, indicating that MSCs originate from perivascular cells and, notably, maintain their immunophenotype in culture. In view of these findings, we suggest that, in addition to MSC markers, PVSC markers should be considered when assessing and characterising equine MSCs.

### Refinement of Preclinical Animal Models

J. T. Easley, R. H. Palmer;

Clinical Sciences, Colorado State University, Fort Collins, CO.

The three R's of animal research are Replacement, Reduction, and Refinement. Research teams' adherence to the guiding principles of the three R's when utilizing translational animal models is a characteristic of humane and ethical study. The first two R's, Replacement and Reduction, are often based on thorough literature review and accurate statistical analysis. Effective Refinement should include specific strategies to design research studies that maximize animal welfare while yielding highly relevant information as well as to most effectively detect and treat pain in research animals in those studies. Conscientious adherence to these guiding principles of humane use of animals in research demands that we continually pursue a goal of alleviation of animal pain and stress in our research programs. This is a lofty goal in research studies that utilize translational animal models to surgically induce specific disease states or to evaluate innovative new biologic or regenerative therapies. Translational animal models are vital to the forward progress of medicine. Discovery of novel therapies for human musculoskeletal diseases relies heavily upon these animal models to ensure safety and efficacy prior to human clinical use. Animals often make the "ultimate sacrifice" to benefit the health of humans. It is, therefore, the responsibility of the research team to REFINED their animal studies to ensure the welfare of the research animals under their care and to maximize the impact of the information gained. Steadfast dedication to refinement is a hallmark of quality in preclinical animal research.

### Comparative Structural and Histological Evaluations of Osteoarthritic Knee Joints after 6 weeks, 4 months and 8 months Implantations of Chondrogenically Induced Cells

C. C. Ude<sup>1</sup>, S. B. Sulaiman<sup>2</sup>, C. H. Chen<sup>3</sup>, R. A. Rani<sup>4</sup>, J. Ahmad<sup>4</sup>, N. M. Yahya<sup>4</sup>, A. B. Saim<sup>5</sup>, R. B. Idrus<sup>1</sup>;

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**Objectives:** Improved gross and histological outcomes can be used to predict effective regenerative treatments. We evaluated the

effectiveness of chondrogenically induced adipose stem cells (ADSCs) and bone marrow stem cells (BMSCs) in the treatment of surgically induced osteoarthritis.

**Methodology:** Osteoarthritis was induced at the right hind knee joints of thirty uncastrated male sheep (Siamese long tail) divided into 6 Controls, 12ADSCs and 12BMSCs by the complete resections of the anterior cruciate ligament and medial meniscus, followed by exercise regimen. Stem cells labelled with PKH26 were induced into chondrogenic lineage before autologous implantation into the defects. Evaluations were made after 6 weeks, 4 months and 8 months post-treatments.

**Results:** The arthroscopic images before treatments showed that all groups had developed severe subchondral lesions. From the 6th week post treatment, the treated knee joints revealed roughly arranged regenerated new cartilages. These regenerated cartilages improved in structure at the 4th month, with the greatest maturity in appearance seen at the 8th months post implantation. Their International Cartilage Repair Society (ICRS) scores depicted the gross and histological outcomes; though there was no significant differences among the groups at different durations, the treated groups had significantly lower grades than the controls in all durations ( $P < 0.05$ ). The resected portions of the regenerated cartilages in the treated groups revealed the fluorescence of PKH26 dye, while the chondrogenic protein analyses via immunohistochemistry proved positive.

**Significance:** The chondrogenically induced multipotent stem cells provided evidence of structural regenerations of osteoarthritis, which did not degenerate, rather increased in maturity and durability with time.

### Longitudinal Cell Tracking by Magnetic Resonance Imaging following Treatment of Induced Tendon Lesions

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*In vivo* cell tracking is of major importance to gain a better understanding on the biodistribution of cells applied as therapeutic agents. Magnetic resonance imaging (MRI) offers the opportunity to visualize iron-oxide labeled cells and the surrounding tissues at the same time. Furthermore, it is a gold standard technique for soft tissue imaging.

In this study, iron-oxide/rhodamine labeled autologous multipotent mesenchymal stromal cells (MSC) were injected into induced tendon lesions in 6 horses. Animals were examined by MRI prior to and immediately after cell injection, as well as 1, 2, 3, 4, 8, 12 and 24 weeks later. During the first week following cell injection, peripheral blood samples were collected and assessed by flow cytometry. Tendon biopsies taken at week 3 and tendon samples collected at week 24 were assessed by histology.

Hypointense artefacts corresponding to the applied cells were distinctively visible at the injection site. They decreased over time, but still remained visible over the whole follow-up period. Best traceability of the cells was achieved combining T1 and T2\* weighted sequences and using the magic angle effect, positioning the limb in a 55° angle to the magnetic field. Very few rhodamine labeled cells were detectable in the peripheral blood. Histology showed that labeled cells were integrated in the tendon tissue.

This study demonstrates the feasibility of MRI cell tracking in the equine tendinopathy model. The results obtained give insight into MSC biodistribution and support the hypothesis that a major part of applied cells remains at the injection site.

### Biomimetic Porous Titanium Scaffolds or Large Bone Critical Defect Reconstruction: an Experimental Study

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The goal of the treatment of large bone defect is directed to guarantee a precocious loading of the affected limb. In the present paper the authors propose a new technique to reconstruct through the use of Biomimetic porous titanium scaffold custom made with Electron Beam melting technology that appears to be suitable to reach the purpose.

A complete resection was practiced in the diaphysis of the right tibia of six sheep and replaced with a five centimeters framework of EBM-sintered titanium. The outcome was followed-up by periodical X-ray and clinical investigations with a follow-up of 12 months. At 9 months the plates were removed, at 12 months the sheep were euthanized and the bones were subjected to histological and immunohistochemical investigations. The post operative X-ray showed a good position of the plate and the proximal and distal border of the scaffolds were perfectly adherent to the bone to permit the process of osteointegration. After surgery the sheep were allowed to move freely in the stables. Nine months after the X-ray showed a remodelling of periosteal callus with a well-defined cortical bone and the scaffolds were completely integrated in the diaphysis. The histological investigations were executed on bone-metal interface and showed bone growth among the titanium bars, bone trabeculae have bridged the titanium trabeculae suggested a very good tissue-metal interaction. In conclusion this implant, used to repair large bone critical defects in a large animal model, can guarantee the immediate body-bearing, a precocious functional recovery and a good osteointegration.

### Session: Nanostructured Biomaterials and Functional Surfaces (Part 1)

**Date and Time: Wednesday, September 9, 2015, 1:00 PM - 2:15 PM**

#### A One-step Approach for Multimodal Functionalization of Electrospun Nanofibers and their Application for Immune-regulatory Surfaces

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Electrospinning is a well-established cost efficient method to produce polymer meshes with high surface area composed of fibers with diameters from micro to nanometer scale. We have described before that a hexafunctional reactive macromolecular additive, six-armed star-shaped poly(ethylene oxide-*stat*-propylene oxide) prepolymers with isocyanate groups at the distal ends of polymer chains, can be used during electrospinning of biodegradable polyester solutions for selective enrichment at and bioactivation of the fiber surface.

In this study we demonstrate that the isocyanate reactive groups can be exploited for multimodal fiber surface functionalization within one fiber preparation step through adding cell-adhesion mediating peptide sequences into the polymer solution before spinning, followed by subsequent incubation of freshly spun mesh into a solution that contains the biomolecules of interest to be immobilized.

Besides the published reduced protein adsorption and controllable cell attachment we show a three-fold functionalization method illustrating the results with fluorescence staining accompanied with specific, controlled protein linkage via biocytin-streptavidin system. Furthermore, we use this approach to generate biochemical activity by dual immobilization with different TNF $\alpha$ -antibodies and detection over *Gaussia Luciferase* activity, and we demonstrate altered interleukin-8 production of HT1080 cells as response to the presented immobilized antibodies.

In summary, we establish a simple and robust strategy to independently tune cell adhesiveness and biochemical activity of electrospun meshes with only one fiber preparation step by using a

reactive macromere additive. With the demonstrated effect on IL-8 production of adherent cells *in vitro* we envision that such meshes could be used as immunomodulatory wound dressing materials.

#### The Effect of Transplantable and Biodegradable Nanopatterned Patches to Study Tissue Engineering and Regenerative Medicine

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To fabricate biologically responsive biomaterials with physiological environment for regeneration of specific tissue structure, we focused on the nanotopographical cue that has been used to enhance cell migration and differentiation. In this study, we manufactured that biodegradable nanopatterned patch (BNP) which was applied as biomimetic scaffold or drug delivery carrier was prepared with poly (lactic-co-glycolic acid) by capillary force lithography. It is believed that the highly sophisticated physicochemical environment below demonstrated the potential of creating nanotopographical guidance cues to cells functions *in vivo*. Firstly, we employed BNP with surface modification using 3,4-dihydroxy-L-phenylalanine (LD) as an adhesion molecule for fibroblast growth factor-2 (FGF2) delivery. Then, FGF2 loaded LD-BNP was implanted a full-thickness wound ( $\phi$  18 mm) of mouse skin. We confirmed that FGF2 loaded LD-BNP showed much faster wound closure at day 14 in terms of synergetic effect by FGF2 and guidance of nanopatterned patch. Secondly, we implanted only BNP and biodegradable flat patch (BFP) as a scaffold on the mouse calvarial defect ( $\phi$  4 mm) because of it provides biophysical cues similar to extracellular matrix to stimulate osteoblast migration and proliferation. After 8 weeks later, BNP had more broad new bone formation and more compact bone regeneration at defect area compared to BFP. The BNP should be suitable utilized for treating chronic and acute disease such as myocardial infarction, and hind limb ischemia disease. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2055348).

#### Micro-Patterning Directional Extracellular Matrix Cues in Hydrogel Scaffolds for Cardiac Tissue Engineering

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Cardiac tissue engineering seeks to use scaffolds to direct cardiomyocytes (CMs) to form cardiac tissue *in vitro*. While there is significant evidence that spatially well-defined ECM cues (e.g. fibronectin/FN patterns) control CM structure and function on 2D substrate, current 3D scaffold fabrication techniques do not allow the integration of microstructural ECM patterns within natural hydrogels (e.g. fibrin, collagen type I/COL1, Matrigel). We developed a technique to micropattern directional FN cues into hydrogel scaffolds. We hypothesized that hydrogel composition modulates CM response to micropatterned FN cues. To test this, we compared CM alignment in response to FN lines on COL1 or fibrin hydrogels, with or without Matrigel. FN-patterned scaffolds were prepared by microcontact printing FN lines on a sacrificial poly(N-isopropylacrylamide) substrate, which enables transfer onto hydrogels by surface-initiated assembly. Pattern transfer fidelity was assessed by measuring line width. Chick primary CMs were cultured for 4 days onto the patterned hydrogels, then fixed and stained for nuclei, actin, and  $\alpha$ -actinin. Alignment of the actin cytoskeleton was measured using Matlab. We found that micrometer-wide features were preserved after transfer. CMs formed beating sheets with striated sarcomeres. CM alignment along the direction of the FN lines was highest on COL1 vs. fibrin hydrogels and was similar to 2D FN-patterned PDMS controls. Addition of Matrigel resulted in a dramatic loss of anisotropy for both COL1 and fibrin scaffolds. Future work will focus on optimizing the

function of cardiac tissue made from human pluripotent stem cell-derived CMs by using ECM patterns inspired by the embryonic heart.

### Functional Nanofibrous Scaffolds Combined with Stem Cells for Bone and Cartilage Tissue Engineering

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The use of temporary scaffolds in Advanced Therapies and in particular of Tissue Engineering is one of the key issues to regenerate tissue defects. The scaffolds should be specifically designed to create environments that promote tissue development and not merely to support the maintenance of communities of cells. Many biomaterials have been proposed to produce scaffolds aiming the regeneration of a wealth of human tissues. We have a particular interest in developing systems based in nanofibrous biodegradable polymers<sup>1,2</sup>. Those demanding applications require a combination of mechanical properties, processability, cell-friendly surfaces and tunable biodegradability that need to be tailored for the specific application envisioned. In our approach, we combine the temporary scaffolds populated with therapeutically relevant communities of cells to generate a hybrid implant. We are exploring the use of adult MSCs<sup>3</sup>, namely obtained from the bone marrow for the development of autologous-based therapies. We also develop strategies based in extra-embryonic tissues, such as amniotic fluid (AF) and the perivascular region of the umbilical cord<sup>4</sup> (Wharton's Jelly, WJ). Those tissues offer many advantages over both embryonic and other adult stem cell sources. The comparatively large volume of tissue and ease of physical manipulation facilitates the isolation of larger numbers of stem cells. Fetal stem cells seem having more pronounced immunomodulatory properties than adult MSCs. This allogeneic escape mechanism may be of therapeutic value, because the transplantation of readily available allogeneic human MSCs would be preferable as opposed to the required expansion stage (involving both time and logistic effort) of autologous cells.

### Session: Nanostructured Biomaterials and Functional Surfaces (Part 2)

**Date and Time: Thursday, September 10, 2015, 3:00 PM - 4:30 PM**

#### Chondrogenic Differentiation within Magnetic-responsive Multilayered Liquefied Capsules Containing Collagen II/TGF- $\beta$ 3 Coated Microparticles

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The use of stem cells is a promising therapeutic approach for the substantial challenge to regenerate cartilage. Considering the two prerequisites, namely the use of a 3D system to enable the chondrogenic differentiation and growth factors to avoid dedifferentiation, the diffusion efficiency of essential biomolecules is an intrinsic issue. We already proposed a liquefied bioencapsulation system containing microparticles as cell adhesion sites<sup>1</sup>. Here, we intend to use the optimized system towards chondrogenic differentiation by encapsulating stem cells and collagen II-TGF- $\beta$ 3 coated PLLA microparticles. As a proof-of-concept, magnetite-nanoparticles were incorporated into the multilayered-membrane. This can be a great advantage after implantation procedures to fixate the capsules *in situ* with the help of an external magnetic patch and for the follow-up through imaging. Results showed that the production of glycosaminoglycans and the expression of cartilage-relevant markers (collagen II, Sox9, aggrecan, and COMP) increased up to 28 days, while hypertrophic (collagen X) and fibrotic (collagen I) markers were downregulated. The presence of nanofibers in the newly deposited ECM was visualized by SEM, which resembles the collagen fibrils of native cartilage. The presence of the

major constituent of cartilage, collagen II, was detected by immunocytochemistry. Safranin-O and alcian blue stainings revealed a basophilic ECM deposition, which is characteristic of neocartilage. These findings suggest that the proposed system may provide a suitable self-governing environment for chondrogenic differentiation.

**Acknowledgments:** We acknowledge the Portuguese FCT (Ph.D.grant-SFRH/BD/69529/2010-ClaraCorreia) and the European Research Council (grant agreement-ERC-2012-ADG 20120216-321266-ComplexiTE).

### Reference

1. Correia CR *et al.* Multilayered Hierarchical Capsules Providing Cell Adhesion Sites. *Biomacromolecules*14;743,2013.

### Development of a Hydrogel Nanocomposite Scaffold System for Sustained Delivery of Angiogenic Peptides

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A critical criterion for the clinical success of hydrogel scaffolds is their ability to promote rapid and stable neovascularization upon implantation prior to material degradation. Previous studies have shown that controlled and sustained growth factor presentation in hydrogels enhances scaffold neovascularization. A promising alternative to growth factors are synthetic growth factor mimetic peptide sequences designed to interact with the specific receptors growth factors use to regulate neovascularization pathways while possessing lower susceptibility to degradation *in vivo*. A peptide mimetic sequence based on vascular endothelial growth factor (VEGF), QK, has been previously developed which activates the signaling pathway involved in VEGF-mediated neovascularization. Recent 3D cell culture studies indicate that continuous replenishment of soluble QK significantly enhances endothelial cell outgrowth compared to immobilized QK presentation. We have developed a hydrogel-based nanocomposite platform that allows for sustained release of the QK peptide within protease-sensitive hydrogel scaffolds. Specifically, QK peptide was loaded within poly(ethylene glycol) (PEG) hydrogel nanoparticles (NPs) which were subsequently encapsulated within protease-sensitive PEG scaffolds. QK loaded NPs were formed using inverse phase miniemulsion polymerization and allowed peptide release up to 63 days. Alterations in QK release from hydrogel NPs was achieved through increases in PEG crosslinker macromer size or by rendering the macromer hydrolytically degradable. Nanoparticle tracking analysis revealed an average particle diameter of  $155.0 \pm 77.9$  nm; zeta potential was  $-29.6 \pm 5.84$  mV; and NP swelling ratio was  $17.2 \pm 5.0$ . Current studies are investigating the role of variations in QK release kinetics on scaffold neovascularization *in vitro*.

### Unnatural Killer Cells: TRAIL-Coated Leukocytes that Kill Cancer Cells in the Circulation

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**Problem:** Metastasis contributes to over 90% of cancer-related deaths. For metastasis to occur, cancer cells detach from the primary tumor and invade the bloodstream as circulating tumor cells (CTCs). Adhesive interactions between selectins on the blood vessel wall and selectin ligands on the CTC surface facilitate metastatic progression.

**Objective:** Here, we describe a novel approach to functionalize human and murine leukocytes with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), along with E-selectin (ES), to capture and induce cancer cell apoptosis both *in vitro* in human blood and *in vivo* in mouse circulation.

**Methodology:** ES/TRAIL liposomes and cancer cells suspended in human blood were subjected to shear flow, and assessed for viability using flow cytometry (FC). For *in vivo* studies, mice were

injected with ES/TRAIL retro-orbitally, followed by injection of tumor cells. Mouse blood was removed via cardiac puncture, and cancer cell viability was assessed using FC. Cancer cells lodged in lung were quantified using multiphoton microscopy.

**Results:** ES/TRAIL liposomes in human blood functionalized leukocytes, as confirmed by microscopy and FC. Exposure of functionalized ES/TRAIL leukocytes into cancer cell-spiked blood successfully targeted and killed cancer cells under shear flow. M ES/TRAIL-functionalized leukocytes captured and induced apoptosis in cancer cells in mouse circulation. Cancer cells lodged within the mouse lung were apoptotic after treatment with ES/TRAIL leukocytes.

**Significance:** ES/TRAIL-functionalized leukocytes are a novel method to target CTCs in the bloodstream to prevent cancer metastasis. Clinically, this therapeutic strategy can serve as preventive measure upon diagnosis of metastatic hematogenous cancers.

### Immunomodulatory Extracellular Matrix Nanoparticles

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Extracellular matrix (ECM) scaffolds composed of decellularized tissues promote regenerative responses in part by polarizing the wound immune environment. Injectable nanoparticles, traditionally synthetic, can be efficiently delivered with a wide biodistribution. Thus, ECM nanoparticles would rapidly present bioactive ECM components to sites of inflammation. Porcine cardiac and bladder ECM were isolated by acid/detergent decellularization as confirmed histologically. ECM nanoparticles (ECM-NPs) were prepared by high pressure homogenization resulting in z-average diameters of  $464 \pm 28$  and  $720 \pm 12$  nm for cardiac and bladder ECM-NPs, respectively. ECM-NP zeta potential ranged between  $-22$  and  $-35$  mV, and electron microscopy showed globular shapes. ECM-NPs induced mixed classical and alternative macrophage phenotypes *in vitro* via elevated IL-1b, TNF- $\alpha$ , IL-10, and Arg-1 gene expression, which were not induced by polystyrene nanoparticles. ELISA showed dose dependent macrophage TNF- $\alpha$  secretion at  $3.9 \pm 3$  ng/ml, which surpassed classical stimulation alone. ECM-NP macrophage activation was partially mediated by NF- $\kappa$ B signaling as shown by nuclear translocation of the p65 subunit with immunofluorescence. Labeled ECM-NPs were distributed systemically within 30 minutes of mouse footpad injection via *in vivo* imaging, and were observed in secondary lymphoid tissues after 6 days. In summary, a method to prepare ECM nanoparticles from decellularized tissues is described. ECM-NPs from both cardiac and bladder sources are immunomodulatory *in vitro*, inducing a mixed classic/alternative macrophage phenotype distinct from polystyrene nanoparticles. ECM-NP retention and trafficking to lymphoid tissues *in vivo* suggests the potential for systemic immunotherapy in addition to local immune polarization.

### Local Delivery of Flavopiridol in PLGA Nanoparticles Improves the Repair of Spinal Cord Injury through Inhibiting Astrocyte Growth and Inflammatory Factor Synthesis

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The cell cycle inhibitor flavopiridol was previously shown in rat models to improve recovery from spinal cord injury (SCI). However, there are side effects in systematic dosage of flavopiridol, and the underlying mechanism of function is still not illustrated. This study aims to develop a strategy for local delivery of flavopiridol and investigate its mechanisms of function as well. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) was used for the sustained delivery of

flavopiridol. The spinal cords were right-hemisectioned and NPs were administered into the injury epicenter. Transparent spinal cord technology was applied for the three-dimensional observation of the anterograde tracing. The results showed that the sustained release of flavopiridol NPs lasted for up to 3 days *in vitro*. Flavopiridol NPs significantly decreased inflammatory factor synthesis of astrocytes, including TNF- $\alpha$ , IL-1 beta, and IL-6, and the anti-inflammatory IL-10 expression was elevated. *In-vivo* study demonstrated that flavopiridol NPs decreased inflammatory expression and glial scarring, and alleviated neuronal survival and neural transmitting. Cavitation volume was decreased by proximately 90%. Administration of flavopiridol NPs also improved the motor recovery of injured animals. These findings demonstrated that local delivery of flavopiridol in PLGA NPs improved SCI recovery through inhibiting astrocyte growth and inflammatory factor synthesis.

### Promoting Bone Fracture Healing using BIO-Loaded Polymersomes

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One-third of the world population will experience a bone fracture in their life-time. 10% of these injuries will not heal adequately requiring multiple reparative surgeries. Bone fracture repair can be pharmacologically enhanced by activating the Wnt signalling pathway, which is essential in stimulating the production of bone from mature osteoblasts. Wnt can be activated using small molecules, including 6-bromoindirubin-3'-oxime (BIO) however controlled delivery is crucial to avoid off-target effects. Polymersomes (PMs) are nanoparticles composed of biocompatible amphiphilic block copolymers that can be loaded with hydrophilic and hydrophobic compounds, allowing spatially and temporally controlled delivery. We hypothesize that BIO-loaded PMs can activate the Wnt pathway at the bone fracture site enhancing bone healing. PMs made of polyethylene glycol-b-polycaprolactone (5k-b-18k) block-copolymer were produced via nanoprecipitation. PMs were loaded with various concentrations of BIO and tested on a reporter cell line. In addition, PMs were loaded with either fluorescein to assess cellular uptake *in vitro* on human skeletal stem cells, or with DiR to determine *in vivo* distribution in mice with a bone injury. We demonstrated that  $99.70\% \pm 0.29\%$  ( $n=4$ ) of the skeletal stem cells internalise PMs within 3 hours' of incubation. We showed that BIO is stably associated with the PMs, and BIO-loaded PMs induce sustained activation of the Wnt pathway compared to the free drug ( $P < 0.001$ ,  $n=3$ ). DiR-loaded PMs injected systemically accumulate in both fractured bone and liver within 24 hours of administration. Given these results we conclude that BIO-loaded polymersomes could represent an innovative approach to promote bone regeneration after fracture.

### Session: Non-Viral Gene Therapy in Tissue Engineering

**Date and Time: Wednesday, September 9, 2015, 1:00 PM - 2:15 PM**

### Biomaterial Delivery of Exogenous Mir-29b to the Infarcted Myocardium Yields Improved Myocardial Recovery

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Heart failure due to adverse remodelling remains a common complication following myocardial infarction (MI) and there exists

excessive and debilitating fibrosis at the peri-infarct region due to cardiac fibroblast deposition of collagen types I and III. Expression of miR-29b is higher in cardiac fibroblasts than in cardiomyocytes, but is downregulated in the border zone of infarcted hearts. It is therefore feasible to assume that delivery of exogenous miR-29b could inhibit production of collagen types I and III, resulting in less fibrosis. It is the hypothesis of this study that exogenous miR-29b can inhibit debilitating fibrosis following MI. We aimed to deliver miR-29b via localized biomaterial intramyocardial delivery to ensure a clinically relevant delivery, and also to eradicate possible off target effects in other organs.

Transient ischemia was induced in C57BL/6J mice via left ascending coronary artery occlusion. Following reperfusion, miR-29b (100 µg/animal) was delivered within a hyaluronan-based hydrogel (crosslinked using thiol-reactive poly(ethylene glycol) diacrylate) via intramyocardial injection at five infarct borderzone locations. miR-239b was employed as a negative control.

At 14 and 35 days following MI, miR-29b-treated mice showed significantly improved ejection fractions and fractional shortening when assessed using echocardiography. Histological and immunohistological analyses also revealed differences in extracellular matrix patterns. miR-29b could be considered as a possible therapeutic intervention when delivered locally to the infarcted myocardium due to its influence on the remodelling myocardium.

**Reference:** van Rooij E. *et al* Dysregulation of micrornas after myocardial infarction reveals a role of mir-29 in cardiac fibrosis. PNAS 2008;105:13027-13032

#### Highly Efficient Delivery of Functional Cargoes by the Synergistic Effect of GAG Binding Motifs and Cell-Penetrating Peptides

J. E. Dixon<sup>1</sup>, G. Osman<sup>1</sup>, G. E. Morris<sup>1</sup>, H. Markides<sup>2</sup>, M. Rotherham<sup>2</sup>, A. El-Hajj<sup>2</sup>, C. Denning<sup>1</sup>, K. M. Shakesheff<sup>1</sup>;

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Protein transduction domains (PTDs) are powerful non-genetic tools that allow intracellular delivery of conjugated cargoes. As these can afford controllable stoichiometry they are attractive for directing cell fate and unravelling protein function. The use of PTDs in biomedicine has been hampered by inefficient delivery to nuclear and cytoplasmic targets. Here we overcame this deficiency by developing a novel fusion protein that couples a membrane docking peptide to heparan sulfate glycosaminoglycans (GAGs) with a PTD. We showed this GET (GAG-binding enhanced transduction) system could deliver enzymes (Cre, neomycin phosphotransferase), transcription factors (NANOG, MYOD), antibodies, native proteins (Cytochrome-C), magnetic nanoparticles (MNPs) and nucleic acids (plasmid (p)DNA, modified (mod)RNA and siRNA) at efficiencies of up to two-orders of magnitude higher than previously reported in cell types considered hard to transduce, such as mouse embryonic stem cells (mESCs), human ESCs (hESCs) and induced pluripotent stem cells (hiPSCs). We demonstrate that the GET system can be harnessed to promote survival, self-renewal or direct the differentiation of pluripotent cells toward a desired lineage. Furthermore other proteins can be delivered by coupling to GET peptides (such as antibodies) and this can be extended to other chemically distinct targets. Our system is not technically complex, as for modified RNA systems, and offers several key advantages over established techniques to deliver the exogenous function of a gene, protein or delivering other reagents intracellularly.

This technology represents an efficient strategy for controlling cell labelling, and directing cell behaviour that has broad applicability for basic research, disease modelling and clinical application.

#### Nanofiber-mediated MicroRNA Delivery for Modulating Foreign Body Reaction and Host-implant Integration

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Foreign body reaction (FBR) inevitably affects the outcomes of scaffold implantations. Fibrous capsule formation is an outcome which affects host-implant integration and alters scaffold designs, e.g. drug/gene release profiles. Hence, modulating fibrous capsule formation is beneficial for more efficient implantations. Although fibrous capsule formation is mainly associated with the proliferation of fibroblast and collagen deposition, fibroblast-macrophage interaction also plays a critical role. Hence, effective transition between pro-inflammatory M1 and pre-healing M2 macrophage by transcriptional modulation may be promising for controlling FBR. In our study, we hypothesized that miRNA-modulated macrophage transition can control fibrous capsule formation. MicroRNA-incorporated nanofibers (387 ± 57 nm) were fabricated for long-term efficient controlled release of microRNAs (up to 88 days). The efficacy of the scaffolds in modulating *in vivo* fibrous capsule formation was evaluated using a rat subcutaneous implantation model. At 2 weeks post implantation, fibrous capsule formation around the nanofiber scaffolds was evaluated by Masson's Trichrome staining. As a result, incorporation of M2-inducing miRs into nanofibers induced less fibrous capsules (200.26 ± 7.18 µm and 203.05 ± 11.98 µm) than scrambled miR treatment (363.16 ± 15.18 µm, p < 0.001) or plain fiber implantation (319.72 ± 10.81 µm, p < 0.001). In addition, both fibrous capsules around M2-inducing implants were thinner than M1-inducing antagomiR implant (293.21 ± 12.72 µm, p < 0.001). Interestingly, M1-inducing antagomiR reduced fibrous capsule more than scrambled miR treatment (p < 0.001). Altogether, we developed a nanofiber-mediated microRNA delivery strategy to control macrophage polarization and reduce fibrous capsule in FBR.

#### Highly Branched Poly(β-Amino Ester)s as New Gene Delivery Vectors

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One of the main bottlenecks for clinical gene therapy is the lack of safe and efficient gene delivery vectors. Over the last decade, linear poly(β-amino ester)s (LPAEs) have emerged as a new class of gene vectors. However, their linear structure has severely limited the further improvement of their performance. In contrast, various dendritic polymers have demonstrated their great potential over their linear counterparts as gene delivery vectors due to their three-dimensional spatial structure with multiple terminal groups. Herein, a novel type of dendritic polymer, highly branched poly(β-amino ester)s (HPAEs), was developed as a new class of gene vector via "A2+B3+C2" type Michael addition from commercially available 4-amino-1-butanol, trimethylolpropane triacrylates and bisphenol A ethoxylate diacrylate. NMR and GPC results indicate that the composition and structure of HPAEs can be easily controlled and adjusted by simply varying the feed ratio of B2 to C3. HPAEs can effectively condense DNA to form small and stable polyplexes. HPAEs have shown very high gene transfection efficiency and low cytotoxicity over twelve different cell types including primary and neural cells, far more efficient and safe than the commercial transfection reagents SuperFect, Xfect and Lipofectamine 2000. Furthermore, *in vivo* transfection studies revealed that HPAEs can carry therapeutic COL7A1 cDNA to restore the expression of type VII collagen along the basement membrane zone in both recessive dystrophic epidermolysis bullosa (RDEB) knockout and human RDEB graft mouse models. HPAEs are highly efficient and safe gene vectors for gene delivery.

#### Three-year Results of an International, Multicenter, Randomized Clinical Trial of a Pcmv-vegfl65 in Progressive Ischemia Caused by Atherosclerotic Peripheral Arterial Disease: Results from 216 Participants

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Human Stem Cell Institute, Moscow, RUSSIAN FEDERATION.

In 2011 Russian regulatory authorities approved "Neovascugen", the first gene product to treat lower limb ischemia in patients with atherosclerotic PAD. After this we have conducted an

international, multicenter, prospective, randomized, open-label, controlled clinical trial of pCMV-vegfl65 intramuscular gene transfer in patients with PAD not considered suitable for successful reconstructive surgery (NVG-03; NCT02369809). The study enrolled 216 patients with stage 2–3 disease according to Fontaine classification modified by Pokrovsky. The primary endpoint was a pain-free walking distance (PWD). An ankle-brachial index (ABI) and a transcutaneous oxygen tension (TcPo2) were chosen as the secondary endpoints. In this paper the data from 25 study centers is presented. PWD in patients who received pCMV-vegfl65 treatment (n=191) increased steadily throughout the 6-month. The highest average PWD among the test group was observed by the end of the trial (288.6 meters,  $p < 0.05$ ), i.e. PWD increased dramatically by 154%. No statistically significant changes were observed in the control group in spite of conventional therapy. ABI tended to increase dramatically by 66% at 6 month follow-up ( $p < 0.05$ ). TcPo2 in test group brought up by 19%, on the other hand, patients of control group didn't exhibit any positive change. 38 of patients enrolled in the trial gave their consent to participate in the long-term 3-year follow-up study. By the end of the 3rd year the parameter increased by 290% compared to baseline ( $p < 0.05$ ). PWD decreased by 27% within a 3-year follow-up period in control patients who received conventional therapy alone. No adverse events connected with pCMV-vegfl65 were observed.

### Session: Novel Injectable Scaffolds for Tissue Regeneration

**Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM**

#### Use of an Injectable Hydrogel for Transplantation of Human Neuroectodermal Cells to Treat Stroke

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Each year 15 million people worldwide will suffer from a stroke, leading to lifelong functional impairments. Cell transplantation aims to deliver an exogenous source of cells to restore function to the patient. While a promising strategy, most cells delivered to the brain will fail to integrate into the host tissue, and undergo cell death. We have developed a hydrogel composed of a physical blend of hyaluronan and methylcellulose (HAMC) which has been previously demonstrated to improve cell delivery and increase survival in a model of retinal regeneration<sup>1</sup> and spinal cord injury<sup>2</sup>. The goals of this work are to determine if the use of HAMC can: 1) enhance cell viability prior to delivery, 2) limit cell loss during delivery, and 3) increase cell survival when transplanted *in vivo*. A population of human neuroectodermal cells (hNECs) was derived from induced pluripotent stem cells (iPSCs) and the differentiation towards a neuronal lineage was characterized. It was determined that when cells were dissociated and stored on ice encapsulation in HAMC significantly increased survival compared to a saline solution. In addition, when cells were injected through a fine gauge needle into an agarose gel (a simple model of brain tissue mechanical properties), a greater number of cells were delivered when first encapsulated in HAMC compared to those in saline. This study provides an *in vitro* basis for the use of HAMC to deliver hNECs into the stroke-injured brain. In ongoing studies, we are studying cell transplantation in the brain of a rat model of stroke.

#### Degradable and Thermoresponsive Hyperbranched Polymers Synthesized via One-pot RAFT Polymerization for Injectable Scaffolds Applications

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In tissue engineering, injectable scaffolds offer the possibility of homogeneously distributing cells and therapeutic molecules throughout the scaffolds, and can be injected directly into cavities with irregular shapes and sizes. However, the challenge lies in finding suitable materials which can solidify *in-situ* to form 3D microenvironments with the desired mechanical and biological properties. Crosslinkable and smart polymers, which change in response to external stimuli such as temperature, pH and enzyme, have attracted much attention for such applications. The objective of this work is to develop injectable biodegradable hydrogels from hyperbranched polymers (HBP) with responsive, crosslinkable and degradable properties. These injectable hydrogels can have tailored mechanical properties and functionalities for cell adhesion via decorating with peptide motifs and for controlled release of therapeutic drugs. Here, we report the synthesis of new thermoresponsive HBP via one-pot reversible addition fragmentation chain transfer (RAFT) copolymerization of poly (ethylene glycol) methyl ether methacrylate (PEGMEMA,  $M_n=475$ ), poly (propylene glycol) methacrylate (PPGMA,  $M_n=375$ ) and disulphide diacrylate (DSDA) using 2-cyanoprop-2-yl dithiobenzoate as RAFT agent. Enzymatic degradable DSDA was used as the branching agent; PEGMEMA and PPGMA were used as macromers to balance hydrophilicity/hydrophobicity thus obtain desired low critical solution temperatures (LCST). The resulted HBP were characterised by NMR and GPC, and LCSTs were also determined. Moreover, the studies on the degradability and swelling properties of hydrogels prepared by the resultant HBP were conducted. The experimental results demonstrated that these degradable and thermoresponsive HBP can be used as tissue engineering injectable scaffolds.

#### Injectable, High Porosity Bone Grafts by Emulsion Templating

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Our laboratory has developed an emulsion templating method that generates high porosity scaffolds that are both biodegradable and injectable. The biodegradable macromers used in these high internal phase emulsions (HIPEs) were designed to polymerize at body temperature and have a low viscosity prior to cure, eliminating the use of toxic solvents common in fabricating biodegradable poly-HIPEs. New methodology was developed to permit the rational selection of these macromers based on prediction of molecular hydrophobicity and structural analysis of surfactant chemical structure in contrast to the traditional trial-and-error approach. As proof-of-concept, we demonstrated that a biodegradable HIPE injected into a porcine bone defect cured to a rigid foam at body temperature with microscale integration with the tissue. Subsequent studies generated a library of polyHIPE grafts with tunable pore sizes (5  $\mu\text{m}$  to 1 mm) and mechanical properties (modulus = 50 kPa–50 MPa). Redox initiation was studied as a means to decrease cure times from hours to minutes, comparable to bone cements used currently in the clinic. This new initiation method also improved mechanical properties with minimal effects on pore structure. The use of a double-barrel syringe permitted storage of the emulsions for up to 6 months prior to cure with no negative effects on pore structure. In summary, this emulsion templating platform can be used to generate injectable porous materials with clinically relevant properties that can be used to improve tissue regeneration.

#### Bioengineered Cell-Based Sensing and Therapy using Smart Hydrogels

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Synthetic hydrogels have been widely investigated as artificial extracellular matrices (ECMs) for cell-based sensing and therapy. Here, we report optogenetic cells encapsulated in poly(ethylene

glycol) hydrogels for *in vivo* optical-sensing and diabetic therapy. The real-time optical readout of encapsulated heat-shock-protein-coupled fluorescent reporter cells made it possible to measure the nanotoxicity of cadmium-based quantum dots *in vivo*. Using optogenetic cells producing glucagon-like peptide-1, we also performed light-controlled therapy and demonstrated improved glucose homeostasis in diabetic mice. In addition, we developed *in situ* supramolecularly assembled and modularly modified hyaluronate hydrogels for genetically engineered mesenchymal stem cell (eMSC) cancer therapy. The long-term expression of mutant interleukin-12 (IL-12M) by eMSCs within the supramolecular hydrogels resulted in effective inhibition of tumor growth with a significantly enhanced survival rate. Finally, we developed silicone hydrogels for a smart contact lens composed of biosensors, drug delivery systems, and power sources for the treatment of diabetes as a model disease. All these systems will be discussed in detail for further clinical applications.

### Controlling the Astrocytic Response to Brain Injury using Self-assembling Peptide Hydrogels

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After traumatic brain injury, the acute phase of astrocytosis is an essential physiological response. It is responsible for demarcating the lesion site, preventing secondary degeneration and arresting growth through the production of pro-inflammatory molecules [1]. This response is detrimental if persistent, as chemical and physical barriers provided by the 'reactive' astrocytes prevent functional recovery. Therefore, attenuation of reactive astrocytosis post-insult is an important biological challenge to promote central nervous system repair and reconstruction.

We have implanted an Fmoc-based self-assembling peptide (SAP), Fmoc-DIKVAV, presenting the laminin sequence IKVAV, into the caudate putamen. This hydrogel self-assembles under physiological conditions, forming an injectable hydrogel with a nanofibrous network [2]. The sequence DIKVAV is presented on the outer surface of the nanofibres, resulting in the high-density presentation of a biologically relevant sequence, whilst also providing morphology reminiscent of the native extra cellular matrix. We functionalised this hydrogel with an anti-inflammatory molecule and show that concomitantly providing physical and biochemical cues into a lesion post-insult significantly reduced astrocyte numbers in the parenchyma at 7 and 21 days. We will further develop this technology to control the persistence of 'reactive' astrocytes after injury, and hence, encourage functional regeneration across the lesion site.

1. Rolls, A., R. Shechter, and M. Schwartz, The bright side of the glial scar in CNS repair. *Nature Reviews Neuroscience*, 2009. 10(3): p. 235–241.

2. Rodríguez, A.L., *et al.*, Tuning the amino acid sequence of minimalist peptides to present biological signals via charge neutralised self assembly. *Soft Matter*, 2013. 9(15): p. 3915–3919.

### Session: Quantitative 3D Micro- and Nano-CT Imaging in Tissue Engineering: Emerging Technologies and Recent Advancements

Date and Time: Friday, September 11, 2015,  
9:15 AM - 10:45 AM

#### NanoCT and Contrast-Enhanced NanoCT as First Line Screening and Quality Control Tools for Robust Production of Tissue Engineered Products: an Overview

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Advanced 3D imaging is one of the enabling technologies that is of increasing importance in the field of tissue engineering (TE) to assess and guarantee high product quality, and to provide better knowledge on the mechanisms behind tissue formation and regeneration. Indeed, TE constructs (such as scaffolds with cells and/or growth factors) are 3D structures with complex spatial heterogeneity, for which traditional 2D imaging techniques are insufficient for comprehensive characterization or to assess their quality. In this overview, we show the potential value of nanofocus computed tomography (nanoCT) and contrast-enhanced nanoCT (CE-nanoCT) as first line screening and quality control tools throughout the entire production process of TE constructs.

Concerning scaffold selection, using nanoCT combined with empirical modelling, we highlighted the important influence of the scaffold material and structure on its bone forming capacity, allowing to select and reverse engineer scaffolds with optimized properties. For bioreactor-driven TE construct development, we showed that CE-nanoCT can be used as a 'whole-construct' imaging technique allowing to quantify *in vitro* formed neo-tissue (cells and extracellular matrix) in large 3D TE constructs. With respect to *in vivo* tissue formation, we have shown that CE-nanoCT allows 3D multi-tissue imaging (using one imaging modality for visualizing multiple tissues) as 'virtual histology' of skeletal tissues (bone, cartilage and bone marrow including fat tissue and blood vessels). For all these steps in the production process, nanoCT and CE-nanoCT also enabled to get a better insight in the mechanisms driving tissue formation both *in vitro* and *in vivo*.

#### Current Research Trends in Micro-CT Evaluation of Biomaterials for Tissue Engineering

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Currently one of the most incisive needs in the industry and in research is the development of non-destructive methods to analyze biomaterial microstructures. Furthermore, the only 2D analysis, provided for example by histology, is totally inadequate in the case of samples which are too fragile to be cut (for example powders) or in the case of connectivity or tortuosity quantification of different material phases. The intrinsic ability to provide a huge amount of data allows the evaluation of biomaterials in two different ways: by studying the 3D structure or by studying the regenerated tissue morphology and thus their efficacy in the event of implantation. Tissues damaged by injury or disease could be replaced using constructs based on biocompatible materials, cells and growth factors. Scaffold design, porosity and early colonization are key components for implant success. Thus, the need to display the architecture of materials and the 3D cellular distribution is a key aspect. In addition, 3D models could be used as a basis for the creation of prototypes (e.g. STL or PLY files, useful in the additive manufacturing technique) or for the creation of 3D meshes useful in the FE analysis. Given the non-destructive nature of the technique, another important aspect is the opportunity to follow the evolution of a microstructure under controlled environmental conditions (e.g. load, temperature and/or corrosive environment). This aspect is sometimes called 4D imaging (3D + time) extending the analysis to dynamic parameters or validating numerical predictions of structural and microstructural evolution (mathematical modeling).

#### Visual Mapping of Computational Shear Stresses Implies Mechanical Control of Cell Proliferation and Differentiation in Bone Tissue Engineering Cultures

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The advantages of longitudinal monitoring techniques are getting more attention in various tissue engineering approaches. They

provide consecutive information about one and the same sample over time and as such may decrease sample numbers tremendously. These techniques also allow taking the actual environmental status of a tissue into account for predicting future development. Micro-computed tomography has been previously shown to be suitable to monitor mineralized extracellular matrix deposition in bone tissue engineering cultures. In this study, shear stresses (SS) acting on human mesenchymal stromal cells (hMSC) seeded on silk fibroin scaffolds in a flow perfusion bioreactor were calculated by computational fluid dynamics. Two different flow rates were investigated, mimicking expected loads on cells during early bone repair (0.001 m/s) and during bone remodeling (0.061 m/s), respectively. The three-dimensional (3D) distribution of these stresses was then visually mapped to the distribution of the mineralized extracellular matrix deposited by the cells. SS values from 0.55–24 mPa were shown to promote osteogenic differentiation of hMSCs, whereas SS between 0.06 and 0.39 mPa were found to induce cell proliferation. Histological and biochemical analyses have confirmed these findings. In the future, these results may allow predicting the behavior of hMSC in 3D tissue culture. The non-destructive nature of this technique may even allow tight control and adaptation of the mechanical load during culture by taking the present status of the tissue into account.

**Acknowledgments:** This study has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 262948 and 336043.

#### Quantification of Mechanical Stimuli and Bone Formation in Fracture Healing Using *In Vivo* Time-Lapsed Imaging

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During bone regeneration, mechanical loading is believed to be responsible for provoking bone formation, however previous investigations into tissue level loading have been limited to cross-sectional studies and relied upon idealized models for mechanics. By applying *in vivo* time-lapse micro-computed tomography (microCT) in concert with imaged based micro-finite element (microFE) analysis we have overcome these limitations and have identified an association between tissue loading and bone formation during fracture healing.

A femoral defect of 1.24[SD=0.13] mm was created in five female mice (C57BL/6); the femur was first stabilized with an external fixator (MouseExFix, RISystem, Switzerland). Weekly scans were performed using microCT imaging (vivaCT 40, Scanco Medical, Switzerland) over a period of 6 weeks, resulting in a series of time-lapsed images. We determined sites of mineralization by registering and overlaying images from the second and third week. Combining this with microFE (Parosol) simulations based upon images of the second week, we separated strains in volumes where mineralization occurred, from volumes where no change occurred.

To assess the efficacy of strain as a predictor of mineralization, receiver operating characteristic analysis was used. The optimum strain level correctly predicted 60[SD=9] % of the mineralization which occurred, and the final state for 86[SD=4] % of the entire volume.

We have for the first time, quantitatively demonstrated that an association exists between local tissue strain and bone formation during fracture healing. This could be used to determine the optimal stiffness for biomaterials intended to promote bone healing.

#### A Staining and Tensioning Method for Micro X-ray CT Scanning of Sutured Tendons

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Suturing has been recommended to re-join tendons and permit tissue regeneration for over a century. However, 25% of flexor tendon patients achieve unsatisfactory postoperative mobility. Traditional *in vivo*, *ex*

*in vivo* and clinical studies have failed to determine an ideal suture arrangement for tendon repair. We aim to employ *in silico* analysis in approaching a conclusion. Tissue acellularity and impaired remodelling may be preceded by stress concentrations and stress shielding respectively. We therefore employed Micro X-ray CT and finite element analysis (FEA) to observe the stress patterns in tendon following suture withdrawal. To ensure characteristic deformation during suture withdrawal, the contrast agents must assert minimal change to tendon and suture mechanical behaviour. Quill barbed suture and porcine flexor digitorum profundus tendon were stained using potassium iodide solutions (0.2%KI, 0.1%I) for 24 hours. This stained their surfaces only, preserving the mechanical interaction between suture and tissue. 10 mm of suture was passed through the tendon centre. Using a tension rig, Micro X-ray CT imaging was permitted whilst samples were submerged in phosphate-buffered saline, and following a 2 mm suture withdrawal a subsequent scan was performed. Using the unloaded reconstructed volume data, a 2 mm suture withdrawal was simulated by FEA. Tendon Constitutive behaviour was described using the anisotropic, hyperelastic Holzapfel model in Abaqus. There was no significant difference in failure load between unstained and surface-stained tendons during suture pull-out, and the surface staining enabled reliable data reconstruction. FEA results and reconstructed volumes showed good agreement, thus validating the method.

**Acknowledgments:** EPSRC, MRC(MRC:G1000788/1, MR/M0007642/1).

#### Optimization of Contrast Enhanced Ct for Neo-tissue Quantification using a Design of Experiment Approach

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To progress the fields of tissue engineering and regenerative medicine, development of quantitative methods for non-invasive 3D characterization of engineered constructs (i.e. scaffolds with cells and/or growth factors) becomes essential. In order to enable the use of X-ray based imaging techniques for quantitative analysis of soft tissue fractions in TE constructs the use of contrast-enhanced nanofocus computed tomography (CE-nanoCT) was optimized.

A fractional factorial 'design of experiments' approach was used to elucidate the influence of the staining time and concentration of two contrast agents (Hexabrix<sup>®</sup> and phosphotungstic acid - PTA) and the neo-tissue volume on the image contrast and dataset quality. Additionally, the neo-tissue shrinkage that was induced by PTA staining was quantified to determine the operating window within which this contrast agent can be accurately applied.

For Hexabrix<sup>®</sup> the staining concentration was the main parameter influencing image contrast and dataset quality. A concentration of 60% and a staining time of 30 minutes were sufficient to allow accurate and fully automated quantification of the neo-tissue formed.

Using PTA the staining concentration had a significant influence on the image contrast while both staining concentration and neo-tissue volume had an influence on the dataset quality. The use of high concentrations of PTA did however introduce significant shrinkage of the neo-tissue indicating that, despite sub-optimal image contrast, low concentrations of this staining agent should be used to enable quantitative analysis. As the staining time did not have any significant influence, this can be kept as low as possible.

#### Metabolic Tracking of Muscle Precursor Cells for Skeletal Muscle Tissue Engineering using PET/CT

D. Haralampieva<sup>1,2,3</sup>, S. Salemi<sup>1</sup>, T. Betzel<sup>3</sup>, I. Dinulovic<sup>4</sup>, S. Kraemer<sup>3</sup>, T. Sulser<sup>1</sup>, C. Handschin<sup>4</sup>, S. M. Ametamey<sup>3,2</sup>, D. Eberli<sup>1,2</sup>;

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Up-to-date organ transplantation is the gold standard for rescuing damaged tissues. Regenerative medicine using autologous stem cells may offer an alternative approach. Despite the progress, there are still crucial challenges for successful therapy. Due to their regenerative capacity muscle precursor cells (MPCs) are investigated for muscle reconstruction. To better understand the engrafting process novel imaging technologies are needed. This study explored the possibility of using PET/CT Imaging for *in vivo* tracking of implanted hMPCs in mice. In addition, we aimed at visualizing the hypoxia in engineered muscle tissue by PET/CT, gaining a metabolic read-out about the implanted cells. hMPCs were isolated from *M.rectus abdominis* and expanded in culture. After genetically modifying them to express a mutated dopamine-D2R, the cells were suspended in collagen carrier and injected subcutaneously in nude mice. The newly-formed muscle tissues were visualized by PET/CT using tracers for D2R and hypoxia and finally, harvested for autoradiography and histological analysis.

The successful transduction of hMPCs to express D2R allowed for detection by PET/CT Imaging *in vivo*. Non-invasive hypoxia imaging provided additional insights regarding their metabolism. Further characterizations of their morphology after harvesting revealed no differences to non-transduced cells. The tissues were also visualized with autoradiography. These results confirm that using genetically modified hMPCs does not alter the muscle phenotype; moreover, it suggests a new possibility for non-invasive read-outs.

Our study demonstrates that PET/CT Imaging may offer a novel method for non-invasive tracking and metabolic visualization of genetically modified hMPCs after injection for treatment of muscle diseases.

#### Non-Invasive Monitoring of Hydrogel Tissue Scaffolds and Nerve Tissue *In Vivo* using a Synchrotron-based X-ray In-Line Phase Contrast Imaging Technique

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Conventional radiography has been widely used in bone Tissue Engineering (TE), but not in soft TE applications especially for *in vivo* studies, due to the fact that the weak X-ray attenuation from soft tissue and low density scaffold materials cannot produce enough image contrast for *in vivo* visualization [1]. A state-of-the-art synchrotron-based in-line Phase Contrast Imaging (in-line PCI) technique, available at the Biomedical Imaging and Therapy (BMIT) facility at the Canadian Light Source (CLS) [2], is able to detect fine features in weakly absorbing biological samples [3]. In the present study, in-line PCI combined with computed tomography (CT) was used for the study of nerve tissue scaffolds *in vivo*. A three dimensional (3D) grid alginate hydrogel scaffold was enclosed in a polycaprolactone stent and implanted at the site of transection of the left sciatic nerve in rats. The animals were sacrificed after 2 and 7 days survival, and the untreated scaffold-implanted hindlimbs were imaged by using in-line PCI-CT. After phase retrieval CT reconstruction [4], the reconstructed images clearly show the 3D structure of the alginate scaffolds and the profile of the nerve conduit *in vivo*. The volume of the scaffold structure can be quantified from the CT images. Based on these findings the synchrotron-based in-line PCI-CT technique shows the potential to monitor nerve tissue regrowth and scaffold degradation over a longer survival period in live animals. This imaging method would greatly assist to monitor scaffold persistence and nerve tissue regeneration, and can also be applied in other TE problems.

#### Session: Regeneration of Dental and Craniofacial Tissues (Part 1)

Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM

#### Periodontal Reconstruction using Decellularized Periodontal Tissue Combined with Artificial Tooth

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The lack of periodontal ligament (PDL) is one of the problems for dental implant. We here propose a tissue engineered periodontal tissue for the reconstruction of the periodontal tissue, including the mandible bone, PDL, and artificial tooth. In this study, we prepared the decellularized mandible bone with PDL matrix and evaluated the reconstruction of the periodontal tissue units using artificial tooth. The units of mouse mandible bone with molar were treated by high hydrostatic pressure and washed by saline to remove the cells. Although the PDL were usually disrupted by molar extraction, the decellularized PDL could be maintained on the mandible bone by SDS treatment. For evaluation for the reconstruction of periodontal tissue units, PDL cells harvested from rat were seeded on the decellularized PDL, and observed not only on but also inside the PDL matrix, although the orientation of the cells was random. They were implanted under the rat renal capsule after seeding of rat PDL cells *in vitro*, the cells oriented along the PDL collagen fibers. As a model of artificial molar, original molar obtained from decellularized unit was used after demineralization and remineralization treatment, instead of preparing artificial tooth fitting decellularized unit. The treated molar was inserted to *in vitro* recellularized PDL and performed the renal capsule implantation. Recellularized PDL were adhered to artificial molar, indicating the decellularized periodontal unit with PDL could be reconstructed with artificial tooth.

#### Increased Osseointegration of Titanium Implants in Challenged Bone Condition

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The increase in population age demands for expansion of age-related healthcare and advancements in medical technology. For instance, oral implantology is considered as the treatment of choice for replacing missing teeth in elderly people. The clinical performance of dental implants has been attributed to their firm osseointegration. However, much research has addressed implant treatment aspects in challenged bone conditions, e.g. osteoporotic conditions. To increase implant osseointegration, new coating strategies involve the development of a dedicated drug-loading ability to locally target bone disorders around dental implants more effectively. In this study, we used an established *in-vivo* femoral condyle implantation model to analyze osseointegration of titanium implants coated with bisphosphonate (BP)-loaded calcium phosphate nanoparticles (nCaP) under osteoporotic and healthy bone conditions. After 4 weeks of implantation, peri-implant bone volume (%BV; by micro-CT) and bone area (%BA; by histomorphometry) were significantly increased within a distance of 500 µm from implant surfaces coated with BP compared to control implants in osteoporotic and healthy conditions. Furthermore, the deposition of nCaP/BP coatings onto implant surfaces increased bone-to-implant contact (%BIC) compared to non-coated implants in osteoporotic and healthy conditions. The results of real-time PCR revealed similar osteogenic gene expression levels to all implant surfaces at 4-weeks post-implantation. In conclusion, using nCaP/BP surface coatings represents an effective strategy for improving implant osseointegration, especially in osteoporotic conditions.



### Electrospun PVA-PCL-HAB scaffold for Craniofacial Bone Regeneration

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Bone tissue engineering for craniofacial region is considered challenging owing to its physiologic and anatomical complexities. A porous bioactive scaffold promoting osteogenesis and angiogenesis is required for clinical applications. We have developed an electrospun polyvinyl alcohol (PVA) poly-caprolactone (PCL)-triphase bioceramic (HAB) scaffold to biomimic native tissue and we tested its ability to support osteogenic differentiation of stromal stem cells (MSC) and its suitability for regeneration of craniofacial defects.

Physicochemical characterizations of the scaffold, including contact angle measurements, showed that PVA-PCL-HAB was more hydrophilic than PCL alone or PCL-HAB combined ( $P < 0.05$ ). Ion release profile studies using ICP-OES of the scaffold showed release of calcium and silica ions, required for initiation of bioactivity. SEM and EDS analyses revealed apatite formation on stimulated body fluid immersed scaffold samples. Culturing human adult dental pulp stem cells (DPSC) and human bone marrow derived MSC seeded on PVA-PCL-HAB scaffold showed enhanced cell proliferation and *in vitro* osteoblastic differentiation. Cell-containing scaffolds were implanted subcutaneously in immune deficient mice. Histologic examination of retrieved implant sections stained with H&E, Collagen Type I and Human Vimentin antibody demonstrated that the cells survived *in vivo* in the implants for at least 8 weeks with evidence of osteoblastic differentiation and angiogenesis within the implants. Our results suggest that PVA-PCL-HAB scaffold can support growth and osteoblast differentiation of MSC and thus should be considered for clinical use in craniofacial tissue regeneration.

### Dental Epithelial-Mesenchymal Cell Sheets for 3D Tooth Regeneration

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Dental epithelial-mesenchymal (DE-DM) cell interactions provide critical functions in tooth development. Our objective was to create biomimetic three dimensional (3D) tooth buds consisting of DE-DM cell sheets created on UpCell thermosensitive plates (CellSeed, Tokyo, Japan), layered over dental cell encapsulated gelatin methacrylate (GelMA) hydrogel scaffolds. Parameters tested included: optimized cell seeding density (0.02, 0.114 and  $0.228 \times 10^6$  cells/cm<sup>2</sup>); *in vitro* culture time (7, 14 and 21 days); and co-cultured porcine DM (pDM), porcine DE (pDE) and HUVEC cell sheets. pDE/HUVEC and pDM/HUVEC co-cultures were established by first seeding either pDM or pDE cells (both at  $0.114$  and  $0.228 \times 10^6$  cells/cm<sup>2</sup>), culturing for 10 days, followed by the addition of  $0.05 \times 10^6$  HUVEC cells/cm<sup>2</sup> and continued culture for an additional 4 days. Harvested cell sheets were then layered over pDM seeded GelMA constructs, and cultured *in vitro*. DM cell sheets were readily obtained after 21, 14 and 11 days at cell seeding densities of 0.02, 0.114 and  $0.228 \times 10^6$  cells/cm<sup>2</sup>, respectively. DE cell sheets were obtained only at the highest cell seeding density after 14 days in culture. H&E and IF analyses of paraffin embedded and sectioned specimens revealed organized multilayered cell sheet formation. We propose our biomimetic 3D DE-DM Cell Sheet + GelMA tooth bud model to optimize DE-DM cell interactions leading to biomimetic tooth formation. *In vivo* implanted constructs are currently being used to assess mineralized dental tissue formation in 3D biomimetic tooth constructs. This work was supported by NIH/NIDCR R01DE016132 (PCY).

### Intravenous Injection of a Bone Marrow Cell Extract Promotes the Regeneration of Irradiated Bone

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Mandibular osteoradionecrosis is a severe side effect of radiotherapy. As an alternative to its treatment by micro-anastomosed free-flaps, pre-clinical studies have shown that a combination of Total Bone Marrow (TBM) and Biphasic Calcium Phosphate (BCP) significantly enhanced irradiated bone formation. One of the mechanisms explaining the bone regenerating capacity of the TBM graft is its paracrine effect. The Bone Marrow Cell Extract (BMCE) that contains soluble factors included in the paracrine effect has shown its regenerative effects in various tissues, but never in irradiated bone. The purpose of this study was to evaluate the effect of BMCE in irradiated bone reconstruction, by intra-osseous or intravenous delivery. Twenty rats were irradiated on their hind limbs with a single dose of 80 Gy. Three weeks later, femoral and tibial bone defects were created. The effect of BMCE *in situ* was studied in the intra-osseous group ( $n = 12$ ) with six combinations (empty, BCP, TBM, BCP-TBM, BMCE, BCP-BMCE). The intravenous group ( $n = 8$ ) received, after four different combinations of implantation (empty, BCP, TBM, BCP-TBM), four intravenous injections of BMCE during two weeks. Five weeks after implantations, samples were analyzed. Intravenous injections of BMCE led to a significant increased new bone formation in combination with TBM inside the defect, with or without biomaterials. Intra-osseous BMCE delivery failed to enhance bone formation. This study demonstrated the capacity of intravenous injections of BMCE in combination with TBM inside the bone defect to repair irradiated bone. The precise mechanisms of action of the BMCE have to be fully understood.

### Effects of Dehydrothermal Treatment of Collagen/ $\beta$ -TCP Scaffold on Bone Regeneration in Critical Size Bone Defects

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Patients annually undergo surgical procedures to have their bone fractures treated, to alleviate severe back pain through spinal fusion procedures, or to promote healing of non-unions. There remains a substantial shortfall in successful treatments of severe skeletal injuries. Autologous bone grafting, which is the current gold standard, requires grafting from the same patient and has many undesirable side effects such as donor site morbidity. For this reason, we have engineered a scaffold constructed of collagen type I and  $\beta$ -TCP sponge using dehydrothermal treatment for crosslinking. Scaffolds were implanted into critical-sized defects in rat crania and femora. To see their effects on bone regeneration in addition to scaffold group three other groups were tested, which contained BMP-2, TGF- $\beta$ 1 and both as additions. The scaffolds were fixed at 4 and 6 months after implantation, respectively. Successful bone regeneration was defined by the absence of scaffold deformation and the presence of new bone formation within the biocomposites. Bone fill percentage in the defect region was assessed by micro-computed tomography (CT) and mammography. A histological assessment was made of the tissue response, the presence of new bone formation, and the appearance of the scaffold. Based on the present findings, we conclude that the engineered tissue scaffolds described herein have great potential as an alternative treatment option when cost, donor region morbidity, and duration of hospitalization are considered.

### Use of Autologous Adipose Stem Cells to Reconstruct 24 Patient Cases with Cranio-maxillofacial Hard-tissue Defects

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This study aimed to review our experience with 24 consecutive cases of cranio-maxillofacial hard-tissue defects at four anatomically different sites, namely frontal sinus (3 cases), cranial bone (5 cases), mandible (10 cases), maxilla (4 cases) and nasal septum (2 cases). Autologous adipose tissue was harvested from the abdominal wall, and adipose-derived stem cells were cultured, expanded, and then seeded onto porous resorbable scaffold materials for subsequent re-implantation into defects. The defects were reconstructed with either bioactive glass or  $\beta$ -tricalcium phosphate scaffolds seeded with adipose-derived stem cells (ASCs), and in some cases with the addition of recombinant human bone morphogenetic protein-2. Production and use of ASCs were done according to good manufacturing practice guidelines. The ASCs were expanded for 3–5 weeks. Multipotency (adipogenic, osteogenic and chondrogenic differentiation) and mesenchymal stem cell surface marker profile (CD14, CD19, CD34, CD45, CD73, CD90, CD105, HLA-ABC-PE, and HLA-DR) of ASCs was analyzed. Moreover, chromosomal integrity, sterility, endotoxins and mycoplasma were determined before cell transplantation. Follow-up time ranged from 9 to 52 months. Successful ossification and integration of the construct to the surrounding skeleton was noted in 18 of the 24 cases. In unsuccessful cases 1 patient developed infection, 1 nasal septum transplant was lost because of nose picking habit of the recipient and 4 patients did not produce sufficient volume of bone. These cases show that the use of autologous adipose-derived stem cells combined with osteostimulative biomaterial results in generation of new viable bone.

### Octacalcium Phosphate Collagen Composites Facilitates Bony Reconstruction after Canine Mandibular Amputation

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Reconstruction of mandibular amputation is a crucial problem in maxillofacial surgery. Octacalcium phosphate and porcine atelocollagen composite (OCP/Col) accomplished effective bone regeneration without cell transplantation and exogenous osteogenic cytokines. This study examined the efficacy of bone regeneration by OCP/Col if implanted into the defect after canine mandibular amputation.

OCP/Col was prepared as previously described<sup>1</sup>, and sterilized using electron beam irradiation (15 kGy). Six male beagle dogs were used. The amputation of critical sized (15 mm length) bone defect was made in the mandibular premolar region, and 30 ~ 45 disks of OCP/Col (9 mm diameter 1.5 mm thick) was implanted into the defect and fixed with titanium plates. Intra-oral radiography was taken immediately and every month after implantation. After 6 months, the specimens were fixed and radiographed by a micro-CT.

Half of the amputated mandible was reconstructed by newly formed bone. In successful cases, the implanted area revealed distinct radiolucency at immediately after implantation of OCP/Col. The radiolucency of the implanted area was still dominant at one through two month. After three months, lower radiopacity than original bone was occupied throughout the defect. Thereafter it was gradually increased. At six months, the OCP/Col implanted area was mainly occupied by relatively dense bone, and the border between the original bone and the OCP/Col implanted area was indistinguishable.

These results suggest that OCP/Col would be a suitable bone regenerative material for the defect after mandibular amputation.

1. Kamakura S, et al., J Biomed Mater Res B Appl Biomater 79, 210, 2006.

### Session: Regeneration of Dental and Craniofacial Tissues (Part 2)

Date and Time: Friday, September 11, 2015, 12:45 PM - 2:00 PM

### Biohybrid Scaffolds based on Polymers and Strontium Salts for the Regeneration of Bone Tissue

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Strontium salts have become important compounds for the treatment of osteoporosis and the activation of bone regeneration in the last few years. Unfortunately the most popular drug, strontium ranelate presents important secondary effects and its application has been restricted seriously in the clinic. This study deals with the design and application of porous biocomposite scaffolds based on calcium phosphate TCP and strontium folate SF dispersed in a 3D matrix of acrylic poly-ethyleneglycol networks. *In vitro* results using human dental pulp stem cells DPSC, demonstrate an excellent behavior of the biocomposite system with biocompatibility, lack of cytotoxicity, and clear activation of cell proliferation and differentiation to osteoblasts. The application of 3D biohybrid scaffolds to a critical rat calvarial defect model for period of time up to 20 weeks, demonstrates the bioactivity of the composite scaffold containing TCP and SF. Quantitative data of the tissue regeneration "*in vitro*" and "*in vivo*" will be presented.

### Regeneration of Human-ear-shaped Cartilage by Co-culturing Human Microtia Chondrocytes with BMSCs

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Previously we had addressed the issues of shape control/maintenance of *in vitro* engineered human-ear-shaped cartilage. In the current study, we believed the lack of clinically applicable cell source had become a major concern. Autologous microtia chondrocytes (MCs) and bone marrow stromal cells (BMSCs) were both promising chondrogenic cells that did not involve obvious donor site morbidity. However, limited cell availability of MCs and ectopic ossification of chondrogenically induced BMSCs in the subcutaneous environment greatly restricted their applications in external ear reconstruction. In addressing this issue, the current study demonstrated that MCs possessed strong proliferation ability but accompanied with rapid loss of chondrogenic ability during passage, indicating a poor feasibility to engineer the entire ear using expanded MCs. Fortunately, the co-transplantation results of MCs and BMSCs (25% MCs and 75% BMSCs) demonstrated a strong chondroinductive ability of MCs to promote stable ectopic chondrogenesis of BMSCs in subcutaneous environment. Most importantly, a human ear-shaped cartilaginous tissue with delicate structure and proper elasticity was successfully constructed by seeding the mixed cells (MCs and BMSCs) into the pre-shaped biodegradable ear-scaffold followed by subcutaneous implantation in nude mouse. These results may provide a promising strategy to construct stable ectopic cartilage with stem cells (BMSCs) for autologous external ear reconstruction.

### Alveolar Jaw Bone Formation by Human Dental Pulp Cells and E1001(1k)/ $\beta$ -TCP Scaffolds

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**Objectives:** The long-term goal of this research is to provide functional repair of jaws and teeth. This study will characterize

alveolar bone formation by neural crest cell derived human dental pulp cells (hDPCs) seeded onto porous scaffolds made of E1001(1k), a member of a large combinatorial library of tyrosine derived polycarbonates, and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP).

**Methodology:** Cylindrical E1001(1k)/ $\beta$ -TCP scaffolds were prepared by a combination of porogen leaching and freeze drying to fit into a 5 mm critical-sized rat mandible defect. Five groups were examined: 1) scaffolds seeded with  $2.5 \times 10^5$  hDPCs/scaffold; 2) scaffolds seeded with  $5.0 \times 10^5$  hDPCs/scaffold; 3) acellular scaffolds loaded with  $4 \mu\text{g}$  BMP-2; 4) acellular scaffolds alone; and 5) empty defect. Cell-seeded and acellular scaffolds were cultured in osteogenic media for one week before implantation. BMP-2 was loaded onto pre-wetted scaffolds 30 minutes before implantation. Micro-CT was performed on live animals at 3 and 6 weeks post-implantation, prior to jaw harvest. Harvested jaws were embedded for cryosection, and histological and immunohistochemical analyses of alveolar bone formation are currently being performed.

**Results:** Micro-CT indicated that scaffolds loaded with BMP-2 showed the most bone formation. The hDPC seeded, and acellular scaffold alone groups exhibited variable levels of new bone formation, as compared to the empty defect group. Their significance: E1001(1k)/ $\beta$ -TCP scaffolds support the formation of hDPC-derived bone formation. Ongoing studies include qualitative and quantitative assessment of alveolar jaw bone formation. These studies were supported by DoD/AFIRM II Award #W81XWH-14-2-0004 and NIH/NIDCR R01 DE016132 (PCY).

### Gingival Papilla Mesenchymal Stromal Cells and Their Potential Role in Clinical Dentistry

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Recently, great advances have been made in exploring the clinical potential of oral and dental stem cells. In dentistry, the regeneration of tooth and periodontal tissues is now focusing on the use of oral tissue derived Mesenchymal Stem/stromal Cells (MSCs). Thanks to the minimal invasive procedure, oral soft tissues represent a convenient source of MSCs. We have isolated MSCs from gingival papilla (GinPa-MSCs) of donors undergoing oral surgery [1]. All the GinPa-MSCs showed a fibroblast-like morphology and a high proliferation rate ( $DT = 50.4 \pm 16.1$  hours) that allowed us to rapidly collect a large number of cells. GinPa-MSCs displayed a high clonogenic potential ( $\sim 20\%$ ), and expressed on their surface CD73, CD90 and CD105 ( $>99\%$ ), CD14 ( $\sim 32\%$ ) and, as expected, they were negative for CD45. GinPa-MSCs were also induced to differentiate towards osteogenic, adipogenic and chondrogenic lineages: an increase in collagen production and extracellular calcified matrix deposition was observed in osteo-induced cells. Preliminary data also indicated that GinPa-MSCs possess a mild chondrogenic potential, while lipid droplets, following adipogenic stimuli, were poorly detectable. GinPa-MSCs primed with high concentration of Paclitaxel were able to uptake and then release the drug into the culture medium. This property was confirmed by the *in vitro* antitumor activity of their conditioned medium [2] and suggests that, as other stromal cells, also the MSCs from gingival papilla might have clinical application in the future for drug delivery strategies.

1. Sonoyama W, Liu Y. PLoS One. 2006 Dec 20;1:e79.
2. Pessina A, Bonomi A. PLoS One. 2011;6(12):e28321.

### Optimization of Mineralized Tooth Constructs for Whole Tooth Bioengineering

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**Objectives:** Our long term goal is to bioengineer functional, living replacement teeth as an improved alternative to currently used synthetic dental implants. Here we describe a Gelatin Methacrylamide (GelMA) hydrogel based biomimetic three-dimensional (3D) model as a platform for regenerative dental applications. Our previous reports identified GelMA formulas that showed elastic moduli similar to that of natural dental tissues and supported dental epithelial (DE) and dental mesenchymal (DM) cell attachment, metabolic activity, differentiation, and also promoted organized endothelial (HUVEC) network formation. *In vivo* implanted constructs exhibited high vascularity and mineralized tissue formation.

**Methodology:** Currently, we are optimizing our biomimetic 3D tooth model by defining parameters to promote increased DE and DM cell proliferation and differentiation. DE/DM/HUVEC GelMA constructs of varying cell seeding densities were fabricated and cultured for either 1 or 14 days in normal media, and then an additional week in osteogenic media. Constructs were then implanted subcutaneously and grown *in vivo* for 2 and 4 weeks.

**Results:** Histological, immunohistochemical, and quantitative radiographic analyses are being used to characterize DE/DM/HUVEC cell morphology, differentiation and biomineralization of *in vitro* and *in vivo* constructs. We anticipate that dental cell interactions and organized dentin and enamel formation in biomimetic tooth constructs will improve with increased cell density.

**Significance:** The results from this study will further establish dental cell encapsulated 3D GelMA hydrogel constructs as biomimetic 3D models for functional tooth replacement in humans. Acknowledgements: This work was supported by NIH/NIDCR R01 DE016132 (PCY).

### Decellularized Xenogeneic Septal Cartilage Instructs Septal Cartilage Regeneration in a Rabbit Model

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Severe deviations of the nasal septum and external nose require surgical reconstruction using autologous cartilage for correction. Tissue engineering is currently discussed as an alternative therapeutic approach for the reconstruction of the nasal septum. Biomaterials which are derived from extracellular matrices are believed to induce constructive and functional tissue remodeling. Therefore such materials might provide a promising strategy for nasal cartilage replacement.

Decellularized extracellular cartilage matrices (DECM), which have been developed recently were applied for nasal septal cartilage replacement in rabbits. DECM, either unseeded or seeded with autologous auricular rabbit chondrocytes (rCh) were implanted between the mucoperichondrial layers of the nasal septum. Ear cartilage was implanted as a control. After three and six months, small-animal MRI was used to assess morphology with a clinical method. Cartilage formation and inflammatory reactions were assessed with histochemistry and immunohistochemistry.

Seeded as well as unseeded DECM and autologous ear cartilage prevented septal perforations in all cases. 3 and 6 months after implantation small-animal MRI revealed septal deviations when replacement materials had been inserted, furthermore 3 different kinds of neo-cartilage formation were observed: *in vitro*-generated cartilage, cartilage generated by the surrounding perichondrium and cartilage generated directly by the surrounding native septum. The unseeded DECM scaffolds induced neo-cartilage formation while seeded matrices and auricular cartilage seemed to inhibit cartilage regeneration.

In summary, we demonstrated that DECM scaffolds can induce neo-cartilage formation in the rabbit nasal septum. These properties

combined with the effective prevention of septal perforations make DECM a promising tool for nasal septal cartilage repair.

### Regeneration of Human Ear Shaped Cartilage by Coculturing Human Microtia Chondrocytes With Bmscs

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Previously, we had addressed the issues of shape control/maintenance of *in vitro* engineered human-ear-shaped cartilage. Thus, lack of applicable cell source had become a major concern that blocks clinical translation of this technology. Autologous microtia chondrocytes (MCs) and bone marrow stromal cells (BMSCs) were both promising chondrogenic cells that did not involve obvious donor site morbidity. However, limited cell availability of MCs and ectopic ossification of chondrogenically induced BMSCs in subcutaneous environment greatly restricted their applications in external ear reconstruction. The current study demonstrated that MCs possessed strong proliferation ability but accompanied with rapid loss of chondrogenic ability during passage, indicating a poor feasibility to engineer the entire ear using expanded MCs. Fortunately, the co-transplantation results of MCs and BMSCs (25% MCs and 75% BMSCs) demonstrated a strong chondroinductive ability of MCs to promote stable ectopic chondrogenesis of BMSCs in subcutaneous environment. Moreover, cell labeling demonstrated that BMSCs could transform into chondrocyte-like cells under the chondrogenic niche provided by co-cultured MCs. Most importantly, a human-ear-shaped cartilaginous tissue with delicate structure and proper elasticity was successfully constructed by seeding the mixed cells (MCs and BMSCs) into the pre-shaped biodegradable ear-scaffold followed by 12 weeks of subcutaneous implantation in nude mouse. These results may provide a promising strategy to construct stable ectopic cartilage with MCs and stem cells (BMSCs) for autologous external ear reconstruction.

### Fibrin Culture of Bone Marrow Stem Cells in a Perfusion Bioreactor System for Cranial Defect Regeneration

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Three-dimensional (3D), osteo-differentiation of mesenchymal stem cells (MSCs) has previously been established utilizing a tubular perfusion bioreactor (TPS), enabling growth of large volume scaffolds. Fibrin is a suitable scaffold material for MSC delivery as it provides an environment for cell adhesion and proliferation. Fibrin is also a highly angiogenic material, making it a promising candidate for supplementing bone regeneration strategy. This study tests the use of fibrin microbeads within the TPS as a bone tissue scaffold in a rat cranial defect model. Rat bone marrow stem cells (BMSCs) were embedded within fibrin microbeads and cultured using osteogenic differentiation media in the TPS or in static culture for 2 weeks. Scaffolds were then implanted within a critical-size cranial defect and harvested at 4 and 8 weeks. Samples were imaged using  $\mu$ CT to evaluate mineralization volume. Samples were also fixed and processed for histology to evaluate vessel ingrowth and tissue regeneration.  $\mu$ CT image analysis showed an increase in normalized mineralization volume in TPS-differentiated samples compared to undifferentiated controls at 4 weeks and 8 weeks. TPS-differentiated samples also showed an increase compared to static-differentiated samples at 8 weeks. TPS-differentiated samples also showed an increase over undifferentiated controls in bony bridge scoring at 8 weeks. Histology shows maximum tissue ingrowth in TPS-differentiated samples, including increased evidence of collagen type 1 deposition and vascularization. In conclusion, fibrin microbeads provide a suitable scaffold

for use in combination with the TPS for critical sized bone tissue engineering.

### Session: Regenerative Medicine Strategies for the Nervous System

Date and Time: Friday, September 11, 2015, 9:15 AM - 10:45 AM

#### Targeted BDNF Gene Delivery Mediated by TMC-based Non-viral Vectors is Neuroprotective in Nerve Injury

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Peripheral neuropathies are very common diseases that still lack an effective treatment option. Envisaging an intervention in peripheral neuropathies it is important to enhance nerve regeneration as well as prevent nerve degeneration. Neurotrophic factors play a crucial role in promoting neuronal trophic support and survival what make them promising disease modulating therapeutic agents to be used in the development of such therapeutic interventions.

Here we propose a non-viral vector based on trimethyl chitosan (TMC) to deliver therapeutic genes to peripheral neurons in order to efficiently promote neuroprotection/regeneration. TMC-plasmid DNA encoding for the brain-derived neurotrophic factor (BDNF) complexes were formed and subsequently targeted to neurons by grafting the non-toxic carboxylic fragment of tetanus toxin, known as neurotropic and able to be retrogradely transported along axons. We investigated whether enhanced expression of BDNF by a peripheral intramuscular administration of these vectors could protect sensorial and spinal motor neurons in a sciatic nerve crush injury animal model.

Our results demonstrate a positive effect of BDNF treatment in the sensorimotor functional recovery as well as a higher expression of neurofilament and Schwann cell markers in the injured nerves of BDNF-treated animals. Besides neuroprotection and neuroregeneration improvement we also observed that BDNF treatment resulted in gastrocnemius muscle protection to denervation that can be responsible for the functional effects.

Altogether, our data show the potential of TMC-based vectors to be used as non-viral gene carriers to deliver therapeutic genes to peripheral neurons and thus provide an effective therapeutic intervention for peripheral neuropathies.

#### Mesenchymal Stem Cells Secretome as Modulator of Brain Repair: Effects on Neurogenesis and Parkinson's Disease Regeneration

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It has been suggested that effects of MSCs in CNS regenerative medicine are mediated by their secretome. Having this in mind, in the present work we aimed at: 1) assess the effects of MSCs Secretome, in the form of conditioned media (CM), on neuronal differentiation of human neural progenitors; 2) Perform a proteomic analysis of MSCs secretome and 3) Study the potential role of the sole use of the secretome as a therapeutic tool in Parkinson's Disease (PD) regenerative medicine. Results revealed that MSCs induced higher levels of neuronal differentiation (MAP-2+ and DCX+ positive cells) of hNPCs. These levels could be even potentiated by culturing MSCs in

bioreactor based dynamic culturing environments. Importantly this differentiation profile could differ according to the passage of MSCs. *In vivo* experiments showed that the injection of the CM of MSCs in the DG of the hippocampus of Wistar rats increased the number of newborn neurons (DCX+ cells). Also, the injection of MSCs CM in a 6-OHDA rat PD model was able to revert the parkinsonian phenotype from both the histological (increased densities of dopaminergic neurons in the SN) and functional (staircase test) outcomes. Finally, a LC-MS/MS proteomic characterization of MSCs secretome revealed the presence of important neuroregulatory molecules, namely DJ-1, PEDF, Cys C, GDN and Galectin-1. The present data shows the modulatory role of MSCs secretome in Brain Repair, further indicating that cell free therapies based in it could represent the basis of future strategies in PD regenerative medicine.

#### Fibrin-based Hollow Microsphere Reservoirs for Controlled Delivery of Neurotrophic Factors to the Brain

J. Samal<sup>1</sup>, N. Moriarty<sup>2</sup>, D. B. Hoban<sup>2</sup>, C. Naughton<sup>2</sup>, R. Concannon<sup>2</sup>, E. Dowd<sup>2,1</sup>, A. Pandit<sup>1</sup>;

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*In vivo* therapeutic potential of neurotrophic factors to modify neuronal dysfunctions is limited by their short half-life. A biomaterials-based intervention, which protects these factors and allows a controlled release, is required. The hypothesis of this study is to establish that template charge manipulation can lead to fabrication of hollow fibrin microspheres as a reservoir for controlled delivery of neurotrophins *in vivo*. Specific objectives were to fabricate and characterize the fibrin based reservoir system, investigate interaction with cells, impact of neurotrophin encapsulation on its bioactivity and assessing host response in a rodent model. Template charge manipulation(1) was used to fabricate fibrin microspheres. Protein loading and release from the microspheres was demonstrated using nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF). Hollow fibrin microspheres showed high loading efficiency (>80%) with no adverse impact on viability of rat mesenchymal stem cells, PC-12 cells and fetal ventral mesencephalon cells. Encapsulation of neurotrophins into the microspheres did not alter their bioactivity and a controlled release of NGF was observed from hollow fibrin microspheres in an *in vivo* study. The host response to NGF, spheres and NGF- loaded spheres was comparable. Therefore, fibrin hollow microspheres act as a suitable delivery platform for neurotrophic factors with tunable loading efficiency and maintaining their bioactive form after release *in vivo*. The absence of any chemical cross-linkers in this system offers an advantage over other biomaterial systems used in similar applications.

**Reference:** 1. Browne S., Fontana G., Rodriguez B.J., Pandit A. *Mol Pharm* (2012), 9:3099–3106.

**Acknowledgments:** Hardiman Fellowship and CÚRAM (13/RC/2073), NUIG. Baxter Healthcare, Vienna for TisseelR.

#### Local Delivery of FTY720 In PCL Membrane Improves SCI Functional Recovery by Reducing Reactive Astrogliosis

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FTY720 has recently been approved as an oral drug for treating relapsing forms of multiple sclerosis, and exerts its therapeutic effect by acting as an immunological inhibitor targeting the sphingosine-1-phosphate (S1P) receptor subtype (S1P1) of T cells. Recently studies demonstrated positive efficacy of this drug on spinal cord injury (SCI) in animal models after systemic administration, albeit with significant adverse side effects. We hereby hypothesize that localized delivery of FTY720 can promote SCI recovery by reducing pathological astrogliosis. The mechanistic functions of FTY720 were investigated *in vitro* and *in vivo* utilizing immunofluorescence, histology, MRI and behavioral analysis. The *in vitro* study showed that FTY720 can reduce astrocyte migration and proliferation activated

by S1P. FTY720 can prolong internalization of S1P1 and exert antagonistic effects on S1P1. *In vivo* study of SCI animal models demonstrated that local delivery of FTY720 with polycaprolactone (PCL)membrane significantly decreased S1P1 expression and glial scarring compared with the control group. Furthermore, FTY720-treated groups exhibited less cavitation volume and neuron loss, which significantly improved recovery of motor function. These findings demonstrated that localized delivery of FTY720 can promote SCI recovery by targeting the S1P1 receptor of astrocytes, provide a new therapeutic strategy for SCI treatment.

#### Session: Silk Biomaterials for Biomedical Application

**Date and Time: Friday, September 11, 2015, 9:15 AM - 10:45 AM**

#### Development of a Biodegradable Silk Mesh Suitable for Intra-Operative Fast Cell Seeding (eg. for Hernia).

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**Introduction:** The combination of meshes with cells (i.e. fibroblasts) has been shown to stimulate the healing process of abdominal wall defects/hernias. However, the extensive *in vitro* expansion step of the cells onto the mesh presents a major limitation. To avoid such time consuming protocol and to decrease the final cost of the product, the development of a new mesh that can be quickly covered on-site (or in theatre) with autologous cells avoiding an *in vitro* cultivation would bring tremendous advantage.

**Materials and Methods:** Covalent immobilization of pro-adhesive glycoprotein lectin on the silk surface was performed on silk films and on silk meshes. The *in vitro* attachment and viability of mouse fibroblasts NIH/3T3 or human adipose derived stroma cells (ASC) on the surface of the modified silk compared to native silk and to control surfaces (PolyStyrene and PolyPropylene) was investigated.

**Results:** Our experiments based on fibroblast and ASC seeding demonstrated that grafting lectin on the surface of silk films and meshes did not induce *in vitro* cytotoxicity, while it significantly enhanced and accelerated cell attachment within 5 minutes incubation period.

**Discussion:** In this study, we successfully grafted pro-adhesive lectin onto silk meshes. We showed that fibroblasts could quickly attach onto the silk-lectin films and meshes. This new material optimized for intra-operative fast seeding with autologous cells could bring a remarkable benefit in soft tissue repair.

#### 3D Interconnected Macroporous Poly(ε-caprolactone) and Silk Fibroin Nanocomposite Fibrous Matrix for Artificial Dermis

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The ideal skin substitute should be non-toxic and have desirable physical and biological properties. Poly(ε-caprolactone) (PCL) is one of the most favorable synthetic biopolymers for tissue regeneration engineering. However, intrinsic hydrophobicity and lower biocompatibility than natural biopolymers limit its applications. In this work, cold-plate electrospinning was applied to fabricate 3D interconnected macroporous sponge-type nanocomposite fibrous scaffolds of PCL/silk fibroin (SF). SF particles, produced from *Bombax mori* cocoon, were successfully introduced within/on the PCL nanofibers. Precipitation of the SF particles in the PCL solution was prevented by using a custom-designed automixing system, while cold-plate electrospinning. As compared with the PCL nanofibers, the PCL/SF nanocomposite fibrous matrix (PSM) was extensively characterized using the various techniques, such as SEM, TEM, contact angle

measurement, FT-IR and TGA. *In vitro* NIH3T3 fibroblasts studies showed that the cells proliferated on the scaffolds for 7 days, but there was no statistical difference between the scaffolds. Conversely, *in vivo* rat model studies indicated that the wound healing area increased, the cells infiltrated into the scaffolds, and the collagen deposition increased with the SF content within/on the PSM scaffolds. This study confirms that the novel nanocomposite fibrous systems possess great potential to be used as an artificial dermal substitute

#### **Incorporation of Quantum Dots in Silk Biomaterials for Fluorescence Imaging**

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Tracking the distribution and degradation of biomaterials after *in vivo* implantation or injection is important for tissue engineering and drug delivery. Intrinsic and externally labeled fluorescence has been widely used for these purposes. In the present study, 3-mercaptopropionic acid (MPA)-coated CdTe quantum dots (QDs) were incorporated into silk materials via strong interactions between QDs and silk, likely involving the hydrophobic beta-sheet structures in silk. MPA-QDs were pre-mixed with silk solution, followed by ultrasonication to induce silk gelation or by blending with polyvinyl alcohol (PVA) to generate silk microspheres. Silk structural changes and hydrogel/microsphere morphologies were examined by ATR-FTIR and SEM, respectively. The fluorescence of QDs-incorporated silk hydrogels and microspheres remained stable in PBS pH 7.4 for more than 4 days. The amount of QDs released from the materials during the incubation was dependent on loading; no QDs were released when loading was below 0.026  $\mu\text{mol}$  per mg silk. After subcutaneous injection in mice, the fluorescence of QDs-incorporated silk microspheres was quenched within 24 h, similar to that of free QDs. In contrast, the QDs-incorporated silk hydrogels fluoresced for more than 4 days *in vivo*.

#### **Osmotic Pump and Silk Fiber Infusion as a Drug Delivery System in Fat Grafting**

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**Introduction:** Lipoaspirate survival after grafting has been unpredictable. Drug delivery systems are an attractive method of improving lipoaspirate survival. The aim of this study was to test biocompatible, biodegradable porous hollow silk fibers as a conduit for the direct infusion of adipogenic agents into adipose tissue grafts.

**Methods:** Silk perfusion fibers were prepared by gel spinning a highly concentrated silk solution generated from *Bombyx mori* silkworm cocoons. Implantable slow-release osmotic pumps (ALZET) with a flow rate of 0.5  $\mu\text{L/hr}$  were filled with dexamethasone and connected to the silk fibers as a drug delivery system. An athymic nude mouse model was used to study the local delivery of 100  $\mu\text{g}$  dexamethasone, a known adipogenic agent, which was released by silk fiber connected to ALZET pump, and compared with pumps filled with phosphate buffer saline (PBS) and lipoaspirate only. Human lipoaspirate was injected around the drug delivery system that was initially inserted in the dorsal aspect of the mouse. After 21 days, the fat grafts were excised and analyzed for mass, followed by histological analysis to quantify vascularization and cell inclusion.

**Results:** Dexamethasone was released at a controlled rate from the pump through the porous silk fiber. *In vivo*, mass measurements of lipoaspirate that received 100  $\mu\text{g}$  dexamethasone was higher than fat grafts containing silk catheter with PBS and lipoaspirate only. Histological analysis demonstrated healthy adipose tissue morphology.

**Conclusion:** This model presents a drug delivery system with ability to vary dosing over time, and enhances adipose tissue graft survival and volume retention.

#### **Polyoxate/Silk Fibroin Film for Bioregeneration of Neo-corneal Endothelial Cells**

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Corneal transplantation, a common surgical protocol for visual acuity improvement, is limited owing to a shortage of high quality donor corneas and/or its accurate replication of structural and biochemical composition of native cornea in a scaffold. Construction of neo-corneas utilizing novel, biocompatible and biodegradable scaffold source, could address such formidable challenges. Herein, we designed optically transparent silk fibroin film, modified degradable properties with POX as an alternative scaffold source for bioengineering cornea. Silk from *B. mori* and POX (Mw 80 K) were used to fabricate the transparent films. The morphological and structural properties were analyzed by FESEM, FTIR, contact angle, etc. *In vitro* biological compatibility was studied such as initial attachment, proliferation, mRNA expression, and proteins related functions using primary corneal endothelial cells. FESEM images display the surface roughness/thickness by the various ratio mixtures of POX and SF. POX content affected in transparency of each POX/SF films but it is not significant except only POX film. Even though differences occur according to ratio of POX and SF, they do not show any significant differences on morphology and well-expressed their functional proteins which are regulated functions of corneal endothelium. Overall, the results suggest that SF offer good environment on growth of CEnCs and POX plays a role to improve physical properties. POX/SF film may be a suitable alternative for high quality corneal tissue expansion and transplantation. This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government (MEST) (NRF-2012M3A9C6050204) and BK21 PLUS.

#### **The Vessel Injury Repair using the Silkworm-cocoon-derived Membrane**

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The purpose of this study was to evaluate silk membrane (SM) for application in the vessel injury repair. Silk cocoons are composed of several layers. We have developed a simple and green processing technique to obtain the thin membrane from inner layer of cocoons for the vessel injury repair. They were compared with commercially available vessel patch such as polytetrafluoroethylene membrane (PM). For the comparison, scanning electron microscopy, FT-IR analysis, tensile strength evaluation, and the animal study were done. The rat carotid artery was partially removed and repaired with each material. The physical properties of SM were comparable to the commercially available vessel patch. The Doppler sonography showed significantly higher rheological values in the SM group ( $P < 0.05$ ). On the histological exam, there was no foreign body reaction in SM group and showed regenerated endothelial cells. The vascular dimension was well maintained. In conclusion, the SM group could be used for vessel repair.

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#### **Fabrication of Silk Fibroin Biomaterials for Biomedical Applications**

C. Park;

Hallym University, Chuncheon, KOREA, REPUBLIC OF.

Silk fibroin of silkworms has been widely studied as biomaterials. To date, silk fibroin, the main component of silk protein, has

been reported to be a perfect substrate for the proliferation and adhesion of a large variety of cells. Thus, silk fibroin may be resolubilized into an aqueous solution, and then formed into a number of different geometrical forms, including electrospun fibers, films, sponge forms, hydrogels, and microspheres. Meanwhile, porous three-dimensional materials, and the network structure materials, are composed of interconnected or closed pores. They possess some excellent characteristics, such as favorable mechanical properties and optic-electrical properties, good perm-selectivity, selective adsorption and chemical activity. Porous three-dimensional biomaterials provide a microenvironment for attachment, increase surface area, support a large cell mass, form an extracellular matrix and play an important role in manipulating cell functions in tissue engineering. Therefore, three-dimensional silk fibroin porous scaffolds' chemical composition, physical structure, and biologically functional moieties are all important for tissue engineering. Recently, there have been many reports about silk fibroin porous materials which have been widely investigated in various fields of tissue engineering. In this, we will focus on several preparation methods for fabrication of regenerated silk and various biomedical applications for tissue engineering.

### Session: Silk Biomaterials for Tissue Engineering (Part 1)

**Date and Time: Friday, September 11, 2015,  
10:45 AM - 12:15 PM**

#### Glass Transitions in Silk Biopolymers

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Solid silk fibers are semi-crystalline structural materials that can be further processed to rebuild a variety of silk materials and morphologies for bioengineering applications. In order to better develop and utilize the silk biomaterials, we first study the structure-property relations and the glass transition behavior for the source silk fibers. Four types of silk fibers from silkworm species *Bombyx mori*, *Anthearea pernyi*, *Anthearea mylitta* and spider species *Nephila edulis* have been examined using dynamic mechanical thermal analysis (DMTA). The glass transition characteristics including transitional temperature and loss factor for the main "disordered" structure in these silks have been defined and compared. The "disordered" structure in silk fibers that contributes to the glass transition can be quantitatively correlated to the non-repeated segments and the end groups in the silk sequences. These information on the structures of solid silk fibers would benefit the understanding on the structures of biomaterials based on silks.

#### Multi-dimensional Tuning and Biological Modulation With Silk: Tropoelastin Protein Alloys

**A. S. Weiss**;

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Blended polymers are used extensively in many critical medical conditions as components of permanently implanted devices. Hybrid protein polymers containing recombinant human tropoelastin and silk fibroin have favorable characteristics as implantable scaffolds in terms of mechanical and biological properties. A firefly luciferase transgenic mouse model was used to monitor real-time IL-1 $\beta$  production localized to the site of biomaterial implantation, to observe the acute immune response (up to 5 days) to these materials. Significantly reduced levels of IL-1 $\beta$  were observed in silk/tropoelastin implants compared to control silk only implants at 1, 2 and 3 days post-surgery. Subsequently, mice (n=9) were euthanized at 10 days (10D) and 3 weeks (3W) post-surgery to assess inflammatory cell infiltration and collagen deposition, using histopathology and immunohistochemistry. Compared to control silk only implants, fewer total inflammatory cells were found in silk/tropoelastin (~29% at 10D and ~47% at 3W). Also fewer ingrowth cells (~42% at 10D and ~63% at 3W) were observed within the silk/tropoelastin implants compared to silk only. Lower IL-6 (~52%) and MMP-2 (~84%) (pro-inflammatory) were also detected for silk/tro-

poelastin at 10 days. After 3 weeks implantation, reduced neovascularization (vWF ~43%), fewer proliferating cells (Ki67 ~58% and PCNA ~41%), macrophages (F4/80 ~64%), lower IL-10 (~47%) and MMP-9 (~55%) were also observed in silk/tropoelastin materials compared to silk only. Together, these results suggest that incorporation of tropoelastin improves on the established biocompatibility of silk fibroin, uniquely measured here as a reduced foreign body inflammatory response.

#### Non Mulberry Silk Materials for Tissue Engineering

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Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur, INDIA.

Efficient coordination of cells, designed matrices and biochemical factors lead to the well distributed tissue or organ formation. This is dealt in cell based tissue engineering. Silk proteins (fibroin and sericin) are obtained mainly from lepidopteron (mulberry and nonmulberry) species. Indian tropical tasar *Antheraea mylitta* silk fibroin also has RGD motifs in its protein structure making it an efficient biomaterial. The matrices (thin film, sponge, hydrogel, micro/nanoparticle, micro patterns and nanofibrous mats) are explored for their biomedical applications such as bone, liver, cartilage, muscle, nerve, heart, lung, and skin tissue engineering. Controlled delivery of bioactive molecules (growth factor/drug) is studied when embedded/encapsulated in fibroin matrix and it enhances with targeted fibroin nanoparticles. 3D porous fibroin scaffold is an effective construct model for studying the tumor microenvironment. Nanofibrous fibroin based matrices enhances osteoinduction and osseointegration. Silk protein sericin (glue protein), a waste product of silk industries is applied to wide area due to its biocompatible properties. The sericin in biomedical applications is explored majorly in skin tissue engineering. Sericin is engineered for bionic microcapsules; stimulus responsive, injectable photoluminescent hydrogels and photo-crosslinked sericin microstructures; and as a suitable vehicle for the delivery of cell, gene and bioactive molecules. Sericin promotes osseointegration and enhances cellular functions for encapsulated cells. Recent findings of nonmulberry silk proteins as biomaterials will be discussed (Supported by DBT, ICMR and DST, Govt. of India).

#### Biomimetic Design of Silk Materials for Tissue Engineering

**Q. Lu, Sr.;**

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Silk materials have been extensively used in tissue engineering and tissue regeneration. It remains a challenge to design silk materials that could provide preferred microenvironments for specific tissues. Here, we describe a feasible strategy in fabricating silk biomaterials based on silk self-assembly regulation. Silk nanofibers formed in aqueous solution are used to prepare silk scaffolds with improved cell biocompatibility. Then the stiffness of the materials is tuned by controlling silk secondary conformations. The preferred cell differentiation into different functional cells suggests the feasibility of silk materials as bioactive matrix in different tissue engineering. The orientation structures are also induced in the above silk materials, further regulating cell behaviors. Furthermore, silk-based delivery systems with designed drug release behaviors are assembled into the silk materials to build better microenvironments for tissue regeneration. All the present studies imply that mulberry silk materials have the capacity in actively controlling cell behaviors, which is critical for successful tissue engineering.

#### An Injectable, Photoluminescent and Biodegradable Sericin/Dextran Composite Hydrogel as a Drug Delivery System for Melanoma Treatment

**Z. Wang**, C. Qi, J. Liu, L. Wang;

Union Hospital, Huazhong University of Science and Technology, Wuhan, CHINA.

Cancer chemotherapy is often challenged by severe cytotoxic side effects, fluctuating blood concentrations, and limited drug access to cancer regions. These limitations motivate the development of controlled, target-specific drug delivery systems. Here we report the fabrication of a novel sericin/dextran injectable hydrogel as a drug delivery system for cancer treatment. Sericin, a natural protein from silk, is modified to be a crosslinkable sericin derivative by adding hydrazide groups to the side chains of sericin. The modified sericin is then crosslinked with oxidized dextran, forming an injectable hydrogel via Schiff base reaction between hydrazide and aldehyde groups. The sericin/dextran composite hydrogel exhibits high porosity, biodegradability and biocompatibility. The chemical and physical properties of the composite hydrogel can be simply tuned by adjusting the formulation ratios of hydrazide functionalized sericin and oxidized dextran. Additionally, the sericin/dextran hydrogel sustains the release of small and macromolecular drugs. Notably, this sericin-based hydrogel exhibits stable photoluminescence *in vivo*, allowing real-time monitoring of hydrogel degradation and drug release. Further, in a B16-F10 melanoma-bearing C57LB/6 mice model, doxorubicin-loaded sericin/dextran hydrogel effectively suppresses tumor growth and prolongs animal survival. Together, our study indicates that the sericin/dextran composite hydrogel can serve as a biodegradable, biocompatible and trackable drug delivery system for treating malignant melanoma. With these integrated functions, this multifunctional hydrogel system offers an alternative way of improving safety of chemotherapeutic agents and optimizing treatment efficacy in conjunction with other cancer regimens.

#### Functional Silk Architectures using Photolithography

R. Pal<sup>1</sup>, N. E. Kurland<sup>1</sup>, S. C. Kundu<sup>2</sup>, V. K. Yadavalli<sup>1</sup>;

<sup>1</sup>Chemical and Life Science Engineering, Virginia Commonwealth University, Richmond, VA, <sup>2</sup>Biotechnology, Indian Institute of Technology, Kharagpur, INDIA.

Precise spatial patterns and micro and nanostructures of peptides and proteins have widespread applications in tissue engineering, bioelectronics, photonics, and therapeutics. Optical lithography using proteins provides a route to directly fabricate intricate, bio-friendly architectures rapidly and across a range of length scales. The unique mechanical strength, optical properties, biocompatibility and controllable degradation of biomaterials from silkworms offer several advantages. Here, we present the biochemical synthesis and applications of a "protein photoresist" synthesized from both the silk proteins, fibroin and sericin. Using light-activated direct-write processes such as photolithography, we show how silk proteins can form high resolution, high fidelity structures in two and three dimensions. Silk fibroin and sericin features can be precisely patterned at sub-microscale resolution ( $\mu\text{m}$ ) at the bench-top over macroscale areas (cm), easily and repeatably with high-throughput. For instance, periodic, microstructured arrays can be patterned over large areas to form functional opto-electronic structures. We further demonstrate how photocrosslinked protein micro-architectures can function for the spatial guidance of cells without use of cell-adhesive ligands as biocompatible and biodegradable scaffolds. The ease of biochemical functionalization, biocompatibility, as well as favorable mechanical properties and biodegradation of this silk biomaterial provide opportunities for otherwise inaccessible applications as sustainable, bioresorbable protein microdevices.

#### The Acoustic Properties of Silk Films for Tympanic Membrane Repair

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Silk fibroin membranes have shown potential as an off-the-shelf alternative to existing graft materials to repair tympanic membrane (TM) perforations due to their excellent mechanical properties, transparency and biocompatibility. However, when developing such

materials, acoustic properties must also be considered since the material used can have a significant impact on patient hearing outcomes. The two most commonly used graft materials for TM repair are temporalis fascia and cartilage, both harvested from the patient. Silk membranes over a range of thicknesses ( $5\ \mu\text{m}$  to  $100\ \mu\text{m}$ ) were tested alongside animal cartilage and fascia as well as with paper, another graft material used for TM repair. All silk membranes showed excellent acoustic properties, with a peak amplitude of at least 10 dB higher than that of cartilage or fascia. The resonance peak of the silk films increased from 2.5 kHz to 7.9 kHz as the film thickness increased from  $5\ \mu\text{m}$  to  $100\ \mu\text{m}$  while the peak amplitude decreased from 32.3 to 5.5 dB rel 1 mm/s/Pa. The paper showed similar properties to silk membranes of a similar thickness. The resonant frequency of silk membranes was much higher than thicker cartilage samples. This is presumably due to differences in stiffness, with the silk membranes showing a Young's modulus up to 150 times greater than that of cartilage. Future work will focus on modifying the silk membranes to bring the mechanical properties and resonant frequency closer to that of the native tympanic membrane while maintaining the other superior properties of the material.

#### Session: Silk Biomaterials for Tissue Engineering (Part 2)

Date and Time: Friday, September 11, 2015,  
12:45 PM - 2:00 PM

#### Turning a Silk Purse into a Sow's Ear: The Importance of Processing When using Silk in Regenerative Medicine

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Processing defines silk, for unlike all other biological materials they are spun, not grown. Silks are biological polymers that have evolved to be processed by controlled protein denaturation. This makes them ideal inspiration and a "gold standard" for comprehending and controlling the solidification of natural materials. This presentation will provide an overview of Nature's 400 million years of R&D into silk and our recent studies into the importance of processing in this fascinating material.

Furthermore, lessons we have learnt from developing tests to characterise silks, essentially small samples which are extremely sensitive to their environment, have proven useful in testing other biopolymers, namely collagen. This has led us to turn evolutionary constraints into design criteria for the use of biomaterials in regenerative medicine under the paradigm of Compliance Matching and Material Property Led Engineering Technologies: 'CoMMPLETe'. Learning from the challenges presented in testing and repairing small tissues (e.g. rotator cuff tendon) and with silk-based biomedical textiles as our goal, we propose a fundamentally new approach towards biomechanical testing that has wide applicability to all musculoskeletal tissues. Finally I will discuss silk's potential in medicine and how fundamental research is being translated into the development of implantable biomedical devices whose performance may be tuned in terms of both degradation rate and mechanical performance simply by altering the processing of the material.

#### Silk-based Macro Encapsulates for Sustained Insulin Release

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The current study describes the fabrication of cell-encapsulating 3D silk scaffolds as bio-artificial pancreas towards sustained insulin release. Developed scaffolds provided pancreas like niche to re-establish the microenvironment of islets lost during isolation. Within optimised silk scaffolds, insulin producing islet cells were encapsulated in combination with alginate and agarose as immune-isolation barriers for enhanced graft survival and function *in vivo*[1]. Scaffolds are extensively characterized for pore architecture, porosity, swelling



index, density and matrix degradation. Further, scaffold suitability was assessed through diverse *in vitro* tests, including measurement of cell viability, glucose consumption and time dependant insulin secretion [2]. Enhanced cellular viability and proliferation using rat primary islets and RIN-5F rat  $\beta$  cell line suggested successful encapsulation and compatibility. Rin-5F cells increased 2.5 folds in 4 weeks of culture while primary islets maintained their native morphology within silk-macro-encapsulates. Islets showed better cell adherence and insulin production with glucose stimulation index of 1.5–2 after 14 days of culture. Live cell imaging confirmed formation of 3D pancreatic spheroid like structures within scaffolds *in vitro*. Further, improved glucose consumption index of nine (09) after 4 weeks of culture suggested metabolically active cells. *In vivo* studies revealed fewer inflammatory cells surrounding implants after 4 weeks of culture [3]. In conclusion, this novel 3D silk platform demonstrated successful encapsulation of insulin producing islet cells with enhanced cellular viability and function. Further development may have potential towards clinically viable bio-artificial pancreas.

### Silk Protein Fibroin and Fibroin Based Nanoparticles Influence Implant Topography, Anti-Bacterial Activity and Osteogenesis

T. Dey<sup>1,2</sup>, D. Naskar<sup>2</sup>, S. Sharma<sup>2</sup>, S. Nayak<sup>2</sup>, S. Bano<sup>2</sup>, H. Kim<sup>3</sup>, S. Kundu<sup>2</sup>;

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Bare metal (Ti or its alloy with Cr, Ni and Ta) orthopedic implants are accepted as standard for bone/dental tissue replacement treatment throughout the World. However, sub-optimal osseointegration and microbial infections are considered as the major challenges in this field. Modifications of the implant surfaces are employed as an opportunity to control tissue-metal interactions and to reduce the bone fixation period. The present study reports the performance of non-mulberry silk protein fibroin for *in vitro* osteogenesis. Primarily, silk fibroin molecules are immobilized on Ti surface and modified surfaces are characterized by SEM, AFM, XPS, EDX, and FTIR. Non-mulberry fibroin facilitates initial cell adhesion followed by improved cell spreading and better mineralization in comparison to the fibroin of mulberry origin, which can be explained by the presence of inherent RGD motifs on the non-mulberry fibroin. Additionally non-mulberry fibroin shows reduced immunological responses (TNF and IL1 $\beta$  secretion) and enhanced osteoblast adhesion. Secondly, the problem of microbial infection is answered by nanoparticle mediated delivery of antibiotics. Fibroin of non-mulberry silk is used to fabricate biodegradable and cytocompatible nanoparticle (100nm) to deliver antibacterial drug (gentamicin). Deposition of fibroin nanoparticles enhances the nano-topography and hydrophilicity of the implant surface significantly as observed by SEM, AFM and contact angle measurement. The antibacterial activities of nanoparticle deposited surfaces are investigated against *Streptococcus aureus*. Presence of gentamicin shows no detectable effect on osteoblast proliferation and function. Osteogenic properties of the nanoparticle deposited surfaces are found to be superior in comparison to bare Ti surface.

### Immune Tolerance of Silk Proteins

B. Kundu<sup>1,2</sup>, J. Kim<sup>3,4,5</sup>, S. C. Kundu<sup>2</sup>, H. Kim<sup>1,4,5</sup>;

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Silk of silkworms is chiefly composed of two protein components; fibroin and sericin. Co-existence of fibroin-sericin evokes immune re-

sponse; therefore, these proteins are extracted separately for biomedical applications. However, critical systematic investigation of immune tolerance of co-existed fibroin-sericin is yet elusive. The present study is conducted to investigate how the concentration of sericin in blends affects the immune tolerance of fibroin in Sprague-Dawley (SD) rats over a period of 4 weeks. In addition, the investigation also includes inter-species comparison of immune responses between fibroins of two different origins; mulberry (*Bombyx mori*) and nonmulberry (*Antheraea mylitta*) in order to elaborate nonmulberry silks as parallel biomaterials to mulberry silk. Natural silk cocoon composition is elucidated by choosing the bio-mimicking blending ratios of silk proteins; fibroin and sericin. The blends form porous interconnected 3D microstructure without any phase separations between the hydrophilic-hydrophobic components and exhibit good *in vitro* compatibility with human dermal fibroblasts. Subsequent subcutaneous implantation indicates no significant inflammatory responses associated with the physico-chemical characteristics of the mulberry and nonmulberry fibroins. However, the blends reveal moderate inflammatory responses with the formation of multi-layer fibrous capsule. Compared to non-mulberry scaffolds, the mulberry scaffolds (pure and blends) show relatively more homogenous cellular infiltration throughout the implants, which results in rapid degradation and absorbance within 4 weeks. Together, the data indicates well immune comparability of pure mulberry and nonmulberry silk fibroins, while the presence of sericin imparts minimal inflammatory responses to implants.

### Exploring Silk Based Biomaterials Functionalized with Antimicrobial Peptides to Prevent Surgical Site Infections

A. R. Franco<sup>1,2</sup>, F. Rodrigues<sup>3,2</sup>, I. Leonor<sup>1,2</sup>, R. L. Reis<sup>1,2</sup>, D. L. Kaplan<sup>4</sup>;

<sup>1</sup>3B's Research Group, University of Minho, Guimaraes, PORTUGAL, <sup>2</sup>ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, PORTUGAL, <sup>3</sup>Life and Health Sciences Research Institute, University of Minho, Braga, PORTUGAL, <sup>4</sup>Departments of Biomedical Engineering, Medford, MA.

Surgical site infections (SSI) often occur after invasive surgery, which is as a serious health problem, making it important to develop new biomaterials to prevent infections. Spider silk is a natural biomaterial with excellent biocompatibility, low immunogenicity and controllable biodegradability. Through recombinant DNA technology, spider silk-based materials can be bioengineered and functionalized with antimicrobial (AM) peptides. The aim of this study is to develop new materials by combining spider silk chimeric proteins with AM properties and silk fibroin extracted from *Bombyx mori* cocoons to prevent microbial infection. Here, spider silk domains derived from the dragline sequence of the spider *Nephila clavipes* (6 mer and 15 mer) were fused with the AM peptides Hepcidin and Human Neutrophil peptide 1 (HNP1). The spider silk domain maintained its self-assembly features allowing the formation of beta-sheets to lock in structures without any chemical cross-linking. The AM properties of the developed chimeric proteins showed that 6 mer + HNP1 protein had a broad microbicidal activity against pathogens. The 6 mer + HNP-1 protein was then assembled with different percentages of silk fibroin into multifunctional films. *In vitro* cell studies with a human fibroblasts cell line (MRC5) showed nontoxic and cytocompatible behavior of the films. The positive cellular response, together with structural properties, suggests that this new fusion protein plus silk fibroin may be good candidates as multifunctional materials to prevent SSI.

**Acknowledgments:** This work was supported by the Portuguese Foundation for Science and Technology under the project PTDC/BBB-BIO/0827/2012.

### Reference

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### Directing Neuronal Projection using Micropatterned Silk Hydrogels

S. E. Anderson, M. Jacobsen, M. L. Smith, J. Y. Wong;  
Biomedical Engineering, Boston University, Boston, MA.

Micropatterned surfaces have proven to be useful for their ability to guide cells into the proper spatial orientation crucial to mimicking the anisotropy of many tissues in the body. However, most culture surfaces that are used for micropatterning are not suitable for implantation directly into the area of interest, which requires a biocompatible scaffolding material. Silk is an attractive material due to its high material strength, biocompatibility, and biodegradability. We developed a technique to form silk fibroin into malleable gels, and we developed an approach to pattern their surface via first microcontact printing lanes of integrin-binding proteins onto coverglass and then transferring this pattern to the silk surface during gelation. Directed growth of neuronal projections was accomplished using fibronectin and laminin as the integrin binding proteins, and the directional alignment of dorsal root ganglion cells seeded onto the micropatterned gels was tracked. Protein concentration and lane geometries were varied to optimize projection potential. Encouraged directional growth is essential in nervous system repair and these patterned gels demonstrate potential as scaffolding elements to restore connectivity in neural injuries for *in vivo* applications. This patterning method can likewise be adapted for use with different proteins suitable for other tissue engineering applications that require directional alignment including muscular, vascular, and bone tissues.

### Sericin Hydrogel as a Neuro-protective Cell Carrier for Neuron Injury Repair

L. Wang, J. Wang, Z. Luo, Z. Wang;

Union Hospital, Huazhong University of Science and Technology, Wuhan, CHINA.

Brain tissue repair remains challenging due to tissue location and neuronal non-renewability. Various biomaterial-based repair strategies have been developed with limited success. Protecting neurons from further damage is key to brain tissue repair. An ideal repairing material should support *in vivo* survival of transplanted neuronal cells that repair the damaged tissue. Sericin, a major component of silk, is a natural protein with excellent biocompatibility and low immunogenicity. Here we develop a neuro-protective genipin-crosslinked sericin hydrogel (GSH) as a cell carrier for neuronal repair. This sericin hydrogel possesses porous structure, brain-tissue resembling mechanical properties, biodegradability and excellent biocompatibility. The genipin-crosslinked sericin hydrogel promotes cell attachment, axon outgrowth and proliferation of primary cortical neurons. Both sericin protein and the degradation products of GSH exhibit the intrinsic capability of protecting neurons from apoptosis induced by hypoxia through altering Bcl-2/Bax balance and down-regulating caspase-3. When a GSH seeded with neuronal cells is subcutaneously transplanted into C57BL/6J mice, the GSH significantly improves the survival of transplanted neuronal cells. By Day 5 approximately 20% of transplanted cells remain alive. Together, we generate a novel sericin hydrogel that possesses a unique neuroprotective activity with an ideal neuronal biocompatibility. Given that it supports long-term neuron survival both *in vitro* and *in vivo*, this sericin hydrogel may serve as a promising carrier for delivering neurons for brain tissue repair.

### Session: Skin Regeneration Form Basic Research to Translational Approaches

Date and Time: Wednesday, September 9, 2015, 1:00 PM - 2:15 PM

### Improved Engraftment and Regeneration of Bio-engineered Autologous Skin Grafts Prevascularized with Adipose-derived Stem Cells

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The engraftment and functionality of a skin graft upon transplantation relies on a rapid and stable formation of microvasculature to connect the graft to the recipient. However, neovascularization of currently used dermal substitutes typically takes 2–3 weeks *in vivo*. To accelerate this process, an innovative human dermo-epidermal skin graft with blood capillaries based on a biodegradable fibrin-hydrogel was bio-engineered. Endothelial and mesenchymal stem cells derived from the stromal vascular fraction (SVF) of human adipose tissue spontaneously formed mature microvascular networks *in vitro*. Their ultrastructural TEM analysis revealed the presence of a lumen, pericyte coverage, and organotypic microstructures, such as a basal lamina and “peg-and-socket” junctions.

Upon transplantation onto full-thickness skin defects in immune-deficient rats, the preformed human blood capillaries anastomosed to the recipient's vasculature within only 3–4 days. This rapid onset of blood perfusion in the graft had profound effects on promoting epithelial and dermal tissue regeneration *in vivo*. As a consequence, the neo-epidermis reached rapidly homeostasis and epidermal cells sustained long-term coverage *in vivo*. The healed neo-dermis showed higher type I collagen deposition with basket-weave organization, consistent with significantly reduced wound contraction and less scar formation.

Thus, such co-culture-based prevascularization strategy emerges as a promising approach to significantly improve wound healing and skin function restoration. Most importantly, our results underline that the development of a complex capillary plexus, acting as a specialized “vascular niche” in the graft, is pivotal for skin regeneration by promoting tissue homeostasis, cell function, survival, and the maintenance of a skin tissue stem cell reservoir.

### Plasma-based Fibrin: A Factor Releasing Matrix for Skin Tissue Engineering

M. Alexaline<sup>1,2</sup>, M. Nivet<sup>1,3</sup>, T. Leclerc<sup>4</sup>, E. Bey<sup>5</sup>, P. Duhamel<sup>5</sup>, J. Lataillade<sup>1,3</sup>, M. Trouillas<sup>1,3</sup>;

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Cultured Epithelial Autografts (CEAs), represent a lifesaving surgical technique in case of full-thickness skin burn covering more than 50% total body surface area. However, CEAs present numerous drawbacks, among them the high fragility of keratinocyte sheets, and the immaturity of the dermal-epidermal junction leading to heavy cosmetic and functional *sequelae*.

To overcome these weaknesses, we developed a new human epidermal substitute (ES) cultured over a human-Plasma Based fibrin Matrix (hPBM). Phenotypical, functional (clonal analysis, long-term culture or *in vivo* graft) and released factors analyses were used to compare ES performed with hPBM, fibrin from purified fibrogen (FPF) or no matrix (CEA).

The use of hPBM for ES production induced more proliferation, better cell organization and clonogenicity, enhanced dermal-epidermal junction protein deposition and prevented their degradation while FPF specifically improved keratinocyte migration potential. Keratinocyte differentiation was decreased using fibrin matrices. The growth factor released from hPBM in culture medium were different than from FPF and their influence on ES properties was investigated. Finally, the use of hPBM as a culture support for ES allowed better engraftment directly on a NOD-SCID model of acute wound.

Together, these results show the positive impact of fibrin matrices, modulated by released growth factor, on ES phenotype and grafting efficiency. We hope that this new strategy could improve the current medical treatment of full-thickness burn patients in the future.

### Skin-derived Cell-sheets as Powerful Tools to Engineer Skin Analogues

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Cell/cell-extracellular matrix (ECM) dynamic interactions appear to have a major role in regulating communication through soluble signaling, directing cell binding and activating substrates that participate in the highly organized wound healing process. Moreover, these interactions are also crucial for *in vitro* mimicking cutaneous physiology. Herein we explore cell sheet (CS) engineering to create cellular constructs formed by keratinocytes (hKC), fibroblasts (hDFB) and dermal microvascular endothelial cells (hDMEC), to target skin wound healing but also the *in vitro* recreation of relevant models. Taking advantage of temperature-responsive culture surfaces, which allow harvesting cultured cells as intact sheets along with the deposited native ECM, varied combinations of homotypic and heterotypic three-dimensional (3-D) CS-based constructs were developed. Constructs combining one CS of keratinocytes as an epidermis-like layer plus a vascularized dermis composed by hDFB and hDMECs were assembled as skin analogues for advancing *in vitro* testing. Simultaneously both hKC and hDMEC were shown to significantly contribute to the re-epithelialization of full-thickness mice skin wounds by promoting an early epithelial coverage, while hDMEC significantly lead to increased vessels density, incorporating the neovasculature. Thus, although determined by the cellular nature of the constructs, these outcomes demonstrated that CS engineering appear as an unique technology that open the possibility to create numerous combinations of 3D constructs to target defective wound healing as well as the construction of *in vitro* models to further mimic cutaneous functions crucial for drug screening and cosmetic testing assays.

**Acknowledgments:** Portuguese Foundation for Science and Technology (FCT) for Mariana Cerqueira Post-doctoral grant (SFRH/BPD/96611/2013)

### Biomimetic Microengineering of Hair Follicles *In Vitro*

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Increased understanding of pathways regulating mesenchymal-epithelial interactions involved in hair follicle development brings forth opportunities for therapeutic development for alopecia and opens new doors for studies in skin regeneration. A lack of physiological *in vitro* models, however, poses major challenges to mechanistic investigation of key biological pathways, as well as identification and screening of hair growth agents. Here we describe a biologically inspired microengineering approach to create a biomimetic *in vitro* model that reconstitutes the *in vivo*-like spatial patterning of the dermal sheath, the dermal papilla (DP), and keratinocytes. Briefly, we fabricated PDMS arrays of cylindrical posts of physiological size and used them to generate microwell arrays in a cross-linked gelatin hydrogel, supportive of cell adhesion and growth. Concurrently, agarose micro-wells were used to engender self-assembly of dermal cells into DP spheroids. With careful inversion, overlay, and alignment with the gelatin scaffold, DP spheres were then transferred from the agarose to the molded gelatin wells. Formation of the dermal sheath was accomplished with overnight attachment of dermal cells seeded in suspension. Finally, keratinocytes were incorporated through passive adhesion from a high density cell suspension. Spatial patterning and cell viability were confirmed with confocal microscopy. Current studies focus on recapitulating the spatial distribution of keratinocytes within a hair follicle *in vivo* by using keratinocyte-laden hydrogel beads to increase filling of void space in the gelatin microwells. This microphysiological culture system will serve as a novel platform to screen cell types and signaling molecules for bioengineering hair follicles.

### Dermal Induction Potential of Mesenchymal Stem Cells Used in a Model of Human Bioengineered Skin

I. Garzón<sup>1</sup>, E. González-Andrades<sup>1</sup>, J. Muñoz-Olmedo<sup>1</sup>, A. Vela-Romera<sup>1</sup>, M. González-Andrades<sup>1</sup>, C. Alfonso-Rodríguez<sup>1</sup>, R. Fernández-Valadés<sup>2</sup>, M. Sánchez-Quevedo<sup>1</sup>, S. Arias-Santiago<sup>1</sup>, M. Alaminos<sup>1</sup>;

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**Objective:** The objective of this work is to evaluate the dermal inductive capability of different mesenchymal stem cells used in a model of human artificial skin.

**Methods:** Primary cell cultures of human adipose tissue (ADSC), dental pulp (DPSC) and Wharton's jelly (HWJSC) stem cells were generated, and 3D fibrin-agarose substitutes of the human dermis were developed. ADSC, DPSC and HWJSC were subcultured on top of the dermal substitutes to reproduce the epidermal layer of the skin. 3D skin substitutes were grafted in nude mice and histological and dermal differentiation was assessed by histochemical analyses after 7, 14, 21 and 28 days of *in vivo* development.

**Results:** Our results revealed that HWJSC and DPSC were able to differentiate in the skin substitute, and an epithelial-like tissue was generated on top of the substitute. The differentiation efficiency of these cell types was higher than ADSC both *ex vivo* and *in vivo*. Staining for collagen and elastic fibers was negative in all *ex vivo* samples. However, *in vivo* grafting was able to induce high collagen synthesis, whose expression tended to increase after 14 days of *in vivo* development, being stronger in HWJSC and DPSC samples. Moreover, the presence of glycoproteins was significant in HWJSC samples *ex vivo* and *in vivo*.

**Conclusions:** These results point out the potential of HWJSC and DPSC to induce dermal differentiation by epithelial-mesenchymal interaction and support their use for skin regeneration.

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### Session: The Effects of Gender and Age on Tissue Engineering

**Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM**

### Age and Gender Dependent Differences in Skeletal Muscle Regeneration after Compression Injury in Rats

T. Criswell, Y. Zhou, S. Soker, B. Yosef;

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The progression of sarcopenia, as well as the decreased ability to repair damaged muscle, in older persons represents a major pathway to disability. Whereas men undergo a gradual decline in muscle mass and function with age, women face a dramatic decrease around the age of 55, corresponding to a menopause related decrease in sex hormones. The objective of this research is to examine age and gender-dependent differences in skeletal muscle regeneration.

Compression injury was induced in the hind limbs of young (3–4 months), adult (10–12 months) and aged (18 months) male and female Lewis rats as previously described (Criswell *et al.*, 2012). Muscle function, as determined via neural stimulation, was used as the measure of muscle function. The tibialis anterior (TA) muscles were collected at days 14 and 28 after injury. RNA was isolated from injured and non-injured TA muscles for quantitative PCR (qPCR) analyses.

Our data demonstrated delayed functional recovery, increased fibrosis and a delayed course of muscle regeneration in the older animals as noted by the delayed activation of myogenic transcription factors. Older female rats demonstrated the most significant reduction in regenerative capabilities. The delayed functional and morphological recovery in the older rats more closely mimics the delayed recovery found in injured patients. Gender differences are evident in this model of muscle injury and regeneration. This model will provide a means for investigating these differences for the treatment of sarcopenia.

**Age and Gender Effects on Regenerative Medicine Therapies**

J. K. Williams, T. Criswell;

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Regenerative Medicine has the potential to supply an unlimited amount of engineered healthy tissue and organs for the repair and replacement of diseased tissue. Great progress has been made in preclinical studies and many applications are now in the clinical stage. However, the effects of age and gender on tissue engineering and regenerative medicine are just beginning to be explored.

Aging reduces the ability of tissue to regenerate and contributes to increased risk of chronic muscle, bone, cardiovascular, urogenital, neuronal and digestive diseases. This is especially true with muscle-associated diseases where aging increases DNA methylation of muscle cells, reduces muscle satellite cell populations and increases the rate of muscle cell apoptosis. This represents a challenge to muscle cell therapies that may work well in younger individuals, but not as well in older individuals who could greatly benefit from such therapies.

Gender too has a profound effect on the risk of chronic diseases and interacts with age to further alter this risk. These include a variety of cardiovascular diseases, cancer, cognitive-associated syndromes, auto-immune, muscle and bone diseases. Dissecting out age from gender as risk factors is often not easy. For instance, one must separate the disease risk associated with changing hormones and age (e.g. menopause) with the efficacy of cell therapy at different ages and hormone concentrations.

By addressing variations of different patient populations, regenerative medicine will be able to better personalize treatment in order to increase the efficacy these promising therapies.

**The Age of hMSC Donor Affects Biomechanical Properties of Tissue Engineered Ligament Constructs**S. S. Novakova<sup>1</sup>, M. J. Smietana<sup>2</sup>, V. Mahalingam<sup>1</sup>, E. M. Arruda<sup>3</sup>, L. M. Larkin<sup>1</sup>;

<sup>1</sup>Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, <sup>2</sup>Biomedical Engineering, University of Michigan, Ann Arbor, MI, <sup>3</sup>Mechanical Engineering, University of Michigan, Ann Arbor, MI.

We have successfully fabricated scaffold-less multiphasic ligament grafts for ACL repair using commercially available and well-characterized human MSCs (hMSC) as a starting material. Using our fabrication process, hMSCs were cultured and harvested at: Day 0 (initial plating), Day 4 (growth phase), Day 8 (differentiation phase), and Day 12 (3-D construct) for qPCR analysis. At day 15, 3-D constructs were tested for biomechanics and histology. The data indicated two significantly different cohorts ( $n > 3$  per donor per time point) that could be explained by age (young vs. old). In the young cohort (22–25 y), the mean tangent modulus of the engineered ligament was significantly stiffer at  $24.66 \pm 5.85$  kPa compared to the old cohort (33–43 y) at  $10.95 \pm 1.01$  kPa. Average gene expression of ligament markers relative to GAPDH was analyzed via qPCR at each time point during fabrication. Compared to undifferentiated hMSCs (Day 0), the young cohort showed significantly increased expression of ligament markers Scx & Ten-C by Day 4, and Col-1 & Col-3 by Day 8. The old cohort showed an upward trend in the expression of Col-1 and TenC, but did not reach significance at any time point, compared to undifferentiated hMSCs. However, the older cohort showed significant expression of Col-3 at Day 12, and Sex at Day 4 and Day 12. In summary, constructs from younger donors had earlier expression of ligament markers, resulting in a greater tangent modulus. Thus, donor age influences gene expression and biomechanics of our tissue-engineered ligament constructs.

**An Osteochondral Microphysiological System to Study the Pathogenesis of Osteoarthritis and the Effect of Hormonal Exposure**R. Gottardi<sup>1,2</sup>, H. Li<sup>1</sup>, T. P. Lozito<sup>1</sup>, P. G. Alexander<sup>1</sup>, K. C. Clark<sup>1</sup>, E. C. Sefton<sup>3</sup>, T. K. Woodruff<sup>3</sup>, R. S. Tuan<sup>1</sup>;

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<sup>3</sup>Department of Obstetrics and Gynecology, Northwestern University, Chicago, IL.

Osteoarthritis (OA), the most prevalent form of arthritis, is characterized by joint articular cartilage degeneration with accompanying subchondral bone lesions. OA incidence in women is 36–45% higher than in men, and osteoporosis in postmenopausal women is twice as high as age-matched men. We hypothesize that restoring hormonal exposure to bone similar to that of the menstrual cycle would stimulate an anabolic response in both bone and cartilage. This hypothesis is tested using a 3D osteochondral (OC) microtissue system maintained in a micro-physiological system (MPS) bioreactor, with separate fluidics for the chondral and osseous components, involving two tissue models - native human OC biopsy plugs, and primary human chondrocytes and osteoblasts seeded in a photocrosslinked gelatin hydrogel. All cells/tissues were harvested from patients undergoing total joint replacement (IRB: Univ Washington). Either the chondral or the bone component was exposed to hormonal stimulation, and the full OC model was then examined to assess bone/cartilage physiology and interaction, using histology, immunohistochemistry, microCT, RT-PCR, and ELISA of culture supernatants. Restoration of hormonal exposure improved bone quality at the OC interface in native OC tissues. Cell/gelatin-based OC model showed a general metabolic decrease in cartilage and bone for both direct and indirect hormonal exposure and Indian Hedgehog signaling modulation by estrogen and progesterone. Responsiveness of the chondral (osseous) component upon hormonal exposure to the osseous (chondral) component suggests active chondrocyte-osteoblast communication in a 3D context.

**Support:** Commonwealth of Pennsylvania, NIH (1U18TR000532, RST; UH2ES022920, TSK), Ri.MED Foundation.

**Effects of Donor Age and Menopausal Status on Proliferation and Osteogenic Differentiation of Human Adipose Derived and Bone Marrow Derived Mesenchymal Stem Cells**E. G. Lobo<sup>1,2</sup>;

<sup>1</sup>Department of Biomedical Engineering, UNC-Chapel Hill, Chapel Hill, NC, <sup>2</sup>Department of Materials Science and Engineering, NC State University, Raleigh, NC.

Human bone marrow derived mesenchymal stem cells (hMSC) and adipose derived stem cells (hASC) are being actively investigated for tissue engineering and regenerative medicine applications. However, a critical issue remaining to be addressed for their effective therapeutic translation is the extensive donor-to-donor variability associated with these cell types. Understanding and elucidation of the donor-associated factors that cause inconsistent proliferation and differentiation capacities of hMSC and hASC remain an active area of investigation. In this presentation, Dr. Lobo will discuss approaches in her lab to elucidate the effects of aging and menopausal status on proliferation and osteogenic differentiation of hMSC and hASC. Methods to create hASC “superlots” based on menopausal status (pre-, peri- or post) will be described along with experiments evaluating the varying proliferation and osteogenic differentiation potential of these groups. In addition, the effect of 10% cyclic tensile strain (a mechanical stimulus we have previously shown significantly promotes and enhances osteogenesis of hASC and hMSC isolated from young donors) on proliferation and osteogenesis of hMSC isolated from osteoporotic donors will be explained. Finally, new approaches to *a priori* assess a donor cell population for potential success in an autologous bone tissue engineering or regenerative medicine application will be discussed.

**Session: The Hype and the Hope of Cardiovascular Tissue Engineering Strategies**

**Date and Time: Thursday, September 10, 2015, 10:30 AM - 12:00 PM**

**An *In Vitro* Model of the Infarct Microenvironment Highlights the Heterogeneity in the Regenerative Potential of c-Kit+ CPC Clones and their Distinct Sensitivities to Infarct Variables**

K. E. Sullivan, L. D. Black;

Biomedical Engineering, Tufts University, Medford, MA.

Cell therapy for the treatment and prevention of heart failure following myocardial infarction (MI) has the potential to benefit 5 million people in the U.S. alone. Although cardiac progenitor cells (CPCs) contribute to a negligible population of cardiomyocytes in the adult heart, the intramyocardial delivery of these cells post-MI has demonstrated clinical efficacy in two Phase I trials. To more clearly understand how CPCs promote function post-MI and to develop implantation strategies which maximize their therapeutic efficacy, our lab has created an *in vitro* model which recapitulates the complexity of the infarct microenvironment by incorporating alterations to matrix composition, substrate stiffness, oxygen tension and inflammatory cytokines. The development of this model system has allowed us to identify specific variables which drive c-Kit+ CPC engraftment, viability, differentiation and paracrine signaling. Our work highlighted how the initial degree of ISL-1 expression in the isolated population was the strongest determinant of cardiomyogenic differentiation. In contrast, oxygen tension and substrate stiffness were significantly correlated with vascular differentiation potential. However, the model system also highlighted the variability which exists across CPC clones isolated from individual rats 1 week post-MI and also discrepancies in their sensitivity to infarct variables. For example, some clones demonstrated a more significant negative correlation with inflammatory cytokines and vascular differentiation, while others possessed a stronger, positive correlation with oxygen tension. This work highlights the importance of characterizing an individual patient's population of CPCs *in vitro* prior to implantation in order to determine their potential for regeneration.

#### **Elastin Reduces Collagen Scaffold Stiffness, Improves Viscoelastic Properties, and Induces a Contractile Smooth Muscle Cell Phenotype in a Multi-layered Vascular Graft**

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Elastin is a key component of native vasculature where it is responsible for vessel elastic recoil and modulation of smooth muscle cell (SMC) behaviour. Thus, we hypothesized that a collagen & elastin composite scaffold would provide a more natural mechanical response and the optimal physiochemical properties to support SMCs. A novel biofabrication method was developed to generate bilayered tubular scaffolds from collagen & elastin through use of controlled dehydration, custom mold freeze-drying, and various physical/chemical crosslinkers. The biomimetic construct contained an outer porous layer having a pore size (~90 µm) and porosity (~98%) in the ideal range to support cells, with a dense film inner layer designed to support a confluent endothelium. Elastin caused a more natural viscoelastic response via increased creep resistance ( $p < 0.05$ ) and improved cyclical strain recovery ( $p < 0.05$ ). Additionally, elastin resulted in enhanced gene expression of mid/late stage contractile SMC proteins (calponin & SM-MHC) necessary for a vasoactive vessel. Following 21 days culture the SMC-seeded construct was remodelled into a dense organised tissue with a homogeneous cell distribution. Carbodiimide crosslinking resulted in mechanically robust vessels with burst pressures up to 109 mmHg and suture retention strengths of 46 g-f, higher than other crosslinkers ( $p < 0.05$ ). Dynamic physical stimulation in a custom-designed pulsatile bioreactor resulted in enhanced tissue maturation with increased tissue compaction and a further 36% increase in burst pressure ( $p < 0.05$ ). We have developed a bioengineered vessel which represents an ideal platform for *in vitro* medical device testing, examining vascular cell interaction in a vasculature-like environment, or further elucidating vascular disease progression.

#### **Activin A is a Key Factor in Communication Between Adipose Stromal and Endothelial Cells in Therapeutic Vasculogenesis**

D. O. Traktuev, S. Merfeld-Clauss, I. P. Lupov, H. Lu, B. R. Lease, K. L. March;

Indiana University, Indianapolis, IN.

Adipose stromal cells (ASC), similar to other types of mesenchymal stem/stromal cells are localized in the perivascular niche of adipose tissue. When delivered to the animals with pathological conditions, freshly isolated or *in vitro* expanded ASC demonstrate therapeutic effects, which are mostly attributed to angiogenic, anti-inflammatory, and tissue preservation factors secreted by ASC. In parallel, ASC exhibit phenotypical and functional properties of pericytes and, together with endothelial cells (EC), form functional multilayered vessels after implantation. ASC form the outer layer of the new vessels and differentiate into mural cells. Analysis of EC and ASC interactions in *in vitro* model of vasculogenesis revealed that ASC have the tendency to migrate to and accumulate around the EC-vascular cords, ASC promote EC to organize into vascular structures and this process is associated with extracellular matrix protein deposition by both cell types. Direct contact between EC and ASC leads to induction of activin A secretion by ASC. Local accumulation of Activin A induces smooth muscle differentiation in ASC that are distal to EC-cords. Moreover, activin A modifies ASC secretome from being pro-angiogenic to angiostatic. While media conditioned by ASC promoted EC survival, proliferation and vasculogenesis, media from EC-ASC co-cultures has neutral effects upon EC responses. These findings suggest that activin A plays a central role in EC-ASC communications leading to the formation of stable vessels. The effect of ASC interaction with EC should be taken into consideration during the development of vascular therapies which are specifically based on ASC paracrine activity.

#### **Pediatric Tubular Heart Valves from Decellularized Engineered Tissue Tubes**

J. Reimer, Z. Syedain, B. Haynie, R. Tranquillo;

University of Minnesota, Minneapolis, MN.

No FDA-approved heart valve prosthesis possesses somatic growth potential, which makes them less than ideal for pediatric patients. Sequential surgeries while the child matures are required to replace outgrown prostheses in these patients. In light of this, we generated a completely biological heart valve using two concentric engineered tubes, attached together using an absorbable suture line. The engineered tubes were composed of an aligned, collagenous matrix, which was produced by dermal fibroblasts entrapped in a sacrificial fibrin gel. The entrapped cells were removed following the *in vitro* culture period via decellularization. Previous studies have demonstrated that similar engineered tubes were amenable to host cell invasion and matrix deposition, which are necessary precursors to somatic growth, when implanted as arterial grafts or aortic valves in sheep.

The absorbable suture line attached the two tubes together in a prescribed pattern that generated three commissure and leaflet regions on the inner tube. The leaflet regions closed the valve by collapsing inward, per tubular valve design, when exposed to reverse pressure/flow. *In vitro* valve performance and fatigue properties were assessed under pulmonary conditions in a custom pulse duplicator system. The valves exhibited excellent opening and closing, minimal regurgitation, low systolic pressure gradients, and no macroscopic tissue damage. Excellent *In vivo* valve performance was observed up to 8 weeks after implantation into the pulmonary position of a growing lamb. One valve showed extensive host cell invasion following explantation at 12 weeks.

**Acknowledgments:** Funding from NIH R01 HL107572 to R.T.T.

#### **The Hype and Hope of Cardiovascular Tissue Engineering Strategies**

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To use a plumbing analogy, the heart and vasculature have a similar function to a household water system, transporting fluid

via a network of pipes and valves, allowing heat transfer and removal of waste products, amongst other functions. As in any other plumbing network, the component parts of the cardiovascular system are subject to wear and tear, in addition to vessel occlusion - two conditions that can compromise the entire system. Current treatments for congenital/advanced heart valve disease, arterial occlusion and myocardial infarction involve surgical replacement and bypass procedures, yet there are numerous limitations with current state-of-the-art materials and prostheses. The present talk will focus on the impact of tissue engineering and regenerative medicine technologies in the treatment of cardiovascular disease. In contrast to implants developed for most other systems in the body, failure of an implant in the cardiovascular system can prove to be catastrophic. We therefore propose to deliver a balanced presentation addressing the issue of whether or not tissue engineering strategies towards the development of better surgical replacement options in the cardiovascular system are proving fruitful, or whether the ultimate goals are potentially beyond reach, drawing on evidence from studies that highlight the successes, drawbacks and failures in this area. The aim of the talk is to generate some much needed, lively debate on this topic. We anticipate discussion with the audience of the key challenges that must be overcome in this field by tapping into existing and emerging technologies to bring this 'pipe dream' to clinical fruition.

**Controlled Delivery of Angiogenic Factor FGF2 and Immunomodulatory Cytokine IL-10 Cocktail by Heparin-based Coacervate Synergistically Enhances Ischemic Heart Repair**

W. Chen<sup>1</sup>, B. G. Lee<sup>2</sup>, D. Park<sup>3</sup>, K. Kim<sup>4</sup>, H. Chu<sup>2</sup>, K. Kim<sup>5</sup>, J. Huard<sup>6</sup>, Y. Wang<sup>2</sup>;

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Myocardial infarction (MI) causes myocardial necrosis, triggers chronic inflammatory responses, and leads to pathological remodeling. Delivery of angiogenic growth factor alone largely yields marginal benefits within the infarct area due to over-activated and prolonged inflammation. Controlled delivery of a combination of angiogenic and immunoregulatory proteins may be a promising therapeutic approach for MI. We investigated the bioactivity and therapeutic potential of an injectable, heparin-based coacervate co-delivering an angiogenic factor, fibroblast growth factor-2 (FGF2), and an anti-inflammatory cytokine, Interleukin-10 (IL-10) in a spatially and temporally controlled manner. A fixed load of FGF2 (500 ng) alone or combined with 100 ng or 500 ng IL-10 was used for coacervate delivery. The loading efficiency was approximately 98% for both FGF2 and IL-10. Coacervate delivery of FGF2 and IL-10 preserved their bioactivities on cardiac stromal cell proliferation *in vitro*. Upon intramyocardial injection into a mouse MI model, echocardiography revealed that FGF2/IL-10 coacervate treated groups showed significantly improved long-term LV contractile function and ameliorated LV dilatation. FGF2/IL-10 coacervate substantially augmented LV myocardial elasticity. Additionally, FGF2/IL-10 coacervate notably enhanced long-term revascularization, especially at the infarct area. In addition, coacervate loaded with 500 ng FGF2 and 500 ng IL-10 significantly reduced LV fibrosis, considerably preserved infarct wall thickness, and markedly inhibited chronic inflammation at the infarct area. These results indicate that FGF2/IL-10 coacervate has notably greater therapeutic potential than coacervate containing only FGF2. Overall, our data suggest therapeutically synergistic effects of FGF2/IL-10 coacervate, particularly coacervate with FGF2 and 500 ng IL-10, for treating ischemic heart disease.

**Session: The Industrialization of Tissue Engineered Products**

**Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM**

**Bioreactors, automation, manufacture of TE constructs**

**S. Wirth;**

Invetech, San Diego, CA.

Developing an industrialized manufacturing capability to produce TE constructs poses a number of challenging questions. Many process steps for clinical trial manufacture are manual, and to transfer laboratory processes to industrial scale processes requires a methodical and collaborative approach from the scientists/engineers and the equipment designers/integrators. Development by Design delivers a rigorous and systematic approach to designing and implementing true industrial manufacturing equipment performing controlled, repeatable production steps for high quality product. To achieve this, key areas to understand are:

- Best practices in planning path-to-market for commercial viability and the company's end game
- Strategies for reducing capital needed for manufacturing
- Manufacturing optimization to achieve cost effective equipment and facilities for high process throughput while minimizing Class A and B clean space requirements.

This presentation will discuss the bioreactor and manufacturing equipment development challenges that are unique to TE constructs.

**Industrialization of Tissue Engineered Products**

**B. Newsom;**

Business Development Leader, ThermoFisher Scientific, Fredrick, MD.

Since the launch of Apligraf<sup>®</sup> in 1997 tissue engineered products have been at the forefront of cell therapy and regenerative medicine and have significantly improved patient quality of life. In more recent years we have seen increasing innovation in the space from cartilage to hollow organs and conduits and ever more sophisticated 3D structures that include vasculature and near native physiology. However, concurrently, we are seeing challenges imposed by regulatory standards and reimbursement issues requiring a closer look at best practices to scale effectively and create efficiencies in manufacturing to ultimately produce durable, cost effective results for patients.

During this 20 year journey there have been successes, struggles and setbacks in the clinical, scientific and regulatory arenas. In this symposium we will discuss issues from the past and how those lessons are leading us to the future of industrializing tissue engineered products.

**Role of the Major Medical Center in the Industrialization of Tissue Engineered Products**

**C. V. Gemmiti;**

Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA.

The tissue engineering and regenerative medicine community saw an unprecedented investment of more than \$6B in 2014, predominantly in early stage companies. As those companies continue to develop their therapies, translational centers in academia and medical institutions must facilitate the movement of the therapeutic into the clinic. This facilitation can take the form of anything from feedback on the product embodiment (e.g., route of administration, delivery method, shelf life), to market adoption and reimbursement, to clinical trial design and execution. Early integration of viewpoints from practicing physicians, nurses and program administrators from major medical centers is critical to ensuring efficient and effective translation. This presentation will review the clinical trial process,

corresponding regulatory framework, and practical considerations for products in development from the perspective of the medical centers.

### Development of Scalable Scaffolds, Biomaterials, Matrices

**J. Johnson;**

Chief Scientific Officer, Columbus, OH.

Focus is shifting to the mass production of tissue engineered scaffolds and products under appropriate regulatory requirements. The scale-up of this translational process will help bring regenerative medicine into the mainstream of clinical practice, but it also drives renewed focus on how earlier research may better enable future therapeutic applications. Manufacturing costs and sourcing of raw materials should be factored into the product development strategy as a critical step towards commercialization. Nanofiber Solutions, Inc. is a regenerative medicine company developing a new class of implants with unrivaled performance for the \$20B soft tissue repair and organ regeneration market. Our nanofiber technology is used to build scaffolds that are critical in the development of life-saving tissue engineered implants. We manufacture the world's first nanofiber tracheal implant which has been used successfully in four European surgeries and are rapidly expanding our product offerings and patent estate based on this proven scaffold technology.

### Industrialization and Manufacture of Cells, Growth Factors

**J. Rowley;**

Chief Executive & Technology Officer, RoosterBio, Inc., Fredrick, MD.

Why Manufacturing Matters: How Today's Cell Therapy Bio-Manufacturing Innovations are Laying the Foundation for a Sustainable Tissue Engineering Revolution. Technology is rapidly moving toward the integration of biologics into products like cell therapies, engineered tissues, bio-robotics, implantable devices, 3D printing, food, clothing, and even toys. This coming decade will see the incorporation of living cells into all these platforms and others not yet imagined. To expedite this biologics revolution, inventors, developers and suppliers will require a limitless, standardized, low-cost supply of cells - and today's cell therapy biomanufacturing innovations are laying the groundwork to make this a reality.

### Session: The Past, Present and Future in Functional Tendon Repair and Regeneration

**Date and Time: Friday, September 11, 2015, 9:15 AM - 10:45 AM**

#### Harnessing Endogenous Stem/Progenitor Cells for Tendon Regeneration

**C. Lee, Y. Jun, K. Kao;**

Columbia University, New York, NY.

Tendon and ligament injuries frequently result in scar-like tissue with poor restoration of biological structure and mechanical properties. In addition to surgical grafting by autologous or allogeneic tissues, previous experimental work has attempted to improve tendon/ligament healing using growth factors, stem/progenitor cells and biomaterials. However, these tissue-engineered grafts have encountered difficulties in clinical translation. Here we identified a rare population (~0.8% of total tendon cells) of tendon stem/progenitor cells (TSCs) with a perivascular origin, consistent to the previous reports. The perivascular TSCs (PTSCs) were highly clonogenic and multipotent. Interestingly, the PTSCs are selectively enriched up to ~20 fold by treatment with connective tissue growth factor (CTGF). In addition, CTGF-treated PTSCs readily differentiated into tenocyte-like cells as evidenced by significant increases in tendon related gene expressions as compared to the other tendon cells. In a patellar

tendon (PT) full-transection model, the number of PTSCs drastically increased in the early phase of CTGF-delivered tendon healing (2 ~ 7 days) in contrast to control. The PTSCs then underwent differentiation into SCX+/COL-I+ tenocyte-like cells in the later healing phase (>7 days) in the CTGF-delivered tendon that consequently led to scar-less tendon healing, featured by highly organized collagen fibrils, normal level of cellularity and mechanical properties. Initial signaling study using siRNA knockdown showed that CTGF-induced proliferation and tenogenic differentiation of PTSCs were regulated via FAK and ERK1/2 signaling pathways. Our approach for tendon regeneration by harnessing regenerative capacity of host stem/progenitor cells requires no cell transplantation, and may offer an alternative approach for endogenous regeneration.

#### Advancing Tendon Regeneration through the Development of Bio-stimulating Tissue Engineering Approaches

**M. Gomes<sup>1,2</sup>;**

<sup>1</sup>University of Minho, Guimaraes, PORTUGAL, <sup>2</sup>PT Government Associate Laboratory, Braga/Guimaraes, PORTUGAL.

Tendon tissue engineering (TE) requires tailoring scaffolds designs and properties to the anatomical and functional requirements of tendons located in different regions of the body. Cell sourcing is also of utmost importance as tendon cells are scarce. Recently, we have found that it is possible to direct the tenogenic differentiation of Amniotic fluid and Adipose tissue derived stem cells (hAFSCs and hASCs), and also that there are hASCs subpopulations that might be more prone to tenogenic differentiation. Nevertheless, biochemical stimulation may not be enough to develop functional TE substitutes for a tissue that is known to be highly dependent on mechanical loading.

These findings trigger our interest on *in vitro* biomechanically-stimulating culture environments that can be achieved modulating the scaffold architecture and composition and the stem cells. Particularly, the incorporation of magnetic nanoparticles (MNPs) within 3D constructs constitutes a novel and attractive strategy towards the development of magnetically-responsive system that may eventually combine therapeutic and diagnostic functionalities. An additional advantage is that cells naturally respond to magnetic forces, and consequently, the application of a magnetic field may enhance cell biological performance, and ultimately stimulate cell proliferation and differentiation. This work reports on recent studies concerning the development of specific scaffolds architectures based on various polymers, doped with MNPs and fabricated by either rapid prototyping technologies or electrospinning, enabling responsive systems for culturing stem cells, stimulating their tenogenic differentiation.

#### Assessment of Tenogenic Induction by Extracellular Tendon Matrix and Cyclic Stretching

**J. Burk<sup>1</sup>, A. Aldag<sup>1</sup>, W. Brehm<sup>1</sup>, S. Heller<sup>2</sup>, B. Pfeiffer<sup>1</sup>, C. Kasper<sup>3</sup>;**

<sup>1</sup>Large Animal Clinic for Surgery, University of Leipzig, Leipzig, GERMANY, <sup>2</sup>Department of Pathology and Laboratory Medicine, Tulane University, New Orleans, LA, <sup>3</sup>Institut für Biotechnologie, BOKU Vienna, Vienna, AUSTRIA.

Treatment of tendon injuries with multipotent mesenchymal stromal cells (MSC) led to encouraging results in animals. However, there is still only limited understanding of the mechanisms underlying the beneficial effects of locally applied MSC on tendon healing.

Tenogenic differentiation induced by the natural tendon environment, accompanied by synthesis of important matrix components, could be part of the complex mechanisms.

The aim of this study was to investigate this hypothesis using a new *in vitro* model system, which allowed combining the use of tendon matrix scaffolds and the application of cyclic strain, imitating crucial natural stimuli.

Adipose-derived MSC from equine donors were seeded on decellularized tendon scaffolds and once subjected to mechanical stimulation with increasing stress-rest-periods. 4, 8 and 24 h post stimulation, samples were assessed regarding their morphology, cell alignment, and integration and expression of musculoskeletal markers.

Significant scaffold-induced effects were observed, and most of them were additionally enhanced by short mechanical stimulation. Scaffold culture induced parallel alignment of MSC and strongly augmented expression of decorin ( $p < 0.05$ ). Moreover, scaffold culture combined with stretching induced upregulation of collagen 3A1 and scleraxis ( $p < 0.05$ ). Furthermore, a time-dependent expression was observed for scleraxis and tenascin-C, with initial decreases but increases at 24 h.

The study shows that natural tenogenic stimuli rapidly induce changes in MSC, which are likely to be related to their beneficial effect. The model system used proved to be highly suitable for further studies on progenitor cells and tendon regeneration.

#### Angiogenic Activity of Angptl4 has a Dual Effects in Tendon Repair and Injury

R. Mousavizadeh<sup>1</sup>, V. Duronio<sup>1</sup>, A. Lu<sup>2</sup>, H. Behzad<sup>2</sup>, G. Safaee Ardekani<sup>1</sup>, R. G. McCormack<sup>2</sup>, A. Scott<sup>2</sup>;

<sup>1</sup>Department of Medicine, University of British Columbia, Vancouver, BC, CANADA, <sup>2</sup>Vancouver Coastal Health Research Institute, University of British Columbia, Vancouver, BC, CANADA, <sup>3</sup>Department of Orthopedic Surgery, University of British Columbia, Vancouver, BC, CANADA.

**Introduction:** Damage to tendon due to overuse or tendon rupture alters the profile of cytokine expression and release, and also modulates pro-inflammatory factors and growth factors which may influence the course of tendon injury or healing. Our recent data introduced a new mechanoreponse protein named Angiopoietin-like 4 (ANGPTL4) which regulates angiogenesis in tendon tissue. In this study we determined the role of ANGPTL4 protein in tendon healing and overuse injury and its regulatory pathways in response to mechanical stimuli.

**Methods:** The angiogenic activity of ANGPTL4 was determined with *in vitro* cell culture models and *in vivo* animal models. The function of ANGPTL4 protein were also studied in ANGPTL4-deficient mice after patellar tendon injury. qPCR, ELISA, dual-luciferase reporter assay and immunohistochemistry were used to measure the expression and activity of proteins.

**Results:** ANGPTL4 protein induces endothelial cell tube formation, and the expression of angiogenic markers and MMP3 in mouse patellar tendon. Cyclic strain induces release of ANGPTL4 through increased activity of TGF- $\beta$  and stabilizing HIF-1 $\alpha$ . The data from immunostaining of human rotator tendon tissue indicate a correlation between the expression of ANGPTL4, HIF-1 $\alpha$  and endothelial cell marker which support the role of HIF-1 $\alpha$  in induction of ANGPTL4 followed by angiogenesis. Lack of ANGPTL4 in ANGPTL4-deficient mouse impaired tendon cell migration and expression of factors involved in tissue vascularization during tendon healing.

**Significance:** The pivotal role of ANGPTL4 in tendon vascularization and its regulatory pathways, HIF-1 $\alpha$  and TGF- $\beta$ , may provide a means for controlling vascularization during tendon injury or healing.

#### Multi-Scale Composite Collagen Biomaterial for Tendon Repair

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<sup>1</sup>Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, <sup>2</sup>Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL.

Injuries to spatially-ordered tissues in the musculoskeletal system such as tendon present unique challenges to the field of tissue engineering. A long-term objective in our lab is to develop biomaterials that address barriers to tendon regeneration. Our efforts concentrate on instructive biomaterials to direct mesenchymal stem cell (MSC) differentiation down osteotendinous lineages (tendon, bone, interface) in a spatially-selective manner. Such a construct could be seeded with the patient's own stem cells then implanted to regenerate tendon and its insertion into bone. We have developed a model collagen-GAG (CG) scaffold with a geometrically-aligned microstructure able to promote long-term tenocyte phenotypic stability. Further, using an *in vitro* bioreactor we show the local scaffold en-

vironment can modulate how MSCs respond to cyclic strain. We report the scaffold can mediate mechanotransduction and integrin subunit ( $\beta 1$ ,  $\beta 3$ ) expression profiles in support of tendinous lineage specification and new matrix synthesis. Given the scaffold will be implanted within an inflammatory wound environment, we describe modification to the scaffold proteoglycan content, and with that the scaffolds inherent capacity to sequester biomolecules within the matrix, to improve tenocyte activity versus a pro-inflammatory challenge. Lastly, given the mechanical strength of the scaffold is a critical design requirement, we report the use of 3D printing to generate bioinspired fiber-reinforced collagen constructs to better balance strength and bioactivity. These multi-scale composites demonstrate both significantly improved stiffness and toughness, but also a pathway to incorporate additional growth factor signals to improve cell regenerative potential.

#### Cross-talk Between Human Tenocytes and Bone Marrow Stromal Cells Potentiates Extracellular Matrix Remodeling *In Vitro*

E. C. Ekwueme<sup>1</sup>, C. A. Ghebes<sup>2</sup>, J. F. Crispim<sup>2</sup>, D. B. Saris<sup>2</sup>, H. A. Fernandes<sup>2</sup>, J. W. Freeman<sup>1</sup>;

<sup>1</sup>Department of Biomedical Engineering, Rutgers University, Piscataway, NJ, <sup>2</sup>MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, NETHERLANDS.

Tendon and ligament (T/L) pathologies account for a significant portion of musculoskeletal injuries and disorders. Tissue engineering has emerged as a promising solution in the regeneration of both tissues. Specifically, the use of multipotent human mesenchymal stromal cells (hMSC) has shown great promise to serve as both a suitable cell source for tenogenic regeneration and a source of trophic factors to induce tenogenesis. Using three donor sets, we investigated the bidirectional paracrine tenogenic response between primary human hamstring tenocytes (HT) and primary bone marrow-derived hMSC. Cell metabolic assays showed that neither cell type experienced significant increases in proliferation during co-culture. Histological staining confirmed that co-culture specifically induced collagen protein levels in both cell types at varying time-points. Gene expression analysis using qPCR showed up-regulation of anabolic and catabolic markers involved in extracellular matrix maintenance for hMSC and HTs. Furthermore, analysis of the hMSC/HT co-culture secretome using a reporter cell line for TGF- $\beta$ , a potent inducer of tenogenesis, showed high TGF- $\beta$  bioactivity. Finally, we showed that the paracrine interaction of these two cell types potentiates matrix remodeling/turnover, which may be attributed to TGF- $\beta$  signaling. These results have significant implications in the clinical use of hMSC for common T/L pathologies.

#### Session: The Regenerative Niche: Activation of Endogenous Mechanisms for Tissue Repair

Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM

#### Involvement of Epithelial Stem Cell in the Regeneration of Endometrial Full-thickness Injury Following Repair by Neonatal Endometrial Cell Engineered Decellularized Uterus Matrix

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Damage to the basal layer of uterus would cause infertility. The purpose of this study was to test the effect of neonatal endometrial cells seeded on decellularized uterus matrix on the regeneration of endometria on uterus full-thickness injury and its potential



mechanisms. Four weeks after transplantation, the recellurized scaffold group exhibited higher regeneration capacities, with thicker regenerated endometria, more regenerated glands compared with the injury group. The existence of endometrial epithelial stem cells responsible for the generation of glands have been reported recently, in order to screen and identify potential bio-marker of endometrial epithelial stem cell, single cell Q-PCR analysis was performed, after clustering and principal component analysis of the expression data, we identified a subgroup of cells that uniquely expressed high level of aldehyde dehydrogenase (ALDH) 1 gene, and the expression pattern of aldh1 gene was highly correlated with the expression of stem cell markers, endometrial transcript factors as well as WNT signaling related genes. Immunofluorescence analysis showed that ALDH1 expressed highly in newly formed gland epithelia as well as invading luminal epithelia during the formation of endometrial gland in postnatal mice, which co-expressed with proliferation marker Ki67 and stem cell marker SSEA1, while the expression of ALDH1 was in the endometrial gland epithelia of the basal layer of the endometrium from the sexual mature mice. These results indicated that ALDH1 positive epithelia may be the candidate stem cell of endometrial epithelia that involved in the regeneration of endometrial epithelial and formation of glands in tissue-engineered repair of uterus injury.

#### Identification of a New Cell Population Endowed with a Healing Capacity and Constitutively Circulating in Healthy Conditions

R. Tasso<sup>1</sup>, C. Lo Sicco<sup>1</sup>, D. Reverberi<sup>2</sup>, M. Cilli<sup>2</sup>, U. Pfeffer<sup>2</sup>, R. Cancedda<sup>1</sup>;

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Stem and progenitor cells are the critical units for tissue maintenance, regeneration, and repair. The activation of regenerative events in response to tissue injury has been correlated with mobilization of tissue-resident progenitor cells, which is functional to the wound healing process. However, until now there has been no evidence for the presence of cells with a healing capacity circulating in healthy conditions. Here, we identified a rare population of cells present in the peripheral blood of healthy mice that actively participates in tissue repair. These Circulating Healing (CH) cells were identified by an innovative flow cytometry strategy as small cells not expressing CD45 and lineage markers. The analysis of their global transcriptome revealed their uniqueness when compared to other cells characterized by varying stemness degree, including Hematopoietic Stem cells (HSCs), Mesenchymal Stem Cells (MSCs), and Very Small Embryonic-Like (VSEL) Stem Cells. Moreover, CH cells presented a high expression of key pluripotency-associated genes and positive selective markers of the epiblast developmental stage. CH-labeled cells derived from healthy Red Fluorescent Protein (RFP)-transgenic mice and systemically injected into syngeneic fractured wild-type mice effectively migrated and engrafted in wounded tissues, and ultimately differentiated into tissue-specific cells. Accordingly, the number of CH cells in the peripheral blood rapidly decreased following femoral fracture. These findings uncover the existence of constitutively circulating cell populations that may represent novel, accessible, rapid and very versatile effectors of therapeutic tissue regeneration.

#### Nano-fibrous Scaffolds and Topological Characteristics Potentiate Paracrine Function of Adipose-derived Stem Cells for Wound Healing

N. Su<sup>1</sup>, K. Wang<sup>1</sup>, Y. Luo<sup>1,2</sup>;

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The immunomodulatory and pro-angiogenesis cytokines secreted by MSCs have been found to play a key role in suppressing inflammation and rendering reparative process *in vivo*. Despite our understanding of the regenerative mechanisms associated with MSCs,

the paracrine function of MSCs under the influence of scaffold materials remains unexplored. Here, rat adipose derived MSCs (rAD-MSCs) were cultured on electrospun scaffolds containing random, aligned or micro-patterned mesh-like fibers and the paracrine function of rAD-MSCs were studied and compared using cell-culture microplate as a control substrate. It is found that when cultured on fibrous scaffolds, rAD-MSCs expressed significantly higher amounts of anti-inflammatory and pro-angiogenesis cytokines in comparison to microplates. In addition, the conditioned media collected from the rAD-MSCs cultured on fibrous scaffolds showed the property to promote the proliferation and tube-formation behavior of human umbilical vein endothelial cells (HUVECs). Moreover, the paracrine factors also modulated LPS-stimulated macrophages toward an anti-inflammatory phenotype. The paracrine behavior of rAD-MSCs varied with the topological characteristics, with the micro-patterned scaffolds showing most beneficial effects. In a rat excisional wound-healing model, the paracrine factors collected from rAD-MSCs on micro-patterned scaffolds accelerated wound closure, enhanced epithelialization and decreased collagen deposition. By inhibiting NFκB pathway, it is demonstrated that the fibrous scaffolds may have promoted the paracrine function of rAD-MSCs via enhancing the NFκB signaling pathway. Our study shows fibrous scaffolds can generate stimulatory cues to potentiate the paracrine function of rAD-MSCs. The results may provide insights into how biomaterials may be designed for MSCs for regenerative applications.

#### High Fat Diet Accelerates Cartilage Repair in DBA/1 Mice

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Microfracture is used to stimulate mesenchymal stem cells (MSCs) to repair cartilage defects. Obese patients are now often excluded from treatment but there is no evidence that intrinsic cartilage repair is negatively influenced by obesity. Here, we investigated effects of a high fat diet (HFD) on cartilage repair in the DBA/1 mouse strain.

Ten-week-old male DBA/1 mice were fed with control diet or HFD (60% energy from fat). After two weeks, a full thickness cartilage defect was made in the trochlear groove of the left knee. Mice were sacrificed after 1, 8, or 24 weeks. Cartilage repair was evaluated on histology. Fasted serum was collected 24 hours before operation and at end points for glucose, insulin and amyloid A. Immunohistochemistry for F4/80, iNOS and CD206 was performed on synovium.

Mice on HFD had higher bodyweight when making the defect. Mice on HFD had more defect filling with fibroblast-like cells one week after making the defect and more cartilage repair after 8 weeks. After 24 weeks, none of the mice had complete cartilage repair and we did not detect a statistically significant difference between the two groups. Serum glucose was significantly elevated in HFD mice 24 hours before and 8 weeks after the operation. HFD did not influence serum amyloid A, insulin and macrophage phenotype.

High fat diet accelerated cartilage repair in DBA/1 mice. Further research needs to be performed to elucidate the complex interaction between high fat diet, metabolic and inflammatory changes, and cartilage repair.

#### Treatment of Experimental Arthritis with Mesenchymal Stem Cell Conditioned Medium

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Intra-articular injection of bone marrow derived mesenchymal stem cells (MSCs) has therapeutic potential for Rheumatoid Arthritis (RA). Secreted trophic factors reduce tissue injury, prevent degradation and promote repair via paracrine signaling. This study aims to

evaluate murine MSC serum-free conditioned medium (SF-CM) as a therapy for antigen-induced arthritis (AIA).

15 µl of concentrated SF-CM were injected intra-articularly 1 day following induction of arthritis, with injection of SF-medium without pre-conditioning applied as controls. Measurements for knee joint diameter represented swelling whilst disease severity was assessed using an arthritis index (AI) calculated from histological scores for synovial infiltrate, hyperplasia, synovial exudate and cartilage depletion. Serum concentration of pro-inflammatory cytokine TNF $\alpha$  was also measured.

In SF-CM treated mice, disease severity was significantly less than in control-treated at 3 and 7 days post-arthritis induction ( $p < 0.001$ , student's t-test). At day 3, synovial infiltrate, hyperplasia and cartilage depletion were significantly reduced from control-treated scores ( $p < 0.05$ , student's t-test). Recovery from peak swelling significantly improved from control-treated animals at days 2, 3, 7 and 14 ( $p < 0.05$ , 2-way ANOVA). TNF $\alpha$  significantly increased from day 3 to day 14 in control animals ( $p < 0.01$ , 2-way ANOVA) but did not significantly change over time in test animals.

We demonstrate therapeutic potential of SF-CM delivered intra-articularly in a pre-clinical RA model, showing ameliorated disease severity, reduced synovial infiltrate, hyperplasia and cartilage depletion and enhanced swelling recovery. Further work is required to identify the mechanisms involved.

Funding was provided by the Dowager Countess Eleanor Peel Trust, the Institute of Orthopaedics Ltd and Oswestry Rheumatology Association.

### The Potential of Human Amniotic Stem Cells to Mitigate Osteoarthritis (OA) Progression

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Osteoarthritis (OA) is a debilitating inflammatory condition resulting in the destruction of articular cartilage. No current therapies successfully mitigate OA progression, but amniotic stem cells offer an intriguing therapeutic alternative due to their anti-inflammatory and immunomodulatory properties.<sup>1-6</sup> To date no study has investigated the effects of human amniotic membrane-derived stem cells (hAMSCs) on human OA cartilage and synovium. We analysed the ability of hAMSCs to mitigate OA progression through an *in vitro* co-culture model using human OA joint tissue explants. Explant co-cultures with (OA+hAMSCs) and without (OA) hAMSCs were evaluated following 3 and 15 days.

OA progression, glycosaminoglycan (GAG) content, collagen release, and cell viability of OA cartilage were assessed via the Osteoarthritis Research Society International (OARSI) Cartilage Histopathology Assessment System, DMMB, hydroxyproline, and Live/Dead Cytotoxicity assays, respectively. Synovial cell viability and modulation of inflammation was also analysed via immunohistochemical staining for M1- and M2-polarized macrophages.

Comparison of co-cultures with hAMSCs showed statistically increased percent change in chondrocyte viability (19.62% OA+hAMSC vs. -17.52% OA), significantly more GAG content (655 µg/mg OA+hAMSC vs. 401 µg/mg OA), and less collagen released to the media as compared to cultures without hAMSCs over the 15 days. Additionally, co-cultures with hAMSCs showed a 51.21% reduction in positive IHC staining for M1-polarized synovial macrophages concomitant with a 23.44% increase in M2-polarized macrophages. Taken together, this data suggests that hAMSCs may be effective at mitigating OA progression via the maintenance of chondrocyte viability and cartilage extracellular matrix while modulating synovial inflammation.

### Session: Therapeutically-Relevant Biomaterials in Musculoskeletal Tissue Regeneration

**Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM**

#### Effects of Hydrogel Stiffness and Biochemical Compositions on Stem Cell-Chondrocyte Interactions *in vivo*

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Cell niche is a multi-factorial environment including biochemical and mechanical cues. We have recently reported an intriguing finding that adipose-derived stem cells (ADSCs) can catalyze cartilage formation by neonatal chondrocytes (NChons) in 3D ECM-containing hydrogels *in vitro*. The goal of this study is to evaluate the effects of hydrogel stiffness and biochemical compositions on modulating such catalyzed cartilage formation *in vivo* using a mouse subcutaneous model. Three methacrylated extracellular matrix (ECM) molecules: chondroitin sulfate (CS-MA), hyaluronic acid (HA-MA) and heparan sulfate (HS-MA), were incorporated into PEG-DMA hydrogels [1]. NChons (25%) and ADSCs (75%) were co-encapsulated in hydrogels with comparable stiffness but varying biochemical compositions (CS, HA or HS), or in hydrogels with same biochemical composition (CS) but varying stiffness (8%, 11% or 14% PEGDMA). All samples were implanted *in vivo* and harvested at 3 or 8 weeks. While soft hydrogels (8% PEG) supported synergistic ADSC/NChon interactions *in vitro*, soft hydrogels degraded prematurely *in vivo* and lost its integrity before harvest. Immunostaining of collagen II showed largest neocartilage nodules and increased GAG deposition in CS-containing hydrogels. In contrast, HS led to undesirable up-regulation of collagen I and collagen X, suggesting the formation of fibrocartilage and hypertrophy phenotype, and decreased GAG retention over time. In sum, here we demonstrated the feasibility of using ECM containing hydrogels to guide catalyzed cartilage formation *in vivo*, which could be further enhanced by tuning the biochemical and mechanical cues of ECM hydrogels.

[1] Wang T. *et al*, Tissue Eng Part A 2014, 20, 2131–2139.

### Therapeutic Tailoring of Matrices for Bone and Surrounding Tissues

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Matrices are on highly demand for the repair and regeneration of skeletal and muscle tissues. They provide active sites for cells to anchor, guide their elongation and migration, and stimulate to a matured state. While the first choice of matrices in musculoskeletal system needs to fulfill the mechanical stability and functionality, bioactive and cell stimulating approaches have shown great promise of achieving regulated constructs of stem / progenitor cells with equivalent properties to native tissues. Here we deliver some technological advances in musculoskeletal regenerative matrices that have been designed / engineered intrinsically or extrinsically to alter the fate of cellular phenotypes in differentiation and even in *in vivo* tissue formation processes. For the bone-surrounding tissues, like ligament, the matrices under mechanical stimulation have proven to preserve better the potency of progenitor cells later to develop their specified lineage. Furthermore, electroactive and nanostructured substrates are considered to provide promising matrix cues to skeletal muscles. Targeting bone, the mineralized step is the key in achieving functional tissues, and the use of stiff matrices with exogenous signaling molecules has largely shown synergistic functions in osteogenesis and cellular maturation. In particular, tailoring scaffolding matrices with engineered extracellular proteins and signaling molecules is a promising strategy to fulfill multiple therapeutic actions required for skeletal-muscle tissue engineering.

### Hypoxia Mimicking Hydrogels Accelerate Chondrogenesis of MSCs *In Vitro* and *In Vivo*

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Chondrogenically primed mesenchymal stem cells (MSCs) are receiving increasing attention for both articular cartilage and endochondral bone tissue engineering strategies. Environmental cues such as hypoxia are key regulators of chondrogenesis, and by controlling oxygen levels *in vitro* it is possible to modulate the phenotype of chondrogenically primed MSCs. Tissue engineering strategies typically necessitate *in vitro* priming of MSCs for several weeks under controlled conditions prior to implantation, adding to the complexity of such therapies and acting as a barrier to clinical translation. To bypass the need for such *in vitro* priming, the goal of this study was to develop a growth factor delivery, hypoxia-mimicking hydrogel to drive the differentiation of encapsulated MSCs. To this end, we incorporated both BMP-2 and TGF- $\beta$ 3 into low molecular weight RGD-modified alginate. To mimic hypoxia, we further incorporated the cell-permeable, competitive inhibitor of hypoxia-inducible factor prolyl hydroxylase, dimethylallyl glycine (DMOG), into the hydrogels. *In vitro*, the incorporation of DMOG into the hydrogels was found to enhance cartilage-specific matrix synthesis by encapsulated MSCs in both normoxic and low oxygen conditions. Following subcutaneous implantation into nude mice, the incorporation of DMOG into growth factor free and growth factor loaded alginate hydrogels was found to accelerate chondrogenesis of encapsulated MSCs. The results of this study demonstrate that hypoxia mimicking hydrogels accelerate chondrogenesis of unprimed MSCs *in vivo*, suggesting that such constructs may overcome the need for the *in vitro* differentiation of stem cells for articular cartilage and endochondral bone tissue engineering strategies.

#### The Spatiotemporal Distribution of Macrophages and Progenitor Cells in Extracellular Matrix Mediated Remodeling of Volumetric Muscle Loss

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Overt loss of skeletal muscle or volumetric muscle loss (VML) overwhelms its inherent regenerative ability and is associated with robust scar tissue deposition and loss of function. Current treatment strategies including cell-centric approaches remain ineffective because they fail to consider the complex microenvironment involved in remodeling.

Alternatively, inductive extracellular matrix (ECM) bioscaffolds obviate the need for exogenous cell delivery, recruit endogenous progenitors, modulate the innate inflammatory response, and cultivate a "friendly" microenvironment. ECM bioscaffolds have been shown to promote functional myogenesis following VML in mice and humans. Previous studies have shown that solubilized ECM promotes an M2 macrophage phenotype. Paracrine factors from these macrophages, in turn, promote skeletal muscle progenitor chemotaxis and myogenesis. The spatial distribution and temporal response of macrophages and progenitor cells within the ECM-treated vs untreated VML injury site, however, has not been characterized. The present study quantitatively analyzes the spatiotemporal myogenic and neurogenic progenitor cell and macrophage response following ECM bioscaffold VML repair.

A VML defect was filled with ECM or left untreated. Animals were sacrificed at 3, 7, 14, and 56 days following implantation. The host remodeling response was characterized by immunolabeling for perivascular stem cells (PVSCs) (CD146+), myoblasts (MHC+), M1/M2 macrophages (F480+iNOS+ or F480+Fizz1+), and innervation (Nestin,  $\beta$ -III tubulin). Results show that ECM facilitates a shift towards a predominant M2 macrophage phenotype, increased PVSC recruitment, and re-innervation. ECM's ability to influence the local microenvironment allows for cross-talk among key immune

regulators and progenitor cells, the spatiotemporal distribution of which correlates with myogenesis.

#### Skeletal Myogenic Differentiation of Human Urine-derived Cells as a Potential Source of Cell Therapy for Urethral Sphincter Muscle Dysfunction

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**Introduction:** The goal of this study was to assess whether human urine-derived stem cells (USCs), obtained through non-invasive procedures, can differentiate into cells of a skeletal muscle lineage and potentially be used for skeletal muscle regeneration when treating urinary incontinence due to urethral sphincter dysfunction.

**Methods:** USCs were harvested from six healthy adults. Growth factors and cytokines secreted from USCs and human bone marrow stroma cells (BMSC, as a control) into the culture supernatant were measured by ELISAs. We selected four different types of myogenic differentiation media to optimize induction of USCs. Differentiated USCs were identified with myogenic markers by gene and protein expression. USCs were then implanted into the tibialis anterior muscles of nude mice for 1 month.

**Results:** Levels of a series of growth factors and cytokines, including IGF1, HGF, FGF, VEGF, and PDGF were significantly higher in the culture supernatant of USCs than in BMSCs. After myogenic differentiation, morphology of the USCs changed from 'rice-grain'-like cells to spindle-shaped cells and then to myotube-like structures. The USCs expressed specific skeletal muscle transcripts and protein markers (myf5, myoD, myosin, and desmin) after being induced with different myogenic culture media. Implanted cells expressed skeletal muscle markers stably *in vivo*.

**Conclusions:** USCs can differentiate into cells with a skeletal muscle lineage *in vitro* and after being implanted *in vivo*. Thus, they might be a potential source for cell injection therapy to induce urethral sphincter skeletal muscle regeneration and thereby be used to treat urinary incontinence.

#### Electrospun Nanocomposite Fibrous Scaffolds with *in vivo* Osteoinductive Potential

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Fibrous scaffolds that can effectively promote bone-like mineral deposition and have the capacity to deliver signaling or therapeutic factors are promising candidates for bone tissue regeneration. Herein, we fabricated electrospun fibrous matrices incorporating various amounts of mesoporous bioactive glass nanospheres (BGn) loaded with osteogenic molecules (OM). The prepared nanocomposite fiber scaffolds showed improved mechanical tensile strength, elasticity, and hydrophilicity when compared to pure fiber scaffolds. The *in vitro* release profile of OM exhibited zero-order release kinetics with sustained release for a month. The proliferation and osteogenic differentiation of stem cells derived from periodontal ligament were significantly enhanced by the BGn incorporation and synergistically stimulated with OM loading, as confirmed by both direct and indirect cell cultures. The *in vivo* bone formation after 6 weeks in rat calvarium model showed significantly enhanced bone regeneration for the OM-loaded scaffolds with respect to the OM-free scaffolds, evidencing the osteoinductive potential of the OM-eluting nanocomposite scaffolds in bone regeneration.

#### Reference

1. El-Fiqi A, Kim HW. RSC Advances, 4, 4444, 2014.

**Session: Three-Dimensional *In Vitro* Tissue Models: A Promising Tool to Study Diseases and Develop New Personalized Tissue Engineering Strategies**

**Date and Time: Thursday, September 10, 2015, 3:00 PM - 4:30 PM**

**An *In Vitro* 3D Model for a Neuromuscular Junction**

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Numerous diseases are associated with dysfunction at the level of the motor unit including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, and Duchenne muscular dystrophy. Advances in our understanding of these diseases and the development of new therapeutic approaches have been hampered by the lack of a realistic 3D model that both recapitulates the function of a neuromuscular junction (NMJ) and provides the capability to monitor its response under a variety of conditions and when subjected to an array of biochemical agents. Our objective was to meet this need by developing a model of a functioning NMJ in a microfluidic platform that would allow selective stimulation of motor neurons in one compartment that connect with and activate muscles in a second, adjacent compartment. The motor neurons are differentiated from mouse embryonic stem cells and genetically engineered to express a light sensitive ion channel, Channelrhodopsin. These are formed into spheroids and introduced to one chamber of a microfluidic system, parallel to a second gel region, and a third region containing muscle strips derived from C2C12 myoblasts differentiated into striated myotubes that form around flexible posts so that the generated force can be quantified. Results will be presented showing the extension of neurites from the neurospheres to the muscle, the formation of synapses, and the activation of muscle contraction by light stimulation of the photosensitive neurons.

1. Sakar MS, *et al.*, Lab Chip. 2012.

**Acknowledgments:** The NSF (CBET-0939511). Mouse ES cells were a gift of H Wichterle and T Jessell.

**A Tissue Engineered Model of the Glial Scar - Matrix Contribution to Astrogliosis**

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Glial scars are widely seen as a (bio)mechanical barrier to CNS regeneration. Inhibitory molecules in the forming scar, which are strongly upregulated and dramatically change the extracellular matrix content and properties, provide a window of opportunity for therapeutic actions. Due to the lack of a screening platform, which could allow *in-vitro* testing of several candidates simultaneously, up to now no comprehensive study has addressed and clarified how different lesion microenvironment components affect astrogliosis.

Using astrocytes cultured in alginate gels and meningeal fibroblast conditioned medium, a simple and reproducible 3D culture system of astrogliosis is here proposed that mimics many features of the glial scar. Cells in this 3D culture model behave similarly to scar astrocytes, showing changes in gene expression (e.g. GFAP) and increased extracellular matrix production (chondroitin 4- sulphate and collagen), inhibiting neuronal outgrowth. Astrocytic reactivity was found to be dependent on RhoA activity, and targeting RhoA using shRNA-mediated lentivirus reduced astrocytic reactivity. Further, we have shown that ibuprofen and chondroitinase ABC diminish astrogliosis.

This simple, controlled and reproducible 3D culture system constitutes a good scar-like system and offers great potential in future neurodegenerative mechanism studies, as well as in drug screenings envisaging the development of new therapeutic approaches to min-

imize the effects of the glial scar in the context of central nervous system disease.

**Acknowledgments**

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**A 3D Printed Human iPSC-derived Hepatic Model that can Improve *In Vitro* Liver Functional Maturation**

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The functional maturation and preservation of hepatocytes derived from human induced pluripotent stem cell (hiPSC) are essential to personalized *in vitro* drug screening and potential *in vivo* transplantation. Major liver functions are tightly linked to the 3D assembly of hepatocytes with the supporting cell types from both endodermal and mesodermal origins in a hexagonal lobule unit. While there are many reports on functional 2D cell differentiation, no study has demonstrated the *in vitro* maturation of hiPSC-derived hepatic progenitor cells (hiPSC-HPCs) in a 3D environment that depicts the physiologically relevant cell combination and architecture.

The application of digital micromirror device-based 3D bioprinting to tissue engineering has allowed 3D patterning of multiple cell types in a pre-defined biomimetic manner that can promote functional improvements of hiPSC-HPCs. Here we present a 3D hydrogel-based tri-culture model that embeds hiPSC-HPCs with human umbilical vein endothelial cells (HUVECs) and adipose-derived mesenchymal stem cells (ADSCs) in a microscale hexagonal architecture. In comparison to 2D monolayer culture and 3D HPC-only model, our 3D tri-culture model shows both phenotypic and functional enhancements in the hiPSC-HPCs over weeks of culture. Specifically, we find improved morphological organization of various cell types, higher liver-specific gene expression levels, and increased metabolic product secretion. The combination of 3D bioprinting technology and tissue engineering enables the development of a 3D hiPSC-HPC maturation model that replicates the native liver module architecture and can potentially be used for personalized preventive medicine.

***In Vitro* Produced Cardiac Extracellular Matrix for Studies of Myocardium Regeneration Potential**

D. Nurzynska<sup>1</sup>, F. Di Meglio<sup>1</sup>, A. M. Sacco<sup>1</sup>, G. Granato<sup>1</sup>, I. Belviso<sup>1</sup>, V. Barbato<sup>1</sup>, M. Di Gennaro<sup>1</sup>, V. Romano<sup>1</sup>, R. Miraglia<sup>1</sup>, M. Vitale<sup>2</sup>, N. Zambrano<sup>2</sup>, A. Scaloni<sup>3</sup>, G. Renzone<sup>3</sup>, S. Montagnani<sup>1</sup>, C. Castaldo<sup>1</sup>;

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The essential requirement for a successful myocardial tissue engineering is that scaffolds and tissue constructs exhibit biomimetic activity toward cells. Our aim was to investigate the significance of cell-matrix interactions in cardiac tissue microenvironment *in vitro*. Cardiac fibroblasts from normal (CF-N) and pathological (CF-P) hearts with ischemic cardiopathy deposited extracellular matrix (Mx) and conditioned medium (Cm), which were then characterized by immunohistology, immunoblotting, ELISA-based protein array or mass spectrometry. Normal and pathological cardiac primitive cells (CPC-N and -P) were cultured on CF-Mx-N and -P or in CF-Cm-N and -P and their proliferation, apoptosis, migration and maturation were evaluated by BrdU, TdT, scratch wound assays or RT-PCR,

respectively. Laminin-1 and tenascin-X were detected in CF-Mx-P. The expression of IGFBP1, II-6, EGF was higher in CF-Cm-P, while that of SCF, TGF- $\beta$ 3, BMP4 was higher in CF-Cm-N. Migration speed of CPC-P peaked at 21.3  $\mu$ m/h on CF-Mx-N. Proliferation of CPCs increased significantly on CF-Mx-P, while apoptosis diminished on both types of matrix. In CPC-N, maturation toward cardiomyocytes was activated by CF Mx-N and toward endothelial cells by CF-Mx-P; in CPC-P the trend toward smooth muscle cell-lineage was evident on both substrates. CF-Cm-N reduced apoptosis of CPC-N (2-fold), while CF-Cm-P reduced apoptosis of CPC-P (3.5-fold) and increased proliferation of CPC-N (1.6-fold,  $n=3$ ,  $p<0.05$ ). These results highlight the influence of microenvironment on cardiac regeneration. Changes that take place in pathological conditions should be taken into consideration when planning cell-based therapy and scaffold functionalization. This work was supported by MIUR grant RBFR10LOGK.

### A 3d Human Neural Cell Culture System for Modeling Alzheimer's Disease

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Advances in stem cell technology have accelerated the development of human neuronal cell culture models of neurodegenerative diseases. We recently developed a novel three-dimensional (3D) human neural stem culture model that recapitulated key events in the pathogenic cascade of AD, including extracellular aggregation of amyloid  $\beta$  peptides and accumulation of hyperphosphorylated/aggregated tau protein (Choi *et al.*, 2014). This unique 3D culture model hold a particular promise for Alzheimer's disease (AD) since existing AD animal models have not been able to fully recapitulate all aspects of AD pathology. In this presentation, we will explain the design and characterization of human 3D culture model of AD and discuss about our ongoing efforts to apply this model system for an experimental drug screening.

### Session: Tissue Engineered Pumps: from Cartoons to Reality

Date and Time: Thursday, September 10, 2015,  
3:00 PM - 4:30 PM

#### Use of Neonatal Cardiomyocyte Transplants into the Aorta and Vena Cava as Biologic Pumps

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We explored the possibility of developing a biologic auxiliary pump that could be implanted as a cuff of beating cells around the aorta or vena cava to eventually improve cardiac output and prevent venous-stasis. 5 million neonatal cardiomyocytes from Fischer rats of both sexes or medium (control) were injected into the wall of the abdominal aorta of adult female Fischer rats and examined at 2 or 6 weeks later. 7/10 cell-treated aortas but none of the medium-treated aortas showed spontaneous rhythmic beating (102 beats/min) at 2 weeks after transplant and after excision of the heart. PCR was positive for the SRY gene (Y chromosome) in cell-treated, but not medium-treated aortas. Histology showed viable striated muscle grafts in the walls of the aorta in 9/10 cell-treated rats at 2 weeks and in 9/9 cell-treated aortas at 6 weeks, but not in medium-treated aortas. With pacing, grafts generated intra-aortic pressures up to 3.8 mmHg (mean of 0.8 mmHg). Neonatal cardiomyocytes were injected into the wall of the inferior vena cava. Three weeks later and with the heart excised, the vena cava demonstrated spontaneous contractions with an intrinsic rate of 101 beats per minute. Histologic analysis confirmed a cuff of striated immature cardiomyocytes. These studies showed that it is feasible to transplant immature cardiomyocytes into the walls of the great vessels. These grafts of neonatal cardiomyocytes survive, differentiate, de-

velop cross striations, contract spontaneously, can be paced and generate some, although limited amount of pressure.

### Designing MiniHearts: Tissue Engineered Tubular Pumps

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Biological fluids include aqueous humor of the eye, milk produced by mammary glands, amniotic fluid, chyme, chyle, gastric juices, lymph, seminal fluid, bile, cerebrospinal fluid and blood. Diminished drainage or decreased flow of these fluids can lead to a wide range of diseases, including peripheral artery disease, chronic venous insufficiency, lymphedema and many others. Tissue engineered tubular pumps can potentially help to alleviate many of these adverse conditions. Many labs are exploring the possibilities of creating such pumps from different cell sources, including stem cells, cardiac myocytes, skeletal and smooth muscle cells with an ultimate goal of creating a rhythmically beating sheath of muscle around vessel of interest, being it thoracic duct, deep vein, a major artery or any other type of tubular structure. Here we will discuss a possibility of creating 'designer' tubular pumps from autologous stem cells differentiated toward either nodal or working ventricular myocyte phenotypes and will deliberate on their ability to alleviate lymphedema or chronic venous insufficiency symptoms.

### Tissue Velcro® for Rapid 3d Assembly of Functional Cardiac Co-cultures

M. Radisic, B. Zhang, M. Montgomery, L. Davenport-Huyer;

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Engineering mature tissues requires a guided assembly of cells into organized three-dimensional (3-D) structures with multiple cell types. Guidance is usually achieved by micro-topographical scaffold cues or by cell-gel compaction. The assembly of individual units into functional 3-D tissues is often time-consuming, relying on cell ingrowth and matrix remodeling, while disassembly requires an invasive method that includes either matrix dissolution or mechanical cutting. Here, we invented Tissue Velcro®, by applying the hooks and loops of the Velcro® system to bio-scaffold design. Assembly of Tissue Velcro® preserved the guided cell alignment realized by the topographical features in the 2-D scaffold mesh and allowed for the instant establishment of co-culture conditions, by spatially defined stacking of fibroblast and cardiomyocyte layers, or through endothelial cell coating. The assembled 3-D tissue constructs were immediately functional, and facile, on-demand, tissue disassembly was demonstrated while preserving the structure, physical integrity and beating function of individual layers.

### Force Generation in the Human Heart Tissue Engineered from Ips Cells

G. Vunjak-Novakovic<sup>1</sup>, K. Ronaldson<sup>2</sup>, T. Chen<sup>2</sup>, S. Ma<sup>2</sup>, D. Sirabella<sup>2</sup>;

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The utility of tissue-engineered cardiac pumps critically depends on the ability of these tissues to generate force. We describe here a protocol designed to generate *in vitro* synchronously contracting and mechanically strong cardiac muscle starting from human iPS cells. Human iPS cells obtained from healthy individuals and those diagnosed with cardiomyopathies were differentiated into cardiovascular lineages by staged molecular induction. By day 8, spontaneously contracting cells were obtained, that displayed properties of immature human cardiomyocytes (iPS-CM). Cardiac tissue organoids were formed by incorporation of iPS-CMs into a native tissue hydrogel that polymerized around two elastic pillars. Electrical stimulation of these organoids over 4 weeks of *in vitro* culture, that made them work against the pillars, resulted in maturation of the cells that

was evidenced by changes in gene expression, ultrastructure, calcium handling, and the frequency and force of contractions. Here we focus on contractile behaviour that was measured using on-line assays, and discuss factors that determined force generation: genetic background of the iPSC cells (healthy donor, or a patient with inherited cardiac myopathy), addition of fibroblasts, duration of culture, and the regime of electrical stimulation. We report that cardiac tissue organoids engineered from healthy iPSC cells and conditioned in culture for 4 weeks develop mechanical force in the mN-range, while displaying positive force-frequency relationship and physiologically relevant calcium handling. We further report specific differences in the development of mechanical force in cardiac tissue organoids engineered using iPSC cells from patients with cardiac myopathies.

### Chitosan-based Neuromuscular Tubular Tissue with Improved Biomechanical Properties Suitable for Gut Lengthening in a Rat Model

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Winston Salem, NC.

**Introduction:** Anastomotic leakage is a common complication in colorectal surgery with high rates of morbidity and mortality. Tissue engineering provides an attractive approach to reduce anastomotic failures using cells and scaffolds. Objective: The objective of this study was to develop cell-seeded scaffolds that can withstand physiological conditions similar to native intestine.

**Methods:** Porous tubular chitosan scaffolds were engineered using the freeze/dry method. Aligned chitosan fibers were engineered using the gelation/extrusion method and embedded within the tubular porous scaffold. The mechanically reinforced scaffold was seeded with an engineered innervated smooth muscle sheet. Seeded and non-seeded scaffolds were implanted subcutaneously in the abdominal wall of rats for 4 weeks. Histological and biomechanical analyses were performed.

**Results:** Fiber-reinforced scaffolds exhibited higher tensile strength and elasticity than scaffolds without fibers. Following implantation, the cell-seeded tubular tissues maintained cell phenotype, luminal patency and demonstrated the capacity to expand and relax following increase in luminal pressure with no signs of leakage. An artificial pellet was manually passed through the lumen of the tissue without obstruction. Implanted seeded scaffolds exhibited appropriate tensile strength ( $0.069 \pm 0.009$  MPa) and tensile strain ( $157.3 \pm 41.8\%$ ), which were higher than implanted non-seeded scaffolds and similar to native intestine. This indicated that the cells remodeled around the scaffolds and contributed to the mechanical properties of the tissue.

**Conclusion:** This study demonstrated the feasibility of developing tubular neuro-muscular tissues that maintain luminal patency and possess biomechanical properties similar to native intestine following subcutaneous implantation. This work was supported by Wake Forest University Institutional Funds.

### Session: Tissue Engineering and Regenerative Medicine Technologies for Functional Restoration of Skeletal Muscle Defects *In Vivo*: From Stem Cells, Imaging and Computer Models to Platform Approaches and Enabling Technologies

**Date and Time: Thursday, September 10, 2015,  
3:00 PM - 4:30 PM**

#### Scaffoldless Skeletal Muscle Tissue Engineering for Repair of Volumetric Muscle Loss

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Multiple pathological conditions including muscle trauma, congenital defects, post-operative damage, and degenerative myopathies can lead to volumetric muscle loss (VML). To address the need for exogenous muscle tissue sources for VML repair, our

laboratory has developed scaffoldless tissue-engineered skeletal muscle units (SMUs), multiphasic muscle-tendon-bone constructs that can produce force spontaneously and in response to electrical stimulation. Our SMU constructs are grown from satellite cells, fibroblasts, and bone marrow stromal cells isolated from Fischer 344 rats and co-cultured to form a multiphasic muscle tissue composed of engineered skeletal muscle joined to bone anchors by myotendinous junctions and entheses. We have recently demonstrated the potential of our SMUs to repair muscle in a 30% VML model in the tibialis anterior muscle of a rat. 28 days following VML repair the average force of the SMU increased to  $1011 \pm 326$   $\mu$ N, a 4.8-fold improvement compared to *in vitro* forces prior to implantation. Histological examination confirms the development of small, aligned muscle fibers with advanced sarcomeric organization, vascular and neural interfaces, and myotendinous junctions. Immunostaining for Pax7-expressing cells confirms the presence of muscle satellite cells in the SMUs prior to implantation, and the number of Pax7-positive satellite cells in the SMU increases during the 28 days *in vivo*. By examining our SMUs' structural and functional development *in vivo*, we have demonstrated that they survive the mechanical stress of their *in vivo* environment, mature in function and phenotype, and may be used to add muscle fibers to muscle recovering from VML.

#### The Role of Stem Cell Exhaustion in Aging and Disease

J. Huard;

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We have demonstrated that a population of muscle progenitor cells (MPCs) isolated from the ERCC1-deficient mouse model of accelerated aging, are defective in their proliferation abilities and differentiation capacity. We have observed that injections of wild-type (WT)-MPCs into *Erc1* knockout (*Erc1*<sup>-/-</sup>) mice resulted in an improvement in age related pathologies. In an attempt to determine whether the defect observed in ERCC deficient MPCs was not exclusive to this progeria model, we isolated MPCs from another progeroid mouse model, the zinc-metalloproteinase (*Zmpste24*) knock-out mouse. We have observed that *Zmpste24*<sup>-/-</sup> MPCs have proliferation and differentiation defects, characteristics also observed in MPCs from normal mice. These results suggest that the defect in MPCs is not specific to a particular model of progeria. We have investigated whether a defect in MPCs can also be observed in skeletal muscle disease, Duchenne muscular dystrophy (DMD). DMD patients lack dystrophin from the time of birth; the onset of muscle weakness only becomes apparent at 4–7 years of age, which coincides with the exhaustion of the MPC pool. There is evidence that supports this concept including the gradual impairment of the myogenic potential of MPCs isolated from DMD patients during aging. Here, we report that dystrophin-trophin double knockout (dKO) mice exhibit a spectrum of degenerative changes in various musculoskeletal tissues including skeletal muscle and bone. In contrast to that observed with MPCs isolated from the *mdx* mice we have recently shown a defect in the MPCs isolated from dKO mouse.

#### A Tissue Engineered Muscle Repair Technology Platform for the Treatment of Volumetric Muscle Loss Injuries

G. Christ;

Biomedical Engineering, University of Virginia,  
Charlottesville, VA.

Skeletal muscle possesses a rather remarkable capacity for regeneration and repair. However, there are injuries where the endogenous regenerative capacity of skeletal muscle is insufficient for functional restoration and structural reconstitution. Among these are volumetric muscle loss (VML) injuries, such as those that can result from trauma, infection, congenital anomalies, or surgery. Such VML injuries produce permanent functional and cosmetic deficits. There are few effective treatment options for VML injuries. Tissue engineering approaches hold great promise toward

this end, and therefore, numerous strategies are currently under investigation. In this regard, we have been developing a Tissue Engineered Muscle Repair (TEMR) technology platform for treatment of VML injuries to civilians and Wounded Warriors. Our first technology involves bioreactor preconditioning of muscle derived cells seeded on decellularized extracellular matrix prior to implantation into relevant rodent models; resulting in restoration of 60–70% of muscle contraction within 2 months of implantation in a surgically created VML injury to the latissimus dorsi or tibialis anterior muscles. This first generation TEMR technology is targeted to treatment of secondary revision of cleft lip in adults. We have since expanded our technology platform to include a variety of other biomaterials and enabling technologies. This strategy recognizes that VML injuries represent a spectrum of functional and cosmetic deficits to a variety of muscles. Thus, a diversity of TEMR technologies will likely be required to address the totality of VML injuries, - especially much larger and more devastating VML injuries. The pipeline of technologies currently being developed will be discussed.

### Creating Interactions between Tissue Engineered Skeletal Muscle and the Peripheral Nervous System

M. P. Lewis;

NCSEM, Loughborough University, Loughborough, UNITED KINGDOM.

3D skeletal muscle constructs provide a test bed for physiological experimentation in a controlled environment whilst better recapitulating native skeletal muscle architecture than conventional *in vitro* culture techniques. Such models could be advanced by the addition of motoneurons in an attempt to develop neuromuscular junctions (NMJs) which would offer the potential for more advanced neuromuscular testing, to both improve our understanding of the underlying physiology of the NMJ, and also allow clinical testing to be undertaken. The latest developments in this area from our laboratory will be discussed.

### Multi-scale Muscle Modeling and *In Vivo* Imaging as Guides to Advancing Muscle Tissue Engineering Research

S. S. Blemker;

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The field of skeletal muscle tissue engineering has made significant strides over the last decade, resulting in tissue engineering solutions that provide promising levels of functional recovery of muscle tissue following an injury. These results are promising, and lead to multiple questions, including: (i) what is the mechanism of action for the observed functional recovery?, and (ii) can the functional recovery be improved? The answers to these questions require consideration of the complex biological and biomechanical functional relationships within muscle tissue. We submit that computational modeling can provide a powerful means to explore these complex relationships in a systematic way that could not be performed with experiments alone, empowering us to answer the two critical questions.

We have developed multi-scale models of skeletal muscle that represent the biomechanical function of muscle at multiple scales - from the macro-scale (meters) to the micro-scale (microns). Models of limb, facial, and breathing muscles have been created to quantify key form-function relationships. In parallel with the biomechanical models, we created agent-based models of muscle tissue that incorporate the behaviors of multiple cell types within muscle (muscle cells, fibroblasts, macrophages) and are capable of predicting tissue adaptive responses to both biomechanical and biochemical cues. Finally, a variety of non-invasive imaging methods are used to inform and validate these models. The goal of this talk will be to describe the modeling and imaging framework, and provide a few examples of how models can be used to inform and optimize the design of muscle tissue engineering solutions.

### Session: Tissues and Materials for Translational Neural Repair

Date and Time: Friday, September 11, 2015, 10:45 AM - 12:15 PM

### Evaluating Growth Factor Effects in Isolated Spinal Interneuron Populations

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During remodeling after spinal cord injury (SCI), long propriospinal interneurons (INs) sprout collaterals that extend into the gray matter to form “detour” circuits with local populations of INs. However, little is known about cues that might promote these local rewiring events, in part because of the difficulty identifying specific contributions of different INs *in vivo*. Here we report an *in vitro* environment to test the efficacy of pro-regenerative factors in isolated IN populations. We employ a genetic strategy to obtain large quantities of high purity embryonic stem cell (ESC)-derived ventral spinal populations without the need for cell sorting. By using homologous recombination to place the puromycin resistance enzyme puromycin N-acetyltransferase into a defining marker locus, after differentiation we can select for desired populations to obtain pure, post-mitotic INs that are not diluted by glia or other proliferating cells, as determined by immunocytochemistry and flow cytometry. We cultured these isolated INs in laminin-coated microdevices that allow us to quantify genetic and morphological changes in multiple neuronal populations in response to chemical gradients or co-culture. Initial results using selected ESC-derived motor neurons and V2a INs demonstrated differential and concentration-dependent neurite growth in response to glial-derived neurotrophic factor, brain derived neurotrophic factor, and neurotrophin-3. Future studies in this model system will expand into other IN cell types and explore the effects of growth factors on functional synapse formation. This platform will enable more intelligent targeting for therapeutic intervention after SCI.

### Bioengineered, Multi-Lumen Scaffolds for Spinal Cord Injury

J. Koffler<sup>1</sup>, D. Shahriari<sup>2</sup>, J. Sakamoto<sup>2</sup>, M. Tuszynski<sup>1</sup>;

<sup>1</sup>UCSD, La Jolla, CA, <sup>2</sup>UM, Ann Arbor, MI.

Approximately 1.2 million Americans have sustained some form of spinal cord injury (SCI), with an estimated annual economic impact of \$20 billion. Unlike the peripheral nervous system, axons of the central nervous system do not regenerate after injury, leading to neural deficits that are frequently irreversible. To promote axonal regeneration after SCI, we have adopted bioengineering technology to fabricate regeneration-promoting scaffolds containing multiple linear channels in a honeycomb architecture that aims to achieve three goals: 1) provide linear axon guidance across long injury gaps, to replicate the fascicular nature of the intact nervous system; 2) to provide a molecular fill or coating to enable axon attachment and growth, and 3) to allow loading with molecules that recruit and stimulate axonal growth, such as nervous system growth factors. To date we have tested scaffolds fabricated from agarose, chitosan, alginate, PEGDA and PCL in rats with complete spinal cord transection. Anatomical analyses 4 weeks after implantation suggest that scaffold degradation can be delayed, allowing retention of structural integrity while reducing reactive cell accumulations at scaffold interfaces with host spinal cord. Axons penetrate scaffolds and frequently regenerate over their entire length.

### Region-specific Cells and Tissue Models for Hindbrain and Spinal Cord Regenerative Medicine

E. S. Lippmann<sup>1</sup>, G. T. Knight<sup>1</sup>, M. C. Estevez-Silva<sup>1</sup>, C. E. Williams<sup>2</sup>, M. A. Bakooshi<sup>3</sup>, D. A. Ruhl<sup>4</sup>, E. R. Chapman<sup>4</sup>, P. M. Gilbert<sup>3</sup>, J. J. Coon<sup>2</sup>, R. S. Ashton<sup>1</sup>;

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The central nervous system (CNS) consists of diverse tissues containing region-specific cell phenotypes. Recent CNS disease studies have demonstrated that cell therapies and tissue models derived from human pluripotent stem cells (hPSCs) must be patterned with appropriate regional phenotypes to exert a regenerative effect or model tissue-specific disease pathologies. However, derivation of phenotypes from diverse hindbrain and spinal cord regions is limited by an inability to deterministically control neural stem cells (NSC) patterning along the posterior CNS's rostrocaudal axis, which is specified HOX gene expression. Using our chemically defined protocol for deriving homogenous NSC cultures<sup>1</sup>, we discovered a biphasic morphogen regimen able to differentiate hPSCs into NSCs from any hindbrain or spinal cord region with discrete, corresponding HOX profiles<sup>2</sup>. Further, we present the use of 'clickable' culture substrates<sup>3</sup> to derive a spectrum of regional Olig2+ progenitors and motor neurons from hPSCs in 2-D culture at high efficiency (>70%) and without the need for passaging. Finally, we present advances in combining clickable culture substrates with microfluidics to instruct NSC tissue morphogenesis *in vitro* and create regional, organotypic spinal cord slice cultures *de novo*. The derivation of region-specific CNS cells and tissues enables optimal regenerative and disease modeling capabilities. Our chemically defined protocols and engineered culture platforms now expand such capabilities throughout the hindbrain and spinal cord.

1. Lippmann, E. S. *et al.* Stem Cells 32, 1032–1042 (2014).

2. Lippmann, E. S. *et al.* Stem Cell Reports, (in press)

3. Knight, G. T. *et al.* Chem. Commun. (Camb.) 51, 5238–5241 (2015).

### A Functional Human *in Vitro* Model of the Spinal Stretch Reflex Arc

J. J. Hickman, X. Guo;

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Spinal cord injuries and diseases can produce deficits in movement, sensation and autonomic function through damage to one or more of the elements in the spinal reflex arc. We have developed a test-bed, based upon motor control circuits, specifically the reflex arc, to address system level function in a defined, serum-free medium. This specialized circuit translates information from the motor cortex to a motoneuron, which then activates muscle fibers, in turn a specialized part of the muscle, called the spindle, relays information based upon this movement to a sensory neuron back to the original motoneuron. We have created each segment of this system utilizing rat cells and have now repeated multiple segments utilizing components derived from human stem cells. These cellular systems have also been integrated with BioMEMs devices [1]. We have been successful in creating functional human NMJs from stem cell derived motoneurons and from stem cell derived myotubes [2]. In a co-culture of human intrafusal fibers with human DRG neurons, mechanoreceptor formation on the bag fibers with Type II sensory neurons as well as innervation from Type Ia sensory neurons was observed. Rat intrafusal fibers and DRG were also cultured on a cantilever where the flexion of the cantilever stretched the intrafusal fiber and enabled the recording of an electrical signal to the sensory neuron proving a functional synapse [3]. The technology that we have created to date is at the forefront of drug development and human toxicity for the creation of a state-of-the-art human-based, functional *in vitro* neuronal systems.

### Injectable Hydrogels to Deliver and Engraft Schwann Cells in Spinal Cord Lesions

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Approximately 12,000 new spinal cord injuries (SCI) occur in the US each year, primarily affecting young adults. Schwann cells are a promising therapy for SCI and are currently being explored in clinical trials; however, significant limitations in cell delivery and long-term survival decrease their therapeutic potential. The low cell retention post-transplantation is partly attributed to (i) mechanical forces during injection that damage the cell membrane and (ii) the lack of a three-dimensional (3D) matrix to support cell viability post-injection. We hypothesized that the development of a shear thinning, injectable hydrogel would improve cell viability, engraftment, and regenerative capacity after transplantation. This hydrogel, produced from a blend of engineered recombinant proteins and peptide-modified synthetic polymer is termed SHIELD: Shear-thinning Hydrogel for Injectable Encapsulation and Long-term Delivery. SHIELD formulations with storage moduli, G', spanning 10–500 Pa all showed excellent viability of encapsulated Schwann cells (>98%) and significant mechanical protection from membrane damage when exposed to syringe needle flow. After 7 days *in vitro*, 3D cultures of within all SHIELD formulations showed positive immunostaining for Schwann cell markers (p75, S100), but cultures in formulations of intermediate stiffness showed higher proliferation rates and decreased caspase activity. In a rat cervical contusion model of SCI, Schwann cells delivered in SHIELD resulted in smaller lesion volumes compared to cells delivered in saline and injury control groups. Since even a mild functional recovery would mean a vast quality-of-life improvement for SCI patients, developing a regenerative therapy for SCI would be extremely significant clinically.

### Combining Biomaterials and Circulating Neural Progenitor Cells for Spinal Cord Injury Regeneration

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Neural progenitor cells (NPCs) in peripheral blood mononuclear cell (PBMNC) is an autologous cell source proven for its proliferation and neural differentiation potential. This study employed neuron-specific fibrin-based niche for both isolation of rat NPC and transplantation to the spinal cord injury (SCI) model to demonstrate improved cell homing when delivered using carrier matrix.

The multipotent adult progenitor cells (MAPC) in PBNMC from inbred animals were grown on the biomimetic fibrin-based niche for selection and induction into proliferating NPC. On the 6th day of culture NPCs were flushed out, labeled with tracker dye and transplanted in contusion type SCI. Cells injected with fibrinogen-thrombin system was the test; controls were NPCs transplanted in medium and the vehicles alone in separate group of 6 animals each. After 8 days of experiment, fluorescent imaging of gross tissue of SCI-site and histological analysis were carried out.

The niche efficiently selected rat NPC and directed differentiation to neural like cells with stage-by stage expression of progenitor, immature neuron and mature neuron markers such as nestin,  $\beta$ -tubulinIII, MAP-2 and synaptophysin. Upon imaging the tissue and histology sections, presence of PKH26 fluorescence that co-localized with neuronal marker indicating neural differentiation and better retention of transplanted cells near SCI in test as compared to cell-control was observed. Sections stained fibrin+ and rarely CD163+ macrophages suggesting that the transplanted cells were well preserved.

The potential of circulating NPC for regenerative medicine and use of fibrin matrix for successful transplantation & retention in SCI site have been demonstrated.



### Session: Vascular Grafts: Challenges and Current Progress

**Date and Time: Thursday, September 10, 2015, 1:00 PM - 2:30 PM**

#### ***In Vivo* Rapid Endothelialization of Small-diameter Decellularized Vascular Grafts with Bioactive Peptide Modifier**

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Although the good patency of the middle-diameter vascular grafts was achieved, suppression of the thrombosis still have not been overcome in a small diameter graft. We have developed the high hydrostatic pressure technology for preparing the decellularized vascular grafts. However, thrombosis formation was not completely suppressed on the small-diameter decellularized grafts. In this study, we developed a peptide modifier for inducing the rapid endothelialization to suppress the thrombosis formation. The peptide modifier containing collagen binding sequence and integrin ligand was synthesized and immobilized to the luminal surface of the decellularized grafts. The modified surface captured endothelial-like progenitor cells in peptide-sequence and cell-type specific manner. The peptide-modified decellularized grafts with 3.5 mm in length were transplanted into rat abdominal aorta. All grafts were patent for one month. On the other hand, unmodified grafts were rapidly occluded. We then challenged long-bypass decellularized grafts with 20 cm in length, and the excellent patency was also confirmed with a femoral-femoral crossover bypass method in a Göttingen minipig.

**Reference:** Fujisato T, Minatoya S, *et al.* Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches, Springer, Tokyo, Japan, 2005 Edited by Mori H, Matsuda H.

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**Disclosures:** The authors declare that there is no conflict of interests regarding the publication of this research

#### **50-Week Implant of a Tissue-engineered Pulmonary Conduit in a Growing Lamb Model**

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Tetralogy of Fallot and Pulmonary Atresia are just two examples of pediatric cardiac defects that, although long-term survival is excellent, will often require multiple operative procedures to replace the reconstructed connection between the right ventricle and pulmonary artery (right ventricular outflow tract, or RVOT). Currently, homograft pulmonary artery conduits or bovine jugular vein grafts are the only options to create this connection. These conduits have zero ability to grow and remodel with the somatic growth of the child.

We have developed a novel tissue-engineered vascular graft, which is allogeneic upon a decellularization performed prior to implantation. The resulting cell-produced matrix tube possesses physiological strength, compliance, and alignment (circumferential). We have shown excellent results implanting this completely-biological graft into the sheep femoral position at 6 months, including complete recellularization and positive remodeling) without mineralization, dilatation, or immune response.

To assess growth and remodeling potential for pediatric RVOT reconstruction, we implanted three lambs (14–18 kg) with 16 mm allogeneic grafts interpositionally into the pulmonary artery and monitored length and diameter with ultrasound over 50 weeks (to adult size), with assessment of the explanted tissue for normal thickness and collagen concentration. All three lambs have at least

doubled in weight (current ages ranging from 36 to 50 weeks), and all grafts have developed a pronounced curvature, with a length increase exceeding 50%, while maintaining similar thickness as at implantation. Also, there was no obvious mineralization detectable with ultrasound. The study shows promising application of this completely biological allogeneic graft for pediatric RVOT reconstruction.

#### **Development and Evaluation of an *In Vivo* Tissue Engineered Blood Vessel in a Porcine Model**

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**Background:** There's a large clinical need for novel vascular grafts. Tissue engineered blood vessels (TEBVs) have great potential to improve the outcome of vascular grafting procedures. Here, we present a novel approach to generate autologous TEBV *in vivo*. Polymer rods were developed that upon implantation evoke an inflammatory response culminating in encapsulation by a fibrocellular capsule. We hypothesized that, after extrusion of the rod, the fibrocellular capsule differentiates into an adequate vascular conduit once grafted into the vasculature.

**Method:** Rods were implanted subcutaneously in pigs. After 4 weeks, rods with tissue capsules grown around it were harvested. Tissue capsules were grafted bilaterally as carotid artery interposition. One and 4-week patency were evaluated by angiography whereupon pigs were sacrificed. Tissue capsules before and after grafting were evaluated on tissue remodelling using immunohistochemistry, RNA profiling and mechanical testing.

**Results:** Rods were encapsulated by thick, well-vascularized tissue capsules, composed of circumferentially aligned fibroblasts, collagen and few leukocytes, with adequate mechanical strength. Patency was 100% after 1 week and 87.5% after 4 weeks. After grafting, grafts remodelled towards a vascular phenotype. Gene profiles of TEBVs gained more similarity with the carotid artery. Wall thickness and  $\alpha$ SMA-positive area significantly increased. Interestingly, a substantial portion of (myo)fibroblasts present before grafting expressed smooth muscle cell markers. While leukocytes were hardly present anymore, the lumen was largely covered with endothelial cells. Burst pressure remained stable after grafting.

**Conclusions:** Autologous TEBVs were created *in vivo* with sufficient mechanical strength enabling vascular grafting. Grafts differentiated towards a vascular phenotype upon grafting.

#### **Engineering Blood Vessels without Exogenous Cells**

Y. Wang;

University of Pittsburgh, Pittsburgh, PA.

Current arterial tissue engineering research focuses on cell-based approaches. The purpose of this study is to investigate the potential of mammalian host to remodel synthetic polymeric grafts into viable arteries. The idea is to bypass *in vitro* cell seeding and culturing completely.

We designed the graft to have two layers, the inner tubular core is made of the elastomeric poly(glycerol sebacate), the outer sheath is made of polycaprolactone fibers. The sterilized grafts are coated with heparin and implanted as interposition grafts in rat abdominal aorta.

The grafts were quickly infiltrated with cells and polymer degradation led to rapid host remodeling. The graft materials were mostly degraded within 3 months. In its place was a neo-artery that mimicked native artery mechanically, biochemically, and anatomically. The neo-arteries were well integrated with the host, remained

patent and pulsed synchronously with host arteries. At one year post implantation, the graft further remodeled to a structure largely resemble native arteries with regenerated peri-vascular nerves. The amount of crosslinked elastin is the same as that of native arteries. This study indicates that synthetic vascular grafts made from fast degrading elastomeric materials can be remodeled by rodents into viable arteries. It remains to be seen if this is translatable to small arteries in large animal models and humans.

### Engineered Arteries from the Laboratory to the Clinic

L. Niklason;

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We have previously described a method for vascular regeneration that comprises seeding of vascular smooth muscle cells onto PGA scaffolds that are cultured in a biomimetic bioreactor. After culture, engineered vessels are decellularized, and then implanted (either with or without autologous endothelium) into various arterial grafting locations. Tissue engineered vascular grafts have been grown and tested in a set of preclinical models of arterial replacement, including coronary artery bypass grafting and vascular access for hemodialysis. In each of these preclinical settings (swine, canine and primate) the engineered acellular blood vessels were safe, well-tolerated and functioned as intended. The acellular vessels exhibited excellent mechanical properties prior to implant, comparable to those of native human vein and artery. Furthermore, the mechanical properties of the grafts (suture retention and burst pressure) strengthened after implantation, and no graft exhibited hemodynamically significant intimal hyperplasia. Based on the successful and robust preclinical data sets, regulatory bodies both in Europe and the United States have approved first-in-man testing of these bioengineered blood vessels for hemodialysis access. To date, 60 grafts have been successfully implanted in patients as a vascular access graft for use in hemodialysis, with clinical follow-up ongoing. All implanted grafts have been suitable for hemodialysis access with excellent flow rates, with no evidence of structural degeneration or immunologic rejection. This emerging technology is poised to be a real alternative to conventional synthetic vascular prosthesis.

### Session: Vascularization for Tissue Engineering and Regenerative Medicine

Date and Time: Wednesday, September 9, 2015,  
1:00 PM - 2:15 PM

#### Engineering Interactions between Morphogens and the Extracellular Matrix

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In natural situations, growth factors such as VEGF and PDGF are present bound to the ECM, yet therapeutic use of such growth factors has focused on application in soluble forms. To explore ECM immobilization of growth factors, we have explored complexation with recombinant variants of ECM proteins such as fibronectin and engineering growth factors fused to a domain that binds strongly to ECM proteins. To explore noncovalent immobilization upon matrices, we have engineered a fibrin-binding domain of fibronectin, containing the 12<sup>th</sup>-14<sup>th</sup> type III repeat (which was known to bind VEGF-A). In studies of the 12<sup>th</sup>-14<sup>th</sup> type III repeat, we determined that the growth factor binding activity of this domain was promiscuous. Incorporation of this domain into fibrin, through engineered transglutaminase activity, provides a powerful and generalizable method to retain such growth factors into surgical matrices. Examination of promiscuous growth factor binding to ECM proteins revealed broad binding to not only fibronectin but also fibrinogen, vitronectin, osteopontin, and tenascin C, among others. We examined these affinities to determine that placenta growth factor-2 (PIGF-2) binds the mentioned ECM molecules very strongly. We identified the binding domain and engineered

it into other growth factors to confer nM affinity to them, demonstrating enhanced efficacy in the context of angiogenesis, skin repair in chronic wound models, and bone repair. In this way, using variant growth factors that have super-affinity for ECM proteins, either a fibrin matrix or a tissue itself may be turned into a reservoir for growth factor sequestration and presentation.

#### Sustained and Simultaneous delivery of FGF2 and FGF9 from Poly(ester amide) Fibers for Building Stronger Microvessels

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Ischemic vascular diseases attack arteries and small blood vessels, causing vessel blockage, reduced blood flow and subsequent ischemia. Although there are several therapies for large arteries (e.g. thrombolysis, angioplasty, stenting and surgical bypass), when small arteries and microvessels become diseased, the treatment options become limited. Therapeutic angiogenesis has emerged as a novel treatment approach. However, systemic administration of angiogenic factors is usually inefficient for maintaining effective concentration at the site of interest, due to rapid clearance or degradation of the angiogenic factor. Clinical studies of basic fibroblast growth factor (FGF2) induced short-term angiogenesis as the new microvessels regressed over time and therefore did not confer long-term improvements in tissue perfusion. Infusion of fibroblast growth factor-9 (FGF9) *in vivo* generated physiologically mature neovessels that augmented flow into mouse ischemic hind limb. In this study, we are introducing a mixed blend and emulsion electrospinning technique for dual-loading of FGF2 and FGF9 into poly(ester amide) (PEA) fibers. *In vitro* release studies showed controlled co-release of both factors in a bioactive form. Co-released FGF2 and FGF9 from dual loaded PEA fibers enhanced endothelial cell (EC) tube formation, directed-migration of smooth muscle cells (SMCs) towards PDGF-BB and ultimately EC/SMC tube stabilization. FGFs-loaded PEA fibers did not induce inflammatory response *in vitro* or *in vivo*; furthermore, their subcutaneous implantation in mice induced cell niche recruitment. This supports the notion that 'cocktail' of angiogenic and arteriogenic factors is required for generating mature and long lasting vessels, which can offer promising applications in therapeutic angiogenesis and tissue engineering.

#### Vascularization of Multi-Organ-Chips for Tissue Engineering and Regenerative Medicine

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Organ-on-a-chips provide an adequate and reliable system for drug testing and engineering of small tissues. Yet, a big drawback is the lack of an intrinsic vascularization of generated 3D tissues. Here, we demonstrate the incorporation of fibrin scaffolds having endothelial and adipose-derived stem cells embedded into a perfused closed multi-organ-chip (MOC) system. We show that under static conditions vascular network formation is influenced by both fibrinogen and thrombin concentrations used to generate scaffolds. Polymerization of fibrin in MOC inserts results in distribution of fibrin fibers throughout the channel system, however, with no negative effect on vascular tube formation as evidenced by integration of fluorescence-labeled fibrinogen. We furthermore report

the formation of stable vascular networks after two weeks of perfusion with both strong and weak pulsatile flow. 3D-rendered images using two-photon microscopy analysis show organized microvascular structures in samples cultured under flow conditions for two weeks. Moreover, mRNA expression data of genes involved in vascularization reveals the effects of pulsatile flow on fibrin mono- and co-culture in MOCs. We conclude that a vascular network can be established in a perfused organ-on-a-chip model utilizing co-culture and used as a tool in tissue engineering and drug development as additional layers of micro-tissues can be added to this system.

### Vascularization Dynamics in Engineered Tissues

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Vascularization continues to represent a major challenge in the successful implementation of regenerative strategies. Cell organization into 3D tissue vascularized structures involves cell-matrix and cell-cell interactions, some of which occur between the different tissue cell types. During this process, cells further differentiate and assemble into structures resembling the final tissue architecture. We have established that vessel network assembly yielding vascularized 3D tissue structures can be induced *in-vitro* by means of co-culturing endothelial cells, fibroblasts and tissue-specific cells. We have also shown that *in vitro* pre-vascularization of engineered tissues can promote tissue survival and further vascularization upon implantation, via anastomosis of the engineered vessels with host vasculature, forming functional blood vessels *in vivo*. Vascularization of engineered tissues can be enhanced through coordinated application of improved biomaterial systems with relevant cell types. Moreover, we have shown that vessel network maturity and morphology can be highly regulated by both matrix composition and by external mechanical stimulations. Our recent studies have focused on investigation of the degree of the *in vitro* prevascularization required to achieve best postimplantation vascularization of tissue constructs, as well as on understanding the mechanisms underlying host-implant vessel integration and anastomosis. New co-culture approaches for inducing pre-defined vessel structures *in vitro* will also be discussed, as will novel studies on vascularized muscle flaps engineered to reconstruct large soft tissue defects.

### Therapeutic Angiogenesis from Vascular Biology to Regenerative Medicine

**A. Banfi;**

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Therapeutic angiogenesis, i.e. the generation of new vessels by delivery of specific factors, is required both for rapid vascularization of tissue engineered constructs and to treat ischemic conditions. Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis. However, uncontrolled expression leads to vascular tumors (angiomas). Major challenges to fully exploit VEGF potency for therapy include the need to precisely control *in vivo* distribution of growth factor dose and duration of expression. We previously found that the therapeutic window of VEGF delivery depends on the VEGF amount in the microenvironment around each producing cell rather than on the total dose, since VEGF remains tightly localized in tissue and a few "hotspots" of high expression are sufficient to cause angioma growth even if the total dose is rather low. On the other hand, short-term expression of less than about 4 weeks leads to unstable vessels, which promptly regress following cessation of the angiogenic stimulus.

Here I will present recent work aimed at: 1) translating fundamental principles of VEGF function into clinically applicable approaches to induce controlled angiogenesis, through the use of genetically modified progenitors or extracellular matrix engineering with recombinant factors; and 2) investigating the mechanisms that regulate the vascular switch between normal and aberrant angiogenesis *in vivo*, to identify novel and more specific molecular targets.

### Session: Whole Organ Tissue Engineering

**Date and Time: Wednesday, September 9, 2015,  
1:00 PM - 2:15 PM**

#### Bioengineered Porcine Kidney Constructs Seeded with Autologous Cells for Long-Term Survival *In Vivo*

J. Zambon<sup>1</sup>, I. Ko<sup>2</sup>, I. Kim<sup>2</sup>, C. Smith<sup>2</sup>, A. Atala<sup>2</sup>, J. J. Yoo<sup>2</sup>;

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Kidney transplantation is the only definitive treatment for end stage renal disease (ESRD). However, the availability of transplantable kidneys is limited. Recent advances in the field of bioengineering whole kidney constructs have provided a promising solution to address the shortage. Previously, we have developed decellularization and recellularization methods that allowed efficient recellularization including re-endothelialization of vasculatures and repopulation with renal cells using acellular porcine kidney scaffolds, followed by promising outcomes from short-term implantation. To make this technology amenable for clinical translation, this study aimed to evaluate vascular patency of bioengineered porcine kidney constructs seeded with autologous cell sources in a heterotopic implantation pig model. To provide anti-thrombotic capability, the decellularized kidney scaffold from native porcine kidneys was re-endothelialized following conjugation of heparin and CD31 antibody. For renal function, the re-endothelialized kidney scaffold was seeded with the renal cells, followed by bioreactor culture before implantation. The engineered kidney construct was implanted at the iliac site of pigs. During implantation, blood perfusion through the kidney implant was examined by CT scan and at 1 week implantation, the harvested implant was processed for histological analysis. Results of CT scan demonstrated evidences of partial blood perfusion within the implant. The histological and immunochemical analysis confirmed the vascular patency and viability of the seeded renal cells with maintenance of renal phenotype during the implantation. These results demonstrate that long-term implantation of engineered porcine kidney constructs is possible and this approach will lead to the development of an alternative treatment method for patients with ESRD.

#### Comparative Evaluation of Mature and Progenitor Human Renal Tubule Cell Adaptation within Decellularized Renal Extracellular Matrices

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**Objectives:** There is a growing shortage of donor kidneys for transplantation. We utilized decellularized rodent kidney extracellular matrix (ECM) as perfusable, three-dimensional templates for formation of tubular structures by human kidney epithelial and papillary cells as a step toward the long-term goal of engineering a functional nephron.

**Methodology:** We infused human epithelial cell lines derived from renal proximal (RPTE) or distal cortical tubules (RCTE) or primary human renal progenitor cells (RPC) isolated from cadaveric kidneys using continuous antegrade perfusion. We evaluated seeding efficiency, proliferation, and changes in segment-specific cell phenotype during perfusion culture in renal ECM.

**Results:** Resazurin perfusion revealed an increase in total engraftment, but a decrease in seeding efficiency with increasing cell infusion number. Both cell lines demonstrated continuous growth in the renal ECM over 7 days of perfusion culture. Distal tubule-derived cells primarily repopulated cortical tubules, coalescing into polarized, tubular structures with tight junctions and apically-oriented cilia.

Proximal cells distributed in glomeruli and surrounding tubules. qPCR is being used to evaluate segment-specific gene expression profiles. Primary human kidney-derived CD133/1+RPC remained viable, and expressed CD133 and Tamm-Horsfall protein three days after seeding.

**Significance:** We developed a chimeric, heterogenic tissue culture system that supports growth of human kidney cells of multiple origins within rodent ECM scaffolds. Using a panel of analytical techniques, we can comparatively evaluate adaptation of specific cell populations derived from different nephron segments during recellularization of renal matrices. These results show promise for future evaluation of pluripotent stem cell differentiation into nephron components within this system.

#### Implantation of Re-endothelialized Porcine Liver Constructs for Whole Organ Engineering

I. Ko, P. Li, A. Peloso, C. Smith, A. Dhal, C. Clouse, W. Zhao, T. Shupe, S. Soker, J. J. Yoo, A. Atala;

Wake Forest Institute for Regenerative Medicine, Winston Salem, NC.

Liver transplantation is severely limited by the availability of donor organs. Whole organ engineering based on decellularization/recellularization techniques using patient's own cells has been proposed as an alternative approach to liver transplantation. Demonstration of this approach was recently reported in a rodent model, however it was evident that maintenance of vascular patency within the implant has been a major challenge. To address this issue, previously, we have developed an endothelial cell seeding method that results in effective and functional re-endothelialization of vasculatures within decellularized porcine liver scaffolds. The current study aims to test the feasibility of implantation of clinical scale bioengineered whole livers and the vascular patency during *in vivo* implantation. To support retention of the seeded endothelium, the decellularized liver scaffolds were conjugated with CD31 antibody, followed by endothelial cells seeding. After perfusion culture, the engineered liver construct was heterotopically implanted into the pigs by vascular anastomosis after nephrectomy. The blood flow and vascular patency was determined by ultrasound imaging and angiography. One day post implantation, the retrieved implant was characterized by histological and immunohistological methods. Results from vascular imaging demonstrate that the re-endothelialized liver construct maintained better blood flow and vascular patency than that of un-seeded scaffolds. The maintenance of vascular patency is confirmed by the existence of seeded endothelial cells within the vasculatures of the implant. These results are a promising indicator of feasibility of re-endothelialized liver implantation long-term and contribution to alternative treatment for chronic liver failure.

#### Bioengineered Pediatric Lung: The use of Tissue Engineering and Regenerative Medicine Techniques for Pediatric Applications

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In children between the ages of 1–11 the most common causes for a lung transplant are cystic fibrosis and pulmonary hypertension. Transplant teams attempt to match the size of donor lungs to the recipient as closely as possible, but children have small chest cavities limiting the size of lungs that can be transplanted. We have developed a technique to bioengineer pediatric sized replacement lungs using discarded human lungs as both support scaffold and source of cells. Discarded pediatric lungs were decellularized to be used as lung scaffolds. Adult double lung sets were used as a source of viable

lung cells. After seeding the pediatric lungs with cells from adult lung parenchymal or vascular tissue, the tissue-constructs were incubated in medium (Small Airway Growth Medium) containing growth factors for thirty days. Lung epithelial and endothelial cells successfully repopulated the pediatric lung. We found that Type I and Type II pneumocytes and CD31 endothelial cells were distributed throughout the alveoli and had an organized tissue structure similar to that of normal lung. These cells formed alveolar-capillary junctions throughout the lung and type II pneumocytes that were capable of secreting surfactant proteins. CT scans and PET-CT scans showed normal gross anatomy with no tumor formation. The production of these bioengineered lungs has the potential to increase the number of lungs available for pediatric transplantation in the future.

#### Towards Autotrophic Tissue Engineering: Photosynthetic Biomaterials for Tissue Regeneration

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Despite decades of research, the appropriate delivery of oxygen still remains one of the biggest unresolved problems for *in vitro* and *in vivo* tissue engineering. In order to overcome this issue we have proposed the use of photosynthesis as a source of oxygen. In this study, we combined the use of biodegradable scaffolds with photosynthetic cells, hereby creating photosynthetic scaffolds. We could demonstrate that upon light stimulation these new materials are capable of producing enough oxygen to be independent of external supply *in vitro*. Furthermore, when photosynthetic biomaterials were engrafted in a mouse tissue defect, we observed that the presence of the microalgae did not trigger a significant immune response in the host. Moreover, the analyses showed that the algae survived for up to one week *in vivo*, generating chimeric tissues comprised of algae and murine cells. Finally, we also evaluated the feasibility of combining photosynthetic and gene therapy to promote tissue regeneration. In this approach we could demonstrate that scaffolds seeded with gene modified microalgae were able to constantly release oxygen and other therapeutic growth factors *in vitro* and *in vivo*. Our study represents the first step towards engineering fully autotrophic tissues, and suggests the use of photosynthetic therapy for the treatment of several clinical conditions.

#### Integrin Specific Re-endothelialization within Decellularized Lungs

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Re-vascularization of engineered whole lungs is required for proper functionality of the organ. Functional re-vascularization requires establishing a continuous layer of endothelial cells (ECs) lining the tunica intima layer of all blood vessels. We hypothesized that the cell's integrin repertoire could be utilized to optimize region-specific adhesion and allow for the formation of a continuous EC layer. Integrin functionality of rat lung microvessel endothelial cells (RLMVEC) was characterized as we previously reported. The RLMVEC showed significant adhesion to fibronectin (FN), Collagen-I (C-I), Collagen-IV (C-IV) and Laminin. They specifically interact with antibodies against  $\alpha$ -1, 2, and 5, and  $\beta$ -1 integrin subunits, but not with  $\alpha$ -4 or  $\beta$ -3. The inclusion of VLO4 completely abolished adhesion to FN, and the simultaneous application of VP12 and obtustatin, significantly inhibited adhesion to C-I&IV, as

compared to the individual disintegrins alone. A combination of all three snake-venom derived inhibitors of integrins resulted in a 50% decrease in adhesion to decellularized rat lung scaffolds (DLS) in a slice culture model. More importantly, adhesion to the macrovasculature and microvasculature was completely blocked in a whole DLS, and resulted in clogging of the capillaries with no apparent cell spreading. Our results suggest that  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  and  $\alpha 5\beta 1$  integrins strongly control the process of re-endothelialization. We surmise that better understanding and control over the of the recellularization process will contribute to the full revascularization of the entire organ, and could lead to region-specific repopulation of decellularized lungs and other organs of interest.

**Acknowledgments:** CTS is supported by NASA GSRP.

### Whole Organ Tissue Engineering of the Bladder: A Collagen Bladder Scaffold Comprising Anatomical Branching

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Radical cystectomy followed by neo-bladder reconstruction can be applied to treat bladders that are comprised due to e.g cancer or traumatic injuries. Currently, neo-bladders are constructed from autologous tissue, which may result in complications. This study aims to construct collagenous bladder scaffolds with seamless anatomical branching by freezing and lyophilization techniques.

An aluminum mold with tubular appendices was developed mimicking the shape and dimensions of a human bladder and anastomosis sites for the urethra and ureters. The mold was filled with insoluble type I collagen suspension and frozen in a freezing bath for 3 min at  $-80^{\circ}\text{C}$  or 12 min at  $-20^{\circ}\text{C}$ . After removal of non-frozen collagen, the construct was lyophilized and subsequently  $\gamma$ -irradiated. MRI was used to measure bladder volume and scanning electron microscopy to investigate scaffold morphology. The freezing process was assessed by temperature measurements. Cytocompatibility was evaluated using smooth muscle cells and urothelial cells.

The bladder scaffold consisted of a spherical part with a volume of 380 cm<sup>3</sup> and seamlessly attached appendices. The outside was porous whereas the luminal side showed low porosity. A radial pore structure from the outside inwards was observed, due to the growth of ice crystals during the freezing process. Pores were smaller for freezing at  $-80^{\circ}\text{C}$  compared to  $-20^{\circ}\text{C}$ . Expression of cell-specific markers indicated cytocompatibility.

Adaptations in scaffold construction can be easily applied such as the size, volume, wall thickness and pore size. This study shows that organ specific scaffolds can be obtained by applying state-of-the-art lyophilization technology.

### Session: Wound Healing and Inflammation

**Date and Time: Wednesday, September 9, 2015, 2:15 PM - 3:30 PM**

### Reprogramming Diabetic Foot Ulcer-Derived Fibroblasts to Restore Regenerative Potential: From iPSCs to Bioengineered 3D Tissues

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Diabetic foot ulcers (DFUs) are a serious complication that can lead to severe disability and increased mortality in diabetic patients.

Yet, the healing of DFUs and other chronic wounds remains a major challenge for regenerative therapies. Our goal is to use bioengineered 3D human tissue models to study the function and phenotype of DFU-derived fibroblasts following their reprogramming to induced pluripotent stem cells (iPSCs) and subsequent differentiation to healing-competent fibroblasts. To accomplish this, DFU-derived fibroblasts (DFUFs), and site- and age-matched controls, including healthy non-diabetic (NFFs) and diabetic fibroblasts (DFFs), were reprogrammed to iPSCs. Complete reprogramming was verified by teratoma assay and staining for Oct4, Tra-1-81, SSEA-4. iPSCs were differentiated to fibroblasts [1] and lineage fate was confirmed by flow cytometry for fibroblast surface markers. iPSC-derived fibroblasts were then incorporated into 3D tissue models to study cell function in a complex microenvironment. When incorporated into a 3D tissue model of extracellular matrix (ECM) assembly that mimics early wound healing stroma [2], iPSC-derived fibroblasts demonstrated dramatic changes in ECM organization, reflecting altered ECM assembly. We are currently investigating the wound healing potential of these iPSC-derived fibroblasts using gene expression microarrays, methylation analysis and 3D tissue models to evaluate if iPSC reprogramming has improved the wound repair potency of DFU-derived fibroblasts by altering their epigenetic profiles.

Using tissue engineered models to characterize the "reversal" of non-healing DFUFs to wound healing-competent fibroblasts provides a powerful platform to characterize iPSC-derived cells, and to develop novel therapeutic strategies for the treatment of DFUs.

### Burn Injury Does not Alter the Anti-Inflammatory Phenotype of Cultured Adipose-Tissue Derived Stem Cells

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Mesenchymal stem cells (MSCs) have been shown to possess broad immunoregulatory abilities. Our lab is characterizing the effects of burn injury on a select subpopulation of MSCs, the adipose tissue derived stem cell (ASC). As bone marrow-derived MSCs have been shown to possess either a pro- or anti-inflammatory phenotype, we examined ASCs for similar properties. The purpose of this study is to determine whether cultured ASCs display a pro- or anti-inflammatory phenotype and whether burn injury may influence this phenotype.

**Methods:** ASC cultures were established from untreated rats and from rats 72 hours following induction of a 60% total body surface area third degree scald wound. Expression of the following genes was monitored: COX-2, TGF $\beta$ 1, IDO, iNOS, TSG-6, IL-6, IL-10, TNF $\alpha$ , CCL3 (MIP-1a), CCL4 (MIP-1B), CCL5 (RANTES), CXCL9, and CXCL10.

**Results:** Cultured ASCs expressed abundant mRNA levels for COX-2, TGF $\beta$ 1, TSG-6, and IL-6, which are associated with an anti-inflammatory phenotype. Pro-inflammatory genes expressed included CCL4 and CXCL10. mRNA was not detected for IDO, iNOS, IL-10, TNF $\alpha$ , CCL3, CCL5, and CXCL9. There was no difference in the gene expression profiles between ASC cultures isolated from control or burned animals.

**Conclusions:** Due to high level expression of COX-2, TGF $\beta$ 1, TSG-6, and IL-6, which are all capable of negatively regulating effector T cell activation, directly or indirectly, we conclude that the default status of cultured ASCs is predominantly anti-inflammatory. The status of the animal from which the cells were isolated, i.e. unburned versus burned did not affect the phenotype of the resultant cultures.

### In Situ Modulation of Macrophage Phenotype to Control Inflammation and Improve Tissue Regeneration

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**Introduction:** macrophages are present in many tissues and can polarize towards a pro-inflammatory (M1) or anti-inflammatory

(M2) phenotype. With their different phenotypes, they can influence regeneration of (engineered) tissues, either positive or negative. The aim of this study was to modulate the behavior of macrophages present in the capsule lining of joints (i.e. synovium) using compounds on synovial explants and to gain insight in the modulatory capacity of the compounds on specific macrophage subtypes.

**Methods:** Synovial explants obtained from osteoarthritis patients were stimulated for 24h with  $\text{IFN}\gamma + \text{TNF}\alpha$  to simulate acute inflammation before addition of BMP-7, Rapamycin, Dexamethasone or Pravastatin. Primary human monocytes were isolated from buffy coats by CD14+ selection and the macrophage subtypes M( $\text{IFN}\gamma + \text{TNF}\alpha$ ), M(IL-4) and M(IL-10) were obtained, followed by treatment with compounds.

**Results:** Treatment with Rapamycin resulted in a pro-inflammatory profile of the explants based on high expression of *IL1B*, *IL6*, and *TNFA* and low expression of *IL1RA*, *CCL18*, *CD206*, *CD163*. In M(IL-4) and M(IL-10), Rapamycin decreased CCL18 protein, without affecting IL-6. Dexamethasone enhanced the behavior of M(IL-4) and M(IL-10) by increasing CCL18 production while decreasing IL-6 in M( $\text{IFN}\gamma + \text{TNF}\alpha$ ).

**Discussion:** Rapamycin and Dexamethasone can modulate the behavior of macrophages *in situ*. Pro-inflammatory effects of Rapamycin and anti-inflammatory effects of Dexamethasone can be partially explained by the effects seen on isolated macrophages. The modulatory capacity of the compounds seems to depend on macrophage subtypes. These data suggest that macrophage modulation can be applied to guide inflammation in (joint) tissues and thereby improve regeneration of engineered joint tissues.

#### **Burn Injury Induces Inflammation and Cell Death in Adipose Derived Stem Cells (ASCs)**

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The use of autologous adipose derived stem cells (ASCs) as a cell based therapy to generate skin substitutes in burn patients has been suggested, however few studies have addressed that the ASCs may be altered by severe burn injury. Objective: Determine whether severe burn injury leads to alterations in the ASCs. Setting: Murine animals sustaining a scald burn over 60% of the total body surface area (TBSA) were euthanized at 24 hours, 48 hours, one and two weeks following burn injury and ASCs were isolated. Adipocytes, the stromal vascular fraction (SVF), and enriched ASCs (isolated after culturing the SVF for 48 hours) at different point were compared to isolates from non-burned animals. CD marker expression,

comet assay, and pro and anti-inflammatory pathway modulations were studied.

**Results:** Adipocytes and the SVF from burn rats show elevated expression of genes involved in inflammation. Following 48 hours in culture, there was decreased expression of mRNA for inflammatory genes for all post-burn isolation time points. Levels of mRNA for IL-1b, IL-6, MCP-1 and TNF-alpha were significantly higher in the SVF compared to the enriched ASCs ( $p < 0.05$ ). DNA damage was significantly higher in the SVF of 24 and 48 hours post-burn compared to controls ( $p < 0.05$ ).

**Conclusion:** SVF from 24 and 48 hours following burn injury are in an inflamed state. Harvesting at later post-burn time points may yield cells with less burn-induced damage or inflammation. Culturing the cells for 48 hours may decrease the activation of inflammatory state in ASCs isolated from burn patients.

#### **Mathematical Model of Macrophage Polarization in Wound Healing**

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Macrophages play an essential role in regulating the wound healing process. Understanding and modulating the macrophage dynamics could potentially aid in the design of new treatments for dysfunctional wound healing. Therefore, a mathematical model was developed to describe the dynamics of pro-inflammatory M1 and anti-inflammatory M2 macrophages in wound healing. The system of equations that describes the macrophage populations takes into consideration the polarization, transition, proliferation, and death rates of each macrophage phenotype. The parameters and rate constants were calculated based on experiments in which primary human monocyte-derived macrophages were cultured over 11 days *in vitro* under conditions that polarized them to the M1, M2, or sequential M1-to-M2 phenotypes. Macrophages were characterized over time for cell viability and the expression of CCR7 (M1 marker) and CD206 (M2 marker) via flow cytometry. The model accurately described the macrophage behavior in normal wound healing over time, which typically starts with an inflammatory response or a peak in the M1 macrophages, followed by the pro-healing response or the accumulation of the M2 macrophages. This model also described chronic wounds when the M1-to-M2 transition term was deleted, which has previously been posited to play a central role in the impairment of healing. Finally, the model accurately predicted the effects of various wound healing treatments, including the administration of anti-inflammatory drugs and infusion of M2 macrophages in chronic wounds. Thus, this model is useful for increasing understanding of the dynamic role of macrophage phenotype in wound healing.

## Poster Abstracts

### Session: Poster Session 1

**Date and Time: Tuesday, September 8, 2015,  
6:00 PM - 8:00 PM**

#### **Influence of Hematopoietic Components of the Bone Marrow on *In Vitro* Bone Formation Assays**

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<sup>1</sup>Translational Center Würzburg 'Regenerative Therapies for Oncology and Musculoskeletal Diseases', Fraunhofer Institute Interfacial Engineering and Biotechnology (IGB), Würzburg, GERMANY, <sup>2</sup>Department of Tissue Engineering and Regenerative Medicine, University Hospital of Würzburg, Würzburg, GERMANY.

Mesenchymal Stem Cells (MSCs) are characterized by self-renewal potential and multi-lineage differentiation capability. Hematopoietic Stem Cells (HSCs) are progenitor cells able to originate all the hematopoietic lineages in the bone marrow. Though a specific niche for MSCs has not been yet identified, several reports underline MSCs-HSCs cross-talk's importance in maintaining HSCs' stemness and differentiation capability, which may in turn result in MSCs' enhanced viability and delayed ageing. In this work MSCs-HSCs co-culturing's influence on collagenous microbeads and in the BioVasc-TERM™ bone formation models was evaluated.

Human HSCs were injected into the microbeads, later seeded with human MSCs in spinning bioreactors, and cultured for 2 weeks. For the BioVasc-TERM™ model a portion of porcine ileum was decellularized and the vascular system was recolonized with dermal-derived microvascular endothelial cells. After two weeks of vascular system perfusion, the lumen was filled with cell-colonized beads and hydroxyapatite-coated collagenous beads embedded in a collagen hydrogel. The constructs were retrieved for analysis after 2 weeks of culture and assayed for histochemical staining, DNA content and RealTime-PCR analysis of ageing and differentiation markers compared to MSCs monocultures.

DNA content analysis of MSCs-HSCs co-cultures in dynamic conditions revealed improved overall proliferation of the co-cultured cells, as confirmed by immunofluorescent staining.

These results underline the importance of the MSCs-HSCs cross-talk, suggesting a role for MSCs-HSCs interaction during bone formation and maintenance.

#### **A Porcine Model for Studying Stem Cell Therapy in Wound Healing**

**I. B. James, D. Bourne, N. Kostereva, J. Lei, P. Chang, K. G. Marra, J. P. Rubin;**

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**Background:** Adipose-derived stem cells (ASCs) are multipotent stem cells capable of replacing multiple tissue types and are ideal candidates for regenerative cell-based therapy. However, large animal models and dose-related effects have not been well studied. We sought to establish a reliable porcine model for wound healing and to optimize ASC dosing.

**Methods:** Circular, full thickness excisional wounds 4 cm in diameter (n=40) were created on the backs of 6 month old female Yorkshire pigs. Passage 1 porcine ASCs were fluorescently labeled with PKH26 and injected intraoperatively into the wound bed and around the wound perimeter at high ( $3.0 \times 10^{-6}$  cells/cm<sup>-2</sup>; n=8), medium ( $1.0 \times 10^{-6}$  cells/cm<sup>-2</sup>; n=8), and low ( $0.3 \times 10^{-6}$  cells/cm<sup>-2</sup>; n=8) doses. Controls received either saline (n=8) or no treatment (n=8). Dressings were changed twice weekly. Epithelialization and

contraction were quantified. Animals were sacrificed at 1 and 2 weeks postoperative, and wounds were collected for ASC tracking.

**Results:** PKH26-labeled ASCs were abundant in treated wounds and fully integrated into the native tissue architecture by 1 week in a dose-dependent fashion. Full epithelialization was achieved in most wounds by day 14. Wounds receiving high dose ASCs trended toward greater percent contraction at days 10 (48.0% vs 37.9%) and 14 (65.5% vs 48.9%) postoperative compared to control.

**Conclusions:** Our porcine model is highly promising and well suited to assess stem cell-based therapies. ASC doses as high as  $3.0 \times 10^{-6}$  cells/cm<sup>-2</sup> are well-tolerated when injected into incisional wounds and integrate into native tissue architecture by 1 week. At high doses, they may also mediate enhanced wound contraction.

#### **Radical Copolymerization of AEMA and Chitosan for Gene Delivery**

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Gene therapy can provide the best way for the treatment of incurable diseases such as inherited disorders and cancers without any side effects in the body. Owing to the fatal drawbacks of a viral vector, a non-viral vector is promising renewed interest in carrying genes into the target tissue. The modification of a polymer as a non-viral vector is a very important factor for using biomaterials to carry not only genes but also drugs or other bioactive materials into the body. In particular, a gene delivery system using a polycationic polymer shows a low gene expression efficiency, even though a non-viral vector has safe properties in the body. To overcome this problem, several researches have documented the advantages of a specific tissue target ligand modified delivery system. In the present study, 2-aminoethyl methacrylate (AEMA) was grafted onto low molecular weight chitosan (LMWC) backbones (AEMA-LMWC) through radical polymerization using a 60 Co gamma-source irradiation in order to increase the cationic linker, which is able to enhance the binding capacity with anionic nucleic acids. The conjugation of AEMA and LMWC not only reduced the cytotoxicity but also enhanced the transfection efficiency. In addition, we could conduct a simply synthesis using gamma-radiation. The characterization and gene delivery capability of an AEMA-LMWC copolymer was evaluated through NMR, gel electrophoresis, DLS, transfection, and FACS. Based on these results, we confirmed that AEMA and LMWC were successfully grafted through gamma-radiation and are outstanding gene delivery carriers.

#### **Extracellular Matrix Derived Cryptic Peptides Promote an Alternatively Activated, Anti-Inflammatory Macrophage Phenotype**

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Acellular biologic scaffolds composed of mammalian extracellular matrix (ECM) have been used in both preclinical and clinical regenerative medicine applications for the reconstruction of injured or missing tissues including skeletal muscle (1-3). When placed at sites of volumetric muscle loss (VML) these bioscaffolds can facilitate site-appropriate constructive and functional tissue remodeling (4, 5).

The mechanisms by which ECM bioscaffolds promote functional tissue remodeling include the creation and release of bioactive effector molecules, including oligopeptide sequences, during the process of host mediated scaffold degradation. These ECM oligopeptides influence cell behavior within the local microenvironment, including the behavior of macrophages. Specifically, ECM bioscaffolds derived from small intestine and urinary bladder tissue have been shown to promote an anti-inflammatory, alternatively activated (M2) macrophage phenotype (6, 7). Such matricryptic peptides have also been shown to attract endogenous stem/progenitor cells via a direct chemoattractant mechanism (8) and via macrophage mediated paracrine mechanisms (9).

The present study investigated the ability of ECM cryptic peptides, derived from a variety of tissue-types, to directly promote an anti-inflammatory (M2) macrophage phenotype. ECM harvested from small intestine, urinary bladder, skeletal muscle, brain, esophagus, colon, and dermis all induced an alternative, anti-inflammatory profile in mouse primary bone marrow derived macrophages. The study also shows the ability of these ECM derived degradation products to promote an anti-inflammatory secretome associated with both progenitor and adult skeletal muscle cells.

#### **Stromal Vascular Fraction Cell Sheets Angiogenic Potential for Tissue Engineering and Regenerative Medicine Applications**

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One of the biggest concerns in the Tissue Engineering field is the correct vascularization of engineered constructs. Strategies involving the use of endothelial cells are promising but adequate cell sourcing and neo-vessels stability are enduring challenges. In this work, we propose the hypoxic pre-conditioning of the stromal vascular fraction (SVF) of human adipose tissue to obtain highly angiogenic cell sheets (CS). For that, SVF was isolated after enzymatic dissociation of adipose tissue and cultured until CS formation in normoxic (pO<sub>2</sub> = 21%) and hypoxic (pO<sub>2</sub> = 5%) conditions for 5 and 8 days, in basal medium. Immunocytochemistry against CD31 and CD146 revealed the presence of highly branched capillary-like structures, which were far more complex for hypoxia. ELISA quantification showed increased VEGF and TIMP-1 secretion in hypoxia for 8 days of culture. In a Matrigel assay, the formation of capillary-like structures by endothelial cells was more prominent when cultured in conditioned mediums recovered from the cultures in hypoxia. The same conditioned mediums increased the migration of adipose stromal cells in a scratch assay, when compared with the medium from normoxia. Histological analysis after implantation of 8 days normoxic- and hypoxic-conditioned SVF CS in a hindlimb ischemia murine model showed improved formation of neo-blood vessels. Furthermore, Laser Doppler results demonstrated that the blood perfusion of the injured limb after 30 days was enhanced for the hypoxic CS group.

Overall, these results suggest that SVF CS created under hypoxia can be used as functional vascularization units for tissue engineering and regenerative medicine.

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#### **Studying Cardiac Repair after Myocardial Infarction by Immunomodulation of Cardiac Macrophages using miRNA Delivery**

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The wound healing process which follows myocardial infarction (MI), is tightly controlled by different macrophages sub-populations: inflammatory macrophages (M1 type) and reparatory macrophages (M2 type). A delay which often occurs in the M1 to M2 macrophages transition, harms the healing process and leads, amongst others, to heart failure. Nowadays, there is no effective treatment for heart failure. Our hypothesis is that modulation of cardiac macrophages from M1 to M2, at the proper time-point after MI, using miRNA delivery, may attenuate infarct expansion and left-ventricular remodeling.

An appropriate delivery system must be able to protect the miRNA from RNases, get it to the site of interest and to not arouse unwanted immune response. Herein we offer a novel RNAi delivery system; nano-particles (NPs) (~140 nm in diameter, ~-11 mV zeta potential), based on the anionic, sulfated, Hyaluronan polymer. When injected IP into mice, after sterile infection stimulation, almost 40% of peritoneal macrophages showed NPs uptake, within one hour. NPs IV injection 3 days post MI, resulted in NPs at the infarct area, in CD11b-positive cells. While using a laser capture microdissection (LCM) microscope, our preliminary results shows that we are able to isolate macrophage-enriched area from the infarct zone, thus able to quantify the RNAi delivery to post-MI cardiac macrophages.

This multidisciplinary approach presented in this research combines cardiac macrophage biology, state-of-the-art LCM technology and engineering of miRNA delivery system. Ultimately we foresee that this approach will lead to a novel therapeutic modality for immunomodulation and subsequent effective cardiac repair after acute MI.

#### **Macromolecular Crowding Drives Cartilage Formation in Chondrogenic Stem Cell Microtissues**

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Molecular crowding is essential for complex formation, which regulates gene transcription, protein production and tissue formation. We hypothesized we could enhance cellular molecular crowding via the contractile forces present in 3D microenvironments. To this end, we formed condensating microtissues containing 100 human periosteal stem cells in high throughput and cultured them under growth factor free conditions. Over a seven day culture period, the microtissues decreased significantly in volume due to cell shrinking. This change proved irreversible as dissociation and subsequent subculture maintained the phenotype. Blocking Rho Kinase I, a key regulator of the cytoskeleton, significantly decreased both cell and microtissue shrinking. Fluorescent confocal microscopy confirmed the cell shrinkage by observing a significant loss of cytoplasmic volume. Importantly, this drastically elevated the cell's molecular crowding by raising its protein concentration 25 fold within seven days. Indeed, transmission electron microscopy revealed that microtissue formation induced swelling of rough endoplasmic reticulum as well as darkening and elongation of the mitochondria, all indicators of increased molecular crowding. Moreover, a deformable cell-based computational model suggests that the cell shrinkage was induced by the biomechanical forces generated by the cells and was counterbalanced by the increasing cellular viscosity caused by molecular crowding. *In vitro* this process decreased stemness markers, increased nuclear translocation of the chondrogenic transcription factor SOX9 and induced a cartilage gene expression fingerprint. Impressively, microaggregation strongly improved *in vivo* cartilage formation and even resulted in significant cartilage deposition one week post implantation.

#### **Cryopreservation of Primed Microencapsulated Bone Marrow Derived Stem Cells using Electrohydrodynamic Spraying for on Demand Minimally Invasive Tissue Regeneration of the Intervertebral Disc**

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Microencapsulation of BMSCs is an attractive approach for intervertebral disc (IVD) regeneration overcoming challenges associated with mode of delivery and cell leakage and providing protection from the harsh microenvironment of the IVD. In addition, cryopreservation of microencapsulated BMSCs differentiated *in vitro* towards the desired phenotype, may also provide off-the-shelf availability for therapeutic strategies. Therefore, this study aimed to investigate the effects of cryopreservation and priming of microencapsulated BMSCs fabricated using an electrohydrodynamic (EHD) spraying technique. Briefly, BMSCs were combined with 1% alginate at two seeding densities:  $10 \times 10^6$  and  $20 \times 10^6$  cells/ml. Alginate/cell suspension was electrosprayed and ionically crosslinked ( $10^2$  mM  $\text{CaCl}_2$ ) producing microspheres ( $240 \pm 22.04 \mu\text{m}$ ). Microspheres were primed for 14 days whereupon they were either cultured for a further 21 days or were cryopreserved in freezing medium (90% FBS, 10% DMSO) and later thawed and cultured for 21 days with non-primed microspheres serving as controls.

Live/Dead staining revealed that microencapsulated BMSCs remained viable after 21 days for both seeding densities investigated. Cryopreservation effects showed that microencapsulation within alginate protects BMSCs from the freezing process (percentage viability = 83.78%). Furthermore, no obvious difference in cell survival was found between pre and post-cryopreserved microspheres. Priming resulted in differentiation towards a nucleus pulposus-like phenotype with increase sGAG and collagen accumulation. These findings illustrate the potential of EHD spraying as a versatile and effective method to microencapsulate cells for minimally invasive tissue repair. Furthermore, cryopreservation of primed-BMSC microspheres may offer the potential for predesigned deliverability mechanisms through cryobanking for on-demand clinical applications and therapeutic strategies.

### 3D Co-culture of Monocytes/macrophages and Mesenchymal Stem Cells Derived from Human Induced Pluripotent Stem Cells for Bone Tissue Engineering

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hiPSCs are a promising cell source for engineering functional bone substitutes due to their multilineage differentiation capacity constituting the bone (osteoblasts (OB) and osteoclasts (OC)). 3D bone tissues must be captured the coupling of bone formation and resorption in an environment of native bone. We investigated the biomaterial-dependent commitment of mesenchymal cells (MSCs) and monocytes/macrophages (M $\phi$ ) derived from hiPSCs toward the OB and OC. We developed the 3D co-culture model for iPSCs-derived MSCs and M $\phi$  in scaffolds composed of PLGA/PLLA with hydroxyapatite (HA) (0, 1, or 5%). MSCs and M $\phi$  were co-cultured or MSCs were mono-cultured in HA-0, 1, or 5 composite for 4 wks *in vitro* and for 8 wks subcutaneous implantation. Mono-culture group in HA-5 led to more bone formation (Col I 2 fold; ALP 2.5 fold; OCN 2 fold) than those in HA-0. Co-culture group in HA-5 showed decreased OB activity (Runx2 and Col I 0.7 fold; ALP 0.5 fold) compared to mono-culture group in HA-5 *in vitro*. Also, co-culture group in HA-5 resulted in increased in OC activity (CTNR1.5 fold; NFATC1 3 fold; CTPK 2 fold) compared to those in HA-0 *in vitro*. Our data demonstrated an increase in HA led to diminished osteogenesis in favor of osteoclastogenesis and suggest intermediate HA concentration (HA-1) showed a balanced ratio of OB to OC differentiation. Our 3D co-culture systems of MSCs and M $\phi$  derived from hiPSCs offer new therapeutic approaches to treat bone defects by engineering personalized and functional bone substitutes with balanced bone matrix deposition and resorption.

### Clay Gels Reduce the Effective dose of Bone Morphogenic Protein

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**Introduction:** Bone Morphogenic Protein (BMP) has been used to stimulate fracture healing and spinal arthrodesis. However, difficulty in maintaining BMP activity at the target site has necessitated large doses with associated adverse effects. Clay (Laponite) hydrogels can bind growth factors for localised efficacy. Our objective was to investigate if localisation of BMP by clay gels would reduce the dose required to mediate bone formation.

**Methods:** Localisation of BMP-2 by clay gels was assessed *in vitro* via staining for BMP-2 induced alkaline phosphatase activity in C2C12 cells. *In vivo*, 500 ng and 40 ng BMP-2 mixed in Laponite or an alginate control were applied to a collagen sponge before subcutaneous implantation in a mouse model. New bone formation was assessed via micro Computed Tomography and histological analysis.

**Results:** Enhanced BMP-2 induced alkaline phosphatase activity ( $p < 0.0001$ ) was localised to cells seeded upon clay gels in a BMP-2 dose dependent manner. *In vivo*, only Laponite, not alginate, sustained ectopic bone formation at 40 ng BMP. Significantly greater bone volume per ng BMP was achieved with 40 ng doses of BMP-2 in Laponite, compared to alginate and Laponite gels with 500 ng BMP ( $p < 0.0001$ ).

**Conclusion:** Laponite is able localise BMP-2 and reduce, by several orders, the effective dose of BMP-2 required to mediate ectopic bone formation compared to current gold standard methods of BMP-2 delivery. Clinical translation of this finding offers, potentially, great significance to orthopaedic surgery.

**Acknowledgments:** Funded by grants from EU (FP7)Biodesign, Rosetrees Trust, BBSRC and EPSRC.

### Enhancement of Wound Healing with Increased Angiogenesis in a Diabetic Rabbit Ulcer Model by Topical Application of CD362+Human Mesenchymal Stem Cells (Cyndacel-M) Seeded in Excellagen™ Scaffold

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Impaired wound healing is a common complication in diabetic patients. Mesenchymal stem cells (MSCs) are known to promote angiogenesis with improved wound healing. Orbsen Therapeutics has identified a novel antibody (CD362+) which can be used to prospectively FACS-isolate CD362+CD45- MSC from bone marrow with enhanced MSC/MNC purity. Excellagen™ matrix was used to seed the cells as biomaterials are well reported to enhance viability and therapeutic efficacy of cells.

In this study, 1 million of CD362+, CD362- and plastic adherent human MSCs were seeded in Excellagen™ and applied to cutaneous ear wounds in an alloxan-induced diabetic rabbits for a 1 week period. The wounds treated with an Excellagen-CD362+ cell demonstrated increased percentage wound closure with more prominent neovasculature. The wound sections were immunohistochemically stained with CD31 and GSL-B4 lectins to study neovasculature. In stereological analysis, significantly increased surface density, length density and reduced radial diffusion distance was observed in the Excellagen-CD362+ cell treated groups in comparison to control. A subsequent study compared the beneficial effects of a combination treatment (IV delivery of  $2 \times 10^6$  cells/kg plus topical treatment) to topical treatment alone. A slight increase was observed in percentage wound healing in combination versus topical treated animals but this difference was not significant. Preclinical biodistribution studies were also done for tracking the distribution of hMSCs after administration. Hence, with improved wound healing potential and augmenting angiogenesis, topical treatment with these specifically selected CD362+MSCs seeded in an Excellagen™ matrix may lead to a new therapeutic product to treat non-healing diabetic foot ulcers.

### Wound Healing Revealed by a Novel Automated Indentation Technique

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**Problem:** Mechanical characterization of wound healing in skin samples mostly relies on uniaxial tensile rupture tests providing local information along the wound and are disruptive for samples.

**Objectives:** In this study, we wanted to test the ability of a novel automated indentation technique to non-destructively characterize mechanical properties of the entire wound and its integration with the surrounding skin.

**Methodology:** Wounded pig skin samples were placed skin surface up on a platform of a multiaxial mechanical tester (Mach-1v500css, Biomomentum Inc., Canada). Following top-view photodocumentation, a position grid (> 130 positions) was superimposed over the image. At each position, the tester was programmed to precisely measure skin thickness and to perform an indentation ramp of 1.5 mm at 200  $\mu\text{m/s}$  with a spherical indenter (6.35 mm diameter). Subsequently, the sample was reshaped in two adjacent dumbbell-shaped strips (perpendicularly to the wound), mounted in tension grips and tensile rupture tests were performed at 2 mm/s.

**Results:** High-resolution mapping of maximum load and thickness were generated (about 30 s per position). These mappings revealed significant spatial variation of the mechanical properties and thickness over the wound region compared to the uniform properties of the intact skin observed at least 1 cm away from the incision site. Considering the load at rupture in the tension tests, a correlation could be observed with the maximum load in indentation (or thickness) measured at the rupture site.

**Significance:** These results indicate that this indentation technique can provide a novel assessment of mechanical properties revealing the 2-dimensional distribution over the wound and its surrounding areas.

### Donor Factors and Stem Cell Type Affect the Potential and Multipotency of Human Adipose Tissue-Derived Stem Cells

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**Objectives:** Human adipose tissue-derived mesenchymal stem cells (AT-MSCs) have different characteristics depending on donor characteristics. AT-MSCs also differs from MSCs of other origins. This study investigated the differentiation and proliferation characteristics depending on age, body mass index (BMI), gender, and origin of MSC.

**Methodology:** AT-MSCs from 66 human donors were sorted according to donor age (10–19, 20–29, 30–39, 40–49, 50–59, 60–69, 70 years and older), BMI (under 25  $\text{kg/m}^2$ , 25–30  $\text{kg/m}^2$ , and over 30  $\text{kg/m}^2$ ), and gender. AT-MSCs were also compared with bone marrow MSCs and chorionic tissue-derived MSCs. MSC yield, growth rate, colony-forming units, differentiation potency (ELISA analysis after Oil Red O staining for adipogenic, Alizarin Red S staining for osteogenic, and Alcian Blue staining for chondrogenic differentiation), and surface antigens (CD90, 13, HLA-DR, CD105, CD34, CD73, CD45, CD146) were compared.

**Results:** AT-MSC proliferation was greater in cells isolated from donors aged less than 30 years compared to cells from donors over 50 years old. Adipogenic differentiation was more strongly induced in cells isolated from donors less than 30 years compared to other age groups. BMI above 30 was associated with enhanced adipogenic differentiation compared to cells isolated from individuals with a BMI lower than 25. Cells from donors with higher BMI had better osteogenic differentiation potency. Bone marrow MSCs showed stronger osteogenic and adipogenic lineage dif-

ferentiation, while AT-MSCs predominantly differentiated into the chondrogenic lineage.

**Conclusions:** The type of regeneration required, and variations among donors and type should be carefully contemplated when selecting MSCs to apply in tissue engineering or cell therapy.

### A Novel Approach to Study The Immunoregulatory Effect of Exogenous Factors on Macrophages

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Macrophages, acting as both immune cells and osteoclast precursors, play an important role in bone homeostasis. The traditional approaches to study macrophages are based on the stimulation of pathogens which can be rarely found under homeostatic conditions. This study aims to create a novel approach to investigate the immunoregulatory effect of exogenous factors on macrophages under both physiological and pathological conditions. A murine macrophage cell line RAW264.7 was cultured in normal growth medium supplemented with different concentrations of the exogenous vascular endothelial growth factor (VEGF). Our results showed that the expressions of inflammatory cytokines in RAW264.7 cells enhanced consistently with the increasing concentration of VEGF with or without lipopolysaccharide (LPS) preconditioning. The same results were found when the VEGF was replaced with the conditioned medium from the osteogenically differentiated bone marrow-derived mesenchymal stromal cells (BMSCs) without the presence of LPS. The present study demonstrated that the immunoregulatory effect of exogenous factors on macrophages could be observed without pathogenic stimuli. The established method could better reflect the *in vivo* bone remodelling process when no inflammation is involved. It is a feasible approach to study the interaction between the target protein and the immune cells under non-inflammatory conditions.

### Use of Skin Substitutes in a Burn Animal Model

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Available treatments in skin regeneration are insufficient to promote healing. This study has aimed to produce a cutaneous substitute joining mesenchymal stem cells (MSCs), keratinocytes and a PDLA biomaterial constructed by the electrospinning technique for use in nude mice. Five groups were tested: (1) PDLA without cells; (2) PDLA/Lam, hydrolyzed scaffold with laminin binding, without cells; (3) PDLA with cells; (4) PDLA/Lam with cells (n=6/group) and (5) animals injured without scaffolds (control), in which gauze was used (n=4). All the animals had 1  $\text{cm}^2$  cutaneous defect performed on their backs, removing all the skin. The scaffolds were implanted in the mice with imitation burn skin defects for up to 9 days. Photographs were taken on the days of surgery and euthanasia. Part of the skin defect was used for histology analysis and another part for gene expression evaluation. After 9 days, the cutaneous defect size for group 1 was  $0.439 \pm 0.008 \text{ cm}^2$  and  $0.315 \pm 0.003 \text{ cm}^2$  for group 2. The scaffolds in which the cells were seeded presented similar results, with a defect size for group 3 and 4 of  $0.411 \pm 0.017 \text{ cm}^2$  and  $0.342 \pm 0.021 \text{ cm}^2$ , respectively. The control group showed a  $0.319 \pm 0.003 \text{ cm}^2$  defect size. Group 2 presented the best appearance in the lesion, with the softest wound. The histological and gene expression analysis are currently being performed. However, it was clear that the scaffolds, mainly PDLA/Lam, improved the quality of the healing process of the wound in this animal model.

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### Initiating Human Articular Chondrocyte Re-differentiation in a 3D System Post 2D Expansion

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Cartilage damage affects a large population via acute injury and diseases. Since native cartilage cannot self-renew, cartilage tissue engineering has gained traction as a potential treatment. However, a limiting factor is that the primary cell type in cartilage, articular chondrocytes, tend to de-differentiate when grown on 2D surface for *in vitro* 2 expansion. Hence, in this study, an enzymatically degradable polyethylene glycol (PEG) hydrogel with and without the integrin binding sequence Arg-Gly-Asp (RGD) was tested as a potential 3D scaffold, in combination with chondrogenic medium, as an alternative *in vitro* system to recapitulate the cells' native environment.

Human articular chondrocytes (hACs) were expanded in 2D cell culture and then embedded within PEG hydrogels and evaluated for viability, proliferation, glycosaminoglycan (GAG) production and chondrogenic gene expression. A slight drop in viability of the encapsulated hACs was observed after 4 weeks of *in vitro* culture, while the DNA content showed very little change. Interestingly, however, the GAG production per cell displayed a marked increase after 4 weeks, with the hACs in the scaffold without RGD displaying the highest increase. All constructs showed a significant increase in Col2 a gene expression. Furthermore, the hACs in constructs without RGD demonstrated a significant increase of Aggrecan and SOX-9, indicating the hACs were recognizing the ideal 3D conditions and regaining their chondrogenic potential. The significant increase of GAG production and chondrogenic markers after 4 weeks of 3D culture in PEG hydrogels with chondrogenic medium suggests this approach may be suitable for re-differentiating hACs.

### Biphasic Calcium Phosphate Encapsulating Homogenous Microsphere-Based Scaffolds For Bone Regeneration

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Calcium phosphates such as hydroxyapatite (HAp) and tricalcium phosphate (TCP) are widely used in scaffolds for bone tissue regeneration due to their compositional similarity to the bone mineral, bioactivity, and osteoconductivity. Moreover, incorporation of HAp and TCP alone in microsphere-based scaffolds can modulate their osteoinductivity [1]. Therefore, we hypothesized that incorporation of biphasic mixture of HAp and TCP in microsphere-based scaffolds with a 3:7 (HAp:TCP) ratio would provide osteoinductive cues to rat bone marrow stromal cells (rBMSCs) and also render the scaffold osteoconductive. We fabricated poly(D,L-lactic-co-glycolic acid) (PLGA) microsphere-based scaffolds encapsulating HAp and TCP mixtures in two different ratios (3:7 and 1:1) with same net ceramic content to evaluate how incorporation of these ceramic mixtures would affect the osteogenesis of rBMSCs, with analyses including morphological characterization, mechanical integrity, biochemical activity, gene expression, and histology. The PLGA alone microspheres were spherical and had a smooth surface while microspheres in both the ceramic mixture groups had minute pores on their surface with deflated soccer ball shape. Additionally, the scaffolds in the ceramic mixture groups had statistically significantly higher porosities than the PLGA only group ( $p < 0.05$ ). Thus, it can be concluded that encapsulation of HAp and TCP altered the morphology of the microspheres and overall porosities of the scaffold. Biochemical, gene expression and histological analyses are in progress to further test our hypothesis, and will be included in the presentation.

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### Application of Pre-vascular High Cell Density Constructs for Tracheal Tissue Engineering

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Tracheal defects greater than 50% in length cannot be easily treated. While tissue engineering holds promise to address this problem, a key limiting factor is the inability to vascularize larger tissues. Using a high-density cell co-culture system, this work aimed to generate a pre-vascular network that may facilitate rapid perfusion of an engineered construct upon implantation into a defect site. Such pre-vascular, ring-shaped constructs were then fused with cartilage rings to partially mimic native trachea for potential replacement to treat stenosis or diseased tissue.

Co-culture spheroid aggregates were self-assembled from mesenchymal stem cells (MSCs) and human umbilical vein endothelial cells (HUVECs). Vascular cords formed as early as 2 weeks and lumens were observed in some sections. To form tracheal tubes, vascular rings (composed of MSCs and HUVECs) and cartilage rings (composed of MSCs and TGF- $\beta$ 1-releasing gelatin microspheres) were formed in agarose wells. Tissue rings were stacked on glass tubes and cultured in a 1:1 mixture of vasculogenic and chondrogenic media to fuse into tracheal tubes. Histology revealed HUVEC organization into elongated vascular cords in vascular portions of the tubes, while significant production of glycosaminoglycan (GAG), a major component of cartilage extracellular matrix, was evident within cartilage portions.

This high-density cell co-culture system allows for the formation of pre-vascular structures. When fused with cartilage rings, vascular rings maintain their ability to form these structures, while the cartilage maintains its ability to produce GAG. This pre-vascular engineered trachea is a promising step towards a viable tracheal tissue replacement.

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### High-Throughput Microfluidic Screening of Human Mesenchymal Stem Cell Proliferative Responses to FGF-2 and Heparan Sulfates

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Human mesenchymal stem cells (hMSCs) are currently pursued as a cell therapy for both regenerative and immunosuppressive applications. Cost-effective scale-up of quality hMSCs remains a key challenge prior to clinical deployment. FGF-2 is a key mitogen for hMSC growth, and can be supplemented exogenously in addition to autocrine production by hMSCs themselves. A critical component for FGF-2 and signalling through its receptor FGFR1 is cell surface heparan sulfate (HS), being involved both in associating ligand with receptor, and in facilitating export of autocrine FGF-2.

In this work, we utilise a HS fraction affinity-selected for FGF-2-binding capacity (HS8 + ve), and deliver it to bone marrow-derived hMSCs in combination with exogenous FGF-2, whilst tracking growth responses. To gain further insight into autocrine effects under different FGF-2/HS8 + ve stimulation regimes, we utilise continuous-flow, high-throughput microfluidic cell culture arrays (microbioreactor arrays). This platform streamlines high-throughput combinatorial analysis of the various treatments, exploiting image cytometry to offer single-cell resolution data. Microfluidic screening revealed HS8 + ve cooperates with both exogenous and endogenous FGF-2 to increase

Ki67+proliferating hMSCs in early phases of a passage of culture, leading to dramatic and enhanced expansion of hMSC numbers throughout a 6-day culture cycle. We present evidence for HS8+ve altering the autocrine/paracrine responses of the cells under FGF-2 costimulation, likely by increasing FGF-2 availability in the bulk culture medium. Controlling and exploiting the bioactivity of exogenous and endogenous FGF-2 will be beneficial in optimising hMSC expansion strategies for clinical scale-up.

#### The Landmark of Trajectory of ES to EC; Differentiation of hESC to Vascular Progenitor Cells in a Synthetic Matrix

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Recently, numerous efforts have been performed in the field of vascular network formation and angiogenesis *in vitro*. To enable the identification, characterization and network formation analysis of human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC)-derived vascular precursor cells, we established an efficient protocol for mesodermal differentiation, followed by formation of functional vascular cells and investigated synthetic collagen hydrogels and demonstrated that enable human endothelial colony-forming cells (ECFCs) to form efficient vascular networks. hESC and hiPSCs were maintained in (EGM2) medium Human pluripotent cells were differentiated in a 2-step protocol, carried out by sequential treatment with (Chir) for mesodermal induction, and a combination of BMP4, and VEGF in RPMI medium for vascular cell specification. Mesodermal and vascular-inductive factors were eliminated on day 5 of differentiation and were replaced with vascular specification medium (EGM2) supplemented with VEGF. After 6 days these cells were encapsulated in collagen I hydrogels. Under our differentiation protocol, differentiated cells were highly enriched (>70%) for PDGFR $\beta$  and 30% for VEGFR2/KDR. Function of these cells as vascular progenitor cells was verified by tube formation and LDL-uptake test. Histological examination of hydrogel section after 1 week reveals functional microvasculature containing human CD31+ cells and PDGF- $\beta$  cells. We show that the vascular network formation can be accurately regulated in a synthetic matrix, resulting in a functional microvasculature useful for the study of 3-dimensional vascular biology and toward a range of vascular disorders and approaches in tissue regeneration.

#### Additive Manufacturing of Dexamethasone-Releasing Scaffolds with Enhanced Osteoinductivity

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The success of additive manufacturing's widespread integration into tissue engineering and regenerative medicine (TERM) strategies greatly relies on the development of novel 3D printable materials with advanced properties. An example of such properties is the incorporation of chemical/biochemical agents to be delivered in a precise and controlled manner to target cells involved in the regeneration process. In this work we have developed a novel 3D printable material with tunable bioerosion rate and dexamethasone release profile for bone TERM applications. The developed material consisted of a blend of polycaprolactone (PCL) and poloxamine (Tetronic<sup>®</sup>) and was processed into a ready-to-use filament form by means of a simplified melt-based methodology, therefore eliminating the utilization of solvents. The conversion into filament form also enables off-the-shelf utilization of the material in fused deposition modelling which is the most common and accessible 3D printing technology in the market. 3D scaffolds composed of various material

blend ratio formulations were additively manufactured and analyzed revealing blend ratio-specific degradation rates and dexamethasone release profiles. Furthermore, *in vitro* culture studies revealed a similar blend ratio-specific trend concerning the osteoinductive activity of the manufactured scaffolds when these were seeded and cultured with human mesenchymal stem cells. The developed material therefore enables to specifically address different regenerative requirements found in specific tissue defects. The versatility of the developed material/strategy is further increased by the ability of additive manufacturing to accurately fabricate implants matching any given defect size and geometry.

#### Inhibition of Chronic Prostate Inflammation by Hyaluronic Acid through an Immortalized Human Prostate Stromal Cell Line Model

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Benign prostatic hyperplasia (BPH) is the most common urologic disease among elderly men and the inflammation in BPH enhances the up-regulation of pro-inflammatory cytokines and the infiltration of immune cells. Considering the importance of the stromal elements in BPH, we attempted to establish an immortalized human prostate stromal cell line, designated as ihPSC, by transduction of HPV 16E6/E7. The ihPSC possessed a significantly higher proliferation rate, shorter doubling time and anti-senescence ability compared to primary hPSC. The prostate-specific markers and proteins including cytoskeleton ( $\alpha$ (SMA and vimentin) and smooth muscle (calponin), especially the androgen receptor (AR) were also examined in ihPSC, almost identical to the primary hPSC. To create an *in vitro* model featuring chronic prostatic inflammation, ihPSC was stimulated with IFN- $\gamma$  and IL-17 and then treated with the high molecular weight hyaluronic acid hylan G-F 20 as an alternative strategy for inhibiting BPH inflammation. Hylan G-F 20 could dose-dependently diminish the inflammation-induced proliferation in ihPSC. The enhanced expressions of inflammatory molecules including IL-1 $\beta$ , IL-6, IL-8, cyclooxygenase 2 (COX2), inducible nitrogen oxide synthase (iNOS), and Toll-like receptor 4 (TLR4) were all abolished by hylan G-F 20. For inflammatory signalings, the increased expression of iNOS and p65 by IFN- $\gamma$ +IL-17 in ihPSC was also effectively diminished by the treatment with hylan G-F 20. In summary, the ihPSC could provide a mechanism-based platform for investigating prostate inflammation. The hylan G-F 20 showed strong anti-inflammatory effects by decreasing inflammatory cytokines and signalings in the ihPSC, indicating its therapeutic potentials in BPH treatment in the future.

#### Modulation of Cell Patch Transfer from Thermally Expandable Hydrogels by Tuning Cell-Adhesive Properties

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Mussel inspired polydopamine coating is known to be applicable to any materials independent of surface chemistry, and easily performable under basic condition. Although most of studies using polydopamine have focused on cell adhesion, various kind of cell culture substrates including poly(N-isopropylacrylamide) have been invented considering balance of cell attachment and detachment to obtain cell layer. Harvesting and delivering cell layer with intact extracellular matrix (ECM) have been developed not only for therapeutically purpose, but also for re-constructing native-tissue like structure. However, long harvesting time and deformation of sheets are main obstacles. We previously developed cell patch transfer technique based on thermally-expandable hydrogel, and achieved rapid and efficient delivery of cell patch without damage. The hydrogels exhibited favorable cell adhesion by conjugating cell adhesive molecules during crosslinking reaction. Although we could fabricate cell patch with various type of cells by conjugating RGD

peptide and fibronectin, we could not find universal cell adhesive molecule which generally applicable to various cell types. In addition, the presentation of cell adhesive domain within the entrapped hydrogels is questionable and the presence of them weakens mechanical property by their consuming crosslinking site. Hence, we applied polydopamine-inspired coating on thermally expandable hydrogels as a universal platform for cell patch transfer. We optimized coating condition regarding cell adhesion and transfer, and observed that longer coating time induce stronger cell adhesion but lower transfer efficiency. Under optimized condition, we could fabricate various types of cell patches and deliver them to target substrates regardless of surface property.

### The Foreign Body Immune Response to Implanted Materials is Dependent on Size and Shape in Rodents and Non-human Primates

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The role of implanted biomedical devices in modern medicine is rapidly expanding but their efficacy is often compromised by host recognition and subsequent foreign body responses. For example, in the field of islet immunoisolation the fibrosis of encapsulating hydrogels remains a major barrier to clinical translation. Herein, we demonstrate that by tuning the spherical dimensions of biomaterials we can reduce their host recognition. In rodent and non-human primate animal models, spheres 1.5 mm and above in diameter significantly abrogated foreign body reactions and fibrosis when compared to smaller-sized spherical counterparts. Remarkably, these findings translated across a broad spectrum of materials including hydrogels, ceramics, metals, and plastics. To highlight the implications of these findings we studied the effect of hydrogel capsule size on the survival of encapsulated pancreatic islet cells. In a xenogeneic treatment model of transplanting encapsulated rat islets into streptozotocin (STZ)-induced diabetic C57BL/6 mice, islets prepared in 1.5 mm alginate capsules were able to restore blood glucose control in diabetic mice for up to 180 days, a greater than 5-fold longer duration than transplanted grafts encapsulated within conventionally sized 0.5 mm diameter alginate capsules. Combined these findings suggest that by simply tuning the spherical dimensions of biomedical devices we can significantly improve their *in vivo* biocompatibility.

### Hypoxia-induced Therapeutic Angiogenesis in a Rat Model of Myocardial Infarction using Injectable 3D HUVEC/cbMSC Aggregates

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Cell transplantation via direct intramuscular injection is a promising therapy for patients with myocardial infarction (MI). However, following injections, retention of transplanted cells in engrafted areas remains problematic, and can be deleterious to cell-transplantation therapy. In our previous study, a method for constructing spherically symmetric three-dimensional (3D) cell aggregates was developed by using a thermos-responsive methylcellulose (MC) hydrogel system.

With an adequate physical size and well-preserved extracellular matrices, the 3D cell aggregates can entrap and retain in the muscular interstices after intramyocardial transplantation. Additionally, cell aggregates can develop a hypoxic microenvironment in their inner cores at distances that exceed the diffusion capacity of oxygen. By switching on a series of hypoxia-modulated signal transduction mechanisms, hypoxia-inducible factors (HIFs) can cause the transcriptional activation of several pro-angiogenic genes. In this study, 3D hypoxic HUVEC/cbMSC aggregates were transplanted into the peri-infarct zones of rats with MI via direct injection. Based on our pinhole single photon emission computed tomography (SPECT) myocardial-perfusion analyses, positron emission tomography (PET) angiogenesis observations, echocardiographic global-heart-function and regional-mechanics examinations, and histological findings, the engrafted hypoxic HUVEC/cbMSC aggregates significantly enhanced regional therapeutic angiogenesis and blood flow recovery in the ischemic myocardium, thereby reducing the size of perfusion defects, preserving cardiac function and attenuating adverse ventricular remodeling. These results demonstrate that direct intramyocardial delivery of 3D HUVEC/cbMSC aggregates with internal hypoxia can be a valuable cell-based therapeutic strategy for the treatment of MI.

### Decellularized Cardiac Tissue Facilitated Vascular Differentiation of Adipose-Derived Stem Cells

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Decellularized tissues, preserving the microstructure and composition of native extracellular matrix, have shown to promote organ specific differentiation of stem cells.[1] The objective of our study is to investigate the feasibility of using decellularized porcine cardiac tissue (DPCT) to facilitate vascular differentiation of stem cells.

Porcine adipose-derived stem cells(ASCs) were isolated as described previously [2]. Decellularization was carried out using 1% sodium dodecyl sulfate (SDS) with 0.01% antibiotic at room temperature for 1 week. DPCT with various thicknesses were obtained by cryosectioning. ASCs were then seeded on the sterilized DPCT with the optimized seeding density. Cell attachment, morphology and infiltration within the DPCT were examined using confocal microscopy. After days 1 and 3 of culture, vascular marker expression of cells was examined by RT-PCR and immuno-staining.

Our results demonstrated that the thickness of DPCT have significant effects on cell response. We found the optimal thickness range to promote attachment, proliferation and infiltration of ASCs to DPCT. The expression of vascular markers has been detected from ASCs cultured on DPCT. We are working on in-depth analysis of the interaction between ASCs and DPCT. Our long-term goal is to use the obtained information to develop a strategy for vascular regeneration.

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### Adipose Derived Stem Cells Alter Fibroblast Extra Cellular Matrix Production

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Following burn injury, patients suffer from devastating consequences such as hypertrophic scar formation, which can lead to

permanent functional impairment due to excessive dermal fibrosis. Currently, to reduce pruritus and improve cosmesis, hypertrophic scars are injected with autologous cells isolated from adipose tissue.

**Objective:** We are investigating whether adipose derived stem cells (ASCs) reduce collagen formation by the fibroblast. We hypothesize that factor secreted by ASCs can alter in the remodeling of fibroblast generated ECM.

**Setting:** Five fibroblast cell lines were either co-cultured with ASCs or grown in conditioned media from ASCs for 0, 6, 12, and 24 hours. Fibroblasts were obtained from a non-burned volunteer (commercially available) and from a biopsy from non-burned skin and one of the hypertrophic scar from two patients at our institution. Fibroblast RNA was isolated at 0, 6, 12, and 24 hours following treatment. Two types of ASCs were used in the experiment: ASCs obtained from a burn patient, and control ASCs from a non-burned patient to determine whether ASCs or their secretome can alter ECM production by fibroblast.

**Results:** Expression of collagen I, III and V mRNA increased compared to the control especially after 24h of culture with the condition media. Interestingly, there was an increase after 24 hour of culture in the level of macrophage inflammatory protein 2 (MIP-2) which is a potent chemoattractant for macrophages that participate in wound healing and ECM remodeling.

**Conclusion:** This study is to determine whether ASCs can impact fibroblast function and ECM remodeling.

#### **A Nonunion Animal Model to Assess the Role and Mechanisms of Action of Mesenchymal Progenitor Cells in Bone Regeneration**

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In the treatment of nonunions an attractive alternative to bone autografts is the use of autologous mesenchymal progenitor cells (MSCs) in combination with biomaterials (mimetic autografts). Although there are several reports in the clinical use of MSCs their fate, contribution to repair tissue and mechanisms of action during the repair process are not yet determined. Our goal is to determine the therapeutic potential of different sources of mesenchymal stem cells.

The femur of Sprague-Dawley rats was stabilized with an aluminum plate (20 mm long, 4 mm wide, 2 mm thick) and four screws (1.5 mm diameter, 8 mm long). A diaphyseal critical size defect (5 mm wide) was performed. Five groups (5–6 animals each) were created, a nonunion group (Group 1, empty defect), positive control (Group 2, live allograft), Scaffold (Group 3, polycaprolactone scaffold), PMSCs (Group 4, periosteum-derived MSCs) and BMSCs (Group 5, bone marrow-derived MSCs). Donor cells were tagged with GFP for cell fate tracking.

Groups 1, 3 and 5 did not show signs of healing during the radiological follow-up, experimental nonunion was confirmed by  $\mu$ CT and histological analysis. In the Group 2 all the animals showed bone bridging. In Group 4 a reduced number of animals showed radiological healing (2 of 5 animals). Bone volume quantification showed significant new bone formation in Group 2 and 4 when compared with empty defect. Interestingly, only GFP positive cells were detected in Group 2.

In conclusion periosteum-derived progenitor cells are suitable for mimetic autograft design although integration is not yet achieved.

#### **Studies of Exogenous Hyaluronic Acid in a Human Minimal Invasive Wound Healing Model *In Vivo***

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Hyaluronic acid, a large glycosaminoglycan involved in proliferation, migration, and tissue repair, is suggested to play an important role in ideal scarless fetal wound healing. This study aimed to investigate the effect of exogenous hyaluronic acid intradermal during deep dermal wound healing. Study parameters were erythema, re-epithelialization, and protein expression. Standardized deep dermal wounds were created in healthy volunteers. The wound sites were treated with hyaluronic acid or saline solution, prior to wounding, or were left untreated. To quantify changes in red blood cell concentration as a measurement of inflammation, the study sites were photographed using a tissue viability imaging system. Biopsy specimens were taken for histology and proteomics analysis. The inflammatory response was not affected by hyaluronic acid, as measured by tissue viability imaging. Hyaluronic acid significantly induced ( $p < 0.05$ ) accelerated re-epithelialization, and wounds treated with hyaluronic acid showed an altered protein expression. The results from the present study are in concordance with previous *in vitro* findings and suggest that exogenous hyaluronic acid has a positive effect on the healing process of cutaneous wounds. We conclude that hyaluronic acid induces accelerated re-epithelialization and alters protein expression *in vivo* in human deep dermal skin wounds.

#### **A Push and a Prod in Vascular Tissue Engineering: Directed Extracellular Matrix Deposition and Improved Biocomplexity using Aligned Nanofiber Substrates and Tgf- $\beta$ Supplementation**

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The development of tissue-engineered vascular grafts (TEVGs) with a biomimetic extracellular matrix (ECM), including a mature elastic network, remains a key challenge for long-term graft functionality. The aim of the current project was to determine whether or not an aligned biodegradable nanofiber substrate can be used to orientate cells and their ECM as a cellularised sheet for the fabrication of tubular grafts, while combining an elastogenic growth factor to produce an ECM support structure optimised for TEVGs.

Human umbilical artery smooth muscle cells (hUASMCs) were seeded on electrospun nanofibrous fibrinogen substrates with an aligned or randomly-orientated fiber morphology. The cells were cultured for 1, 7 and 14 days on each substrate, with or without TGF- $\beta$ -supplementation (1 ng/ml) (all groups  $n = 18$ ). The resulting ECM development was analysed using both immunofluorescence microscopy and quantitative dye binding assays. Cellular orientation was measured using FFT analysis of actin filaments.

The aligned nanofibrous substrates induced parallel orientation of both cells and key ECM components, including collagen and elastic fiber assembly proteins (tropoelastin, fibrillin, elastin). In groups supplemented with TGF- $\beta$ , statistically significant increases in both collagen and elastin synthesis were measured after both 7 days, and subsequently, 14 days in culture. As proof-of-concept for TEVG fabrication, tubular constructs were fabricated from rolled, cell-seeded electrospun fibrinogen sheets and demonstrated concentrically-organised hUASMCs and ECM synthesis after 7 days in culture. The present study indicates that the combined application of both morphological and chemical cues can potentially be used to develop appropriately structured and orientated ECM for TEVG development.

#### **Effective Treatment of Apical Periodontitis by Adoptive Transfer of CD4+CD25+ Regulatory T Cells**

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Apical periodontitis, due to root canal infection, is one of the most common dental diseases and difficult to treat due to the pulp damage and lesion formation around the apex of the root. Regulatory T cells (Treg cells) play a crucial role in bone remodelling by regulating immune response and suppressing the differentiation/function of osteoclasts. Previous studies have shown that adoptive transfer of Treg cells has a therapeutic effect in several experimental models of immune-mediated diseases, especially the bone destructive diseases such as rheumatoid arthritis. This study aims to evaluate the effect of adoptive transferring CD4+CD25+Treg cells in apical periodontitis. Our results showed that adoptive transfer of Treg cells significantly inhibited apical lesion formation in a rat model via the regulation of Treg/Th17 imbalance caused by inflammation. This study demonstrates that Treg cells, acting like a vaccine that generates host suppressor cells, play a vital role in apical periodontitis. The therapeutic potential of Treg cells in the inflammation induced bone destruction should be further investigated.

#### The Effect of Bone Marrow-Derived Mesenchymal Stem Cells and their Conditioned Media Topically-Delivered in Fibrin Glue on Chronic Wound Healing In Rats

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Bone marrow-derived mesenchymal stem cells (BM-MSCs) represent a modern approach for management of chronic skin injuries. In this work, we describe BM-MSCs application versus their conditioned media (CM) when delivered topically admixed with fibrin glue to enhance the healing of chronic excisional wounds in rats. Fifty-two adult male rats were classified into four groups after induction of large-sized full thickness skin wound; untreated control group, fibrin only group, fibrin+BM-MSCs group and fibrin+CM group. Healing wounds were evaluated functionally and microscopically. Eight days post-injury, number of CD68+ macrophages infiltrating granulation tissue was considerably higher in the latter two groups. Although -later- none of the groups depicted a substantially different healing rate, the quality of regenerated skin was significantly boosted by the application of either BM-MSCs or their CM both: (1) structurally as demonstrated by the obviously increased mean area percent of collagen fibers in Masson's trichrome-stained skin biopsies; (2) functionally as supported by the interestingly improved epidermal barrier as well as dermal tensile strength. Thus, we conclude that topically applied BM-MSCs and their CM -via fibrin vehicle- could effectively improve the quality of healed skin in chronic excisional wounds in rats, albeit without true acceleration of wound closure.

#### Interrogating the Integration of Microenvironmental Factors on Human MSC Differentiation in 3D

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Biomaterial physical and biochemical properties along with external mechanical and soluble chemical microenvironmental cues combine to direct cell fate and function. To date, select combinations of these factors have been studied in 2D, but systematic consideration of the integration of microenvironmental factors in 3D culture has not been explored fully. To address this, we developed a 3D screening platform to systematically study cell responses to microenvironmental stimuli. Permutations of the cell adhesion sequences RGD, DGEA and YIGSR at different concentrations (0–2 mM) were incorporated into 5–11 wt% (5–22 kPa) polyethylene glycol norbornene (PEG-NB) hydrogels. Hu-

man bone marrow-derived mesenchymal stromal cells were cultured in the 3D arrays for 7 days with 0 or 5 ng/mL TGF- $\beta$ 1. The extent of myofibroblastic differentiation (% alpha smooth muscle actin (aSMA) positive cells) and collagen type I deposition (total area of collagen/total area of Hoechst staining), were modeled using least squares estimation and regression. Regression analyses revealed that PEG-NB hydrogel wt% was the most significant parameter affecting myofibroblastic differentiation and collagen deposition without TGF- $\beta$ 1, with more aSMA+ cells and more collagen at lower gel wt%. With TGF- $\beta$ 1, collagen deposition but not aSMA expression was significantly correlated with hydrogel wt%. Collagen deposition showed a biphasic dependence on DGEA concentration, with peak deposition at 1 mM without TGF- $\beta$ 1. Conversely, with TGF- $\beta$ 1, RGD and YIGSR significantly enhanced collagen deposition at higher concentrations. These data suggest that complex interactions between microenvironmental stimuli can be identified with our system to generate non-intuitive hypotheses and identify optimal conditions for tissue engineering.

#### Evaluation of Cultured Epithelial Cell Sheet's Basement Membrane using Temperature Responsive Dish, without Enzymatic Treatment

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Cultured epithelial autograft (CEA) therapy was used for clinical application since 1980s. It has become a major application of severe burn treatment. However many unsolved problems have remained. It is sometimes occurred dropping off or blistering with progress. Enzymatic treatment is used for collection methods of epithelial keratinocyte in many countries. It is thought that enzymatic treatment is one of the reasons of poor take. Our laboratory has developed temperature responsive culture dish which does not need enzymatic treatment. It can collect the cells without any enzyme. We hypothesized that this culture dish could preserve basement membrane proteins. In this study, we cultured rat's epithelial keratinocytes with temperature responsive culture dish and normal cell culture dish, and harvest the cultured epithelial keratinocyte for sheet. We made the transplantation model and evaluate the survival rate of transplanted cells. Both cell culture dishes could culture and harvested as sheet. The major basement membrane proteins, collagen IV and Laminin5, were confirmed the sheet which harvested by without enzymatic treatment. Survival rate of temperature treatment group is significantly superior to enzymatic treatment group at one week after transplantation. Our results show that the basement membrane proteins are important for cell survival and initial attachment. It is important that remain the basement membrane proteins. Our cell sheet engineering approach is useful for cell survival and can be effective for an application of CEA therapy.

#### Immobilization of SMOC1 Extracellular Calcium-binding Domain Peptide onto HA/ $\beta$ -TCP Particles Stimulates Regeneration of Calvarial Bone Defects in Mouse

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SPARC-related modular calcium binding 1 (SMOC1) has been implicated in the regulation of osteoblastic differentiation of human bone marrow mesenchymal stem cells (BMSCs). In this study, we designed a peptide (SMOC1-EC peptide; 16 amino acids in length) residing in the extracellular calcium (EC) binding domain of SMOC1. We found that the SMOC1-EC peptide stimulated osteoblastic differentiation of human BMSCs *in vitro* and calvarial bone regeneration

*in vivo*. The SMOC1-EC peptide showed no significant cytotoxicity and did not hinder the proliferation of BMSCs. However, it significantly stimulated the mineralization of BMSCs in a dose-dependent manner. The expression of osteoblastic differentiation marker genes, including type I collagen and osteocalcin, were also consistently increased in a dose-dependent manner. To examine the osteogenic potential of the SMOC1-EC peptide *in vivo*, the peptide was covalently immobilized onto hydroxyapatite/ $\beta$ -tricalcium phosphate (HA/ $\beta$ -TCP) particles. X-ray photoelectron spectroscopy analysis showed that the peptide was successfully immobilized onto the surface of HA/ $\beta$ -TCP. The implantation of the SMOC1-EC peptide-immobilized HA/ $\beta$ -TCP particles into calvarial defects and subsequent analyses using micro-computed tomography and histology showed significant bone regeneration compared with unmodified HA/ $\beta$ -TCP. Collectively, our data suggest that peptides derived from the EC domain of SMOC1 induces osteogenic differentiation of human BMSCs *in vitro* and efficiently enhances bone regeneration *in vivo*.

### Engineered Human Pluripotent Stem Cell Derived Cardiomyocyte Platforms to Detect Cardiovascular Safety Liabilities

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The development and screening of new therapeutic entities is often a lengthy and costly process, where it has been estimated to take between 10 – 15 years to bring a drug to market at a cost of up to \$1.8 billion. The pharmaceutical industry also relies heavily on the use of animal models for measuring drug safety liabilities, however these have been shown to be unreliable. The ability to differentiate human pluripotent stem cells (hPSC) into cardiomyocytes could provide an alternative approach for drug testing without the need for animal use. We are developing a pseudo-3D muscular thin film (MTF) platform with tuneable elastic properties and customisable surface micro-patterning for use in drug screening assays. The platform will allow for key parameters such as calcium imaging, electrophysiology and contractile force measurement to be measured using a single assay. Our aim is to develop clinically relevant cardiovascular models that are not only cost effective and will help to reduce the reliance on animal testing but will deliver improved patient safety. Current work has focused on developing MTFs with an elastic modulus which replicates healthy (30 kPa) as well as diseased (100 kPa) human adult cardiac tissue, optimisation of the substrate micro-patterning has produced synchronised contraction of anisotropically aligned cardiomyocytes. Future work will focus on the development of an optical system and associated software to allow for image movement analysis as well as contraction force measurements.

### Biomimetic Three-Dimensional Gradient of GAG-Binding Enhanced Transduction Proteins for Stem Cell Differentiation

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The success of biomimetic approaches replicating the three-dimensional (3D) cellular microenvironment is defined by careful design of the scaffold micro-architecture. Biological processes occurring within this microenvironment such as migration, angiogenesis and differentiation are guided via spatially dependant signals. In this context, various micro-scale technologies have been used to create spatio-temporal gradients of biologically active factors on two-dimensional (2D) surfaces and in 3D scaffolds. However, the misrepresentation of the complex 3D microenvironment using 2D models in terms of differences in morphology, cell-cell and cell-matrix interactions, and differentiation potential, limited their applications.

In the current study, a compartmentalized diffusion model of Source-Gel-Sink assembly is employed to generate 3D biomolecule gradients across cell-laden hydrogels. We utilized a novel approach named GAG-binding Enhanced Transduction (GET) that produced a fusion protein comprised of a membrane docking peptide to heparan sulfate glycosaminoglycans (GAG) together with a cell penetrating peptide (CPP) to efficiently deliver proteins. Gradients of reporter monomeric red fluorescent protein (mRFP) and GET-tagged mRFP proteins were created and the respective intracellular transduction was monitored as a function of location and time. The gradient of GET tagged mRFP across the hydrogel was well-maintained over time compared to mRFP. Analysis on the encapsulated cells revealed that the cells acted as sink that retained the GET-mRFP and maintained a stable 3D gradient across the hydrogel with gradual cellular responses.

The ability to spatiotemporally control the intracellular delivery of functional proteins will be a powerful tool for directing cellular behaviour and controlling stem cell differentiation in 3D environments.

### Tissue Engineered Human Scar Models Identify Extracellular Matrix and Inflammatory Cytokine Differences Between Normotrophic, Hypertrophic and Keloid Scars

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**Introduction:** abnormal scars (hypertrophic scars and keloids) can have a significant physiological (limited joint mobility) and psychological impact on quality of life. Since animals do not form adverse scars which are similar to humans, it is difficult to identify and test novel therapeutics in animal tests. Hypertrophic scars and keloids arise from different underlying mechanisms, and therefore the aim of this study was to develop physiologically relevant human tissue-engineered scar models for future drug targeting studies and testing of novel therapeutics specific for each scar type.

**Methods:** epidermal keratinocytes and dermal fibroblasts from normal healthy skin and scars (normotrophic, hypertrophic, keloid) were used to construct skin-equivalents (SE). Keloid scars were further subgrouped into periphery, superficial-center, deep-center and surrounding-normal-skin regions. Read-out parameters were contraction, dermal thickness, myofibroblasts ( $\alpha$ -smooth-muscle-actin), extracellular matrix gene expression (ECM) and cytokine secretion.

**Results:** all SE showed normal epidermal differentiation of fibroblast populated dermal matrices. Both hypertrophic and keloid SE showed increased contraction and dermal thickness compared to normal skin and normotrophic scar. Myofibroblasts ( $\alpha$ -SMA expression) were present in both hypertrophic and keloid SE, but was more abundant in SE containing central deep keloid fibroblasts. Notably, numerous ECM genes and cytokines showed differential profiles between abnormal scars and normal skin and also particularly between hypertrophic scars and keloids.

**Conclusion:** using tissue engineered scar models we have identified quantifiable parameters which can distinguish normal skin from scars. Importantly we have identified parameters which distinguish normotrophic scars from hypertrophic scars and keloids, and also which distinguish hypertrophic scars from keloids.

### MSC Recruitment during Human Immune Responses to Polylactic Acid and Chitosan 3D Scaffolds

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Implanted biomaterials elicit an inflammatory response, whose delicate balance will determine the effectiveness of tissue repair/regeneration. The events that facilitate the shift from detrimental inflammation to constructive tissue remodeling and regeneration involve orchestrated cell recruitment. However, it remains unknown how immune responses triggered by different 3D biomaterials interfere with



the stem cell dynamical behavior. Here, the responses of human peripheral blood mononuclear cells (PBMCs), NK cells, monocytes and macrophages towards polylactic acid (PLA) and chitosan scaffolds and the subsequent MSC recruitment were investigated. PLA and chitosan lead to increased metabolic activity with macrophages but not PBMCs, NK cells or monocytes. This increase was not correlated with cell number. Importantly, while both NK cells and monocytes in TCPS (tissue culture polystyrene) lead to high number of recruited MSC, macrophages were the most effective in recruitment of MSC when differentiated in the presence of either chitosan or PLA scaffolds. Furthermore, imaging of Dendra2 labeled and photo-converted MSC to characterize their motility in 3D microenvironments revealed 43% more mobility when in co-culture with macrophages over 7 days. Overall, distinct immune populations respond differently to diverse biomaterials, which impacts on the extent of MSC recruitment. This study provides insights for the development of strategies modulating host responses to attract specific progenitor cells for a constructive remodelling of implanted biomaterials.

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### Regenerative Therapy using Adipose tissue-derived Stem Cell Sheets Transplanted to the Intestinal Ulcer Lesion in Animal Models

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**Objective:** It has been known that mesenchymal stem cells from adipose tissue as well as bone marrow have an anti-inflammatory and angiogenic actions. Adipose -derived MSCs are easily and less invasively harvested than bone marrow MSCs.

In the present study, first, we developed a novel intestinal ulcer model in rat in which surgical submucosal dissection was performed in the cecum. Second, the allogeneic ASC sheets were fabricated, and transplanted onto the ulcerative lesions. Finally, the wound healing was compared with the control group which was not treated with cell sheets.

**Methodology:** F344/NJcl-rnu/rnu rats (10 weeks, male, n=6) were used to create a isolated cecal pouch model as described previously. Then, Ulcer (2×2 cm) was surgically made.

ASCs were isolated from rats epididymal adipose tissue (1.5–2.0 g in one rat and cell numbers were about  $1.45 \times 10^6$  cells from one tissue). Then, cells were subcultured for three times within 2 weeks. Finally, cells ( $50 \times 10^4$  cells/dish) were seeded in thermo-responsive polymer-grafted dish (UpCell<sup>®</sup>, 35 mm in diameter). After one week culture, cell sheets were harvested and transplanted onto the ulcerative lesions.

**Result:** The macroscopic findings 1 week after the operation identified that ulcerative lesions red in color, and not epithelized in the control group. To the contrary, in the ASC sheet transplanted group, there were some cell sheet structure and color variations within the ulcerative lesions.

**Conclusion:** Transplantation of allogeneic ASC sheets promoted wound healing of intestinal ulcers in a rat model.

### Inhibition of Fibrocontraction by C-phycoyanin through Modulation of Connective Tissue Growth Factor and $\alpha$ -Smooth Muscle Actin Expression

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**Purpose:** C-phycoyanin (C-pc) is a phycobiliprotein from spirulina. Hypertrophic scarring can be essentially related to inflammation-mediated dermal fibrosis. Herein, the effect of C-pc on  $\alpha$ -SMA

and CTGF, pro-fibrotic mediators involved in hyper-tropic scarring were investigated in relation with modulation of trans-differentiation of fibroblast to myo-fibroblast, an icon of scar formation.

**Methods:** C-phycoyanin was isolated from *Spirulina Platensis* extract. *In vitro* wound model with scratched primary human dermal fibroblasts were employed.  $\alpha$ -SMA and CTGF levels were determined by western blot analysis. Fibroblast contractility was assessed by three-dimensional collagen gel contraction assay. Collagen lattices and cells were stained with immunofluorescence  $\alpha$ -smooth muscle actin antibody; then analyzed with a confocal microscope.

**Results:** Significant over expression of  $\alpha$ -SMA and CTGF were observed in scratched wound model. C-pc suppressed the over expression of  $\alpha$ -SMA and CTGF; does-response of down regulation showed a saturation behavior; 0.2 ug/ml of C-pc produced maximum down regulation for  $\alpha$ -SMA (63%) and CTGF (50%) to control, respectively. In confocal assay, normal, un-scratched cells exhibited an absence of  $\alpha$ -SMA staining while in scratched without C-pc treated cells exhibited strong up-regulation of  $\alpha$ -SMA. C-pc treated cells showed less stress fiber levels as compared to un-treated cells ( $25.8 \pm 2.8$  vs  $86.2 \pm 4.6\%$ ) and inhibition of collagen contraction ( $34.9 \pm 0.0\%$  vs  $49.6 \pm 0.1\%$ ). High correlation between  $\alpha$ -SMA levels and collagen lattice contraction rate was observed. Conclusion C-pc isolated from *Spirulina* extract could control the over expression of  $\alpha$ -SMA and CTGF and trans-differentiation of fibroblasts to myofibroblasts in wound model. This study suggests the potential application of C-pc for anti-scarring therapy.

### Homogenous Microsphere-based Scaffolds with High Concentrations of Hydroxyapatite and Tricalcium Phosphate for Bone Regeneration

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We and others have shown that poly(lactic-co-glycolic acid) (PLGA) microsphere-based scaffolds loaded with osteogenic materials enhance bone regeneration. Our previous studies, however, have been limited to concentrations of hydroxyapatite (HAp) and tricalcium phosphate (TCP) below 30% in the PLGA microspheres. The purpose of this study was to determine whether it would be possible to construct a scaffold exclusively of PLGA microspheres that were themselves predominantly composed of TCP and HAp. Although various microsphere sintering methods were ruled out, we are pleased to report that a methylene chloride vapor treatment was an efficient and effective means to sinter scaffolds at HAp:TCP (1:1) concentrations up to 70%. The analysis for the performance of the scaffolds will include scanning electron microscopy, mechanical stress, calcium release, and PCR after a two-week *in vitro* study. The results of this study will allow us to build upon the foundation of microsphere-based scaffolds for osteochondral regeneration, with a predominantly natural material composition in the bone phase, and a PLGA composition to allow for a continuous transition to the chondrogenic side of the scaffold in the future.

### Reference

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### Dermal Scaffold and Bone Marrow Aspirate Concentrate Usage for Severe Facial Burns Management as a New Paradigm for Rescue Procedure

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**Purpose:** Facial burns (FB) often get an inextricable situation in terms of healing and long-term morbidity. Although results were more promising in terms of plastic surgeons, is inadequate for patients. We present the effectiveness of the use of dermal scaffold (DS) and bone marrow aspirate concentrate (BMAC) for severe FB management.

**Methods:** Three in acute, three in subacute phase, a total of six patients with severe FB were treated by using DS (Matriderm®), BMAC and immediate skin grafting after serial debridements. 120 cc of bone marrow was harvested from the anterior iliac crest in adults and 30 cc from anteromedial face of the tibia in children younger than 2 years of age and processed using the SmartPREP BMAC® system (Harvest Technologies Corp.) to obtain BMAC. Lipoinjection procedure was performed in subacute facial burns to contour in addition.

**Results:** Average follow-up period was 16 months. Average operation number per patient was 4. A considerable improvement in skin softness, thickness, elasticity, color was obtained in all patients.

**Conclusions:** DS creates a perfect ground for full-thickness FB to obtain elasticity and acceptable thickness. Grafted body parts undergo pigmentation and contractures as is known. DS usage reduces pigmentation, contracture development and pressure garment time as a result. These features facilitate a return to social life and promote a better quality of life for people living with burns. BMAC may develop graft survival and integration. The results of the study demonstrate that combining the DS with BMAC improves the outcomes of severe FB management.

#### Xenografting of Isolated Human Follicles in Fibrin Scaffold Associated to Hyaluronic Acid: Preliminary Results

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In order to develop a biodegradable artificial ovary, our aim was to verify if human isolated follicles included in a fibrin clot matrix would increase the recovery rate when associated to hyaluronic acid (HA), a natural compound of extracellular matrix. To this end, 50 isolated human preantral follicles and 50,000 ovarian stromal cells (OSC) were encapsulated into a matrix containing fibrinogen (F; mg/mL) and thrombin (T; IU/mL): F50/T10 (control) or F50/T10/HA3 (3% HA) and grafted to the peritoneal pocket of six nude mice. Fibrin clots were analyzed at histology at days 0 (control, 10 minutes after grafting) and 7 after grafting. The average recovery rate of follicles at D0 and D7 was 29% and 17% respectively for F50/T10 and 51% and 20.4% for F50/T10/HA3. Considering follicular structures (when oocyte was not visible in the histological section), at day 0 and day 7 the recovery rate was 52% and 41% respectively for F50/T10 and 76% and 45.5% for F50/T10/HA3. Moreover, when the fibrin was associated to HA, the matrix was more degradable with a higher cell invasion at D7. The present study is the first to report the survival and growth rates of isolated human ovarian follicles 1 week after the transplantation of isolated human follicles and OSCs in a fibrin scaffold associated to HA. The results suggest that the association of fibrin to HA is a promising candidate to be used as a matrix in the artificial ovary. However, further analyses will be necessary to validate the present findings.

#### Fluorescent Monitoring of Poly(lactic-co-glycolic Acid) Degradation and Extracellular Matrix Turnover of Engineered Tissue Constructs

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Development of fluorescent tagging techniques for inert polymers is an advantageous tool for monitoring biomaterial degradation on-line. Here we present a protocol for the fluorescent tagging of poly(lactic-co-glycolic acid) (PLGA) which allows for accurate monitoring of degradation. This was applied to PLGA with various

lactic/glycolic acid ratios to determine cellular responses to degradation. The final aim was to produce a new metric, the turnover index (TI), which assesses cellular responses to biomaterial degradation.

Ammonia plasma treatment led to the formation of rhodamine b isothiocyanate reactive amine groups on the PLGA surface. Characterisation, by x-ray photoelectron spectroscopy and high performance liquid chromatography, demonstrated that post reaction rhodamine was covalently bound to the PLGA surface. Degradation was accurately predicted through reduction in solid phase fluorescence and soluble fluorescent by-product release. Faster degrading PLGA supported extracellular matrix (ECM) production over proliferation *in vitro* and increased healing within an *ex vivo* chick femur defect. TIs were calculated as ratios of degradation to ECM production and proliferation. Faster degrading PLGA led to increased ECM TI and decreased proliferation TI when compared to slower degrading compositions.

Fluorescently labelling inert synthetic polymers allowed for on-line monitoring of biomaterial degradation both *in vitro* and *in vivo*. Online monitoring *in vivo* is becoming increasingly important under the 3Rs strategy. Differences observed in cellular response to various degradation rates of biomaterials enabled the development of a TI. This *in vitro* tool can assess the suitability of biomaterials for particular applications and be utilised to select biomaterials for future clinical applications.

#### Comparison of Different *in vivo* Incubation Sites for Production of Tissue Engineered Intestine (TEI)

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**Objectives:** To compare different *in vivo* incubation sites as bioreactors for the production of tissue engineered intestine (TEI).

**Methods:** Sprague Dawley rat pup (5–7-day-old) intestine was digested and filtered through different sized sieves (200, 70, and 25 µm). Intestinal stem cell (ISC)-containing crypts were located predominantly in the 25–70 µm fraction and were used to seed 1 cm long × 0.5 cm internal diameter tubular polyglycolic acid (PGA) scaffolds. Scaffolds were implanted into the dam of the pups with each dam receiving 4 scaffolds as follows: 1) one wrapped with omentum, 2) one attached to the peritoneal surface of the anterior abdominal wall, 3) one wrapped around native intestine, and 4) one inserted subcutaneously in the left groin. After 4 weeks of *in vivo* incubation, scaffolds (n = 12 from each location) were explanted and histologically examined for the presence of neomucosa. Quantification of neomucosal villous height was performed.

**Results:** Ten of 12 scaffolds in Groups 1 and 2 (83%), 12 of 12 scaffolds in Group 3 (100%), and 4 of 12 scaffolds in Group 4 (33%) were positive for neomucosa. Villous heights were 378 ± 38 µm, 357 ± 48 µm, 412 ± 45 µm, and 115 ± 33 µm for neomucosa from each of the respective groups.

**Conclusion:** The greatest quantity and quality of TEI neomucosa were observed when seeded scaffolds were wrapped around native small intestine during *in vivo* incubation. Continued evaluation is warranted to determine potential future clinical applicability of this observation.

**Acknowledgments:** Ohio Third Frontier.

#### Fabrication and Evaluation of 3D-Printed Polymeric Tubular Scaffolds for *in Vivo* Application

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Recently, three-dimensional (3D) printing technology has been widely used in tissue engineering fields because of its controllable

features and well-defined products. Especially, the application of biodegradable polymer is important to fabricate the scaffolds in 3D bioprinting technology. In the present study, we developed a novel scaffolds fabricated with biodegradable polymer using 3D printing technique. For the present study, we synthesized MPEG-b-(PCL-ran-PLLA) (PCLA) copolymers with various monomer ratio and molecular weight. Tubular morphological scaffolds were fabricated by solid freeform fabrication (SFF) system. The tubular morphologies of PCLA scaffolds were confirmed by optical microscope. The scaffolds were implanted under the dorsal skin of rats and then the scaffolds were removed from the dorsal skin of rats at 2, 4, 8, and 16 weeks after implantation. The degradability of PCLA tubular scaffolds was visualized through decrease of fluorescence and was measured using <sup>1</sup>H-NMR and GPC. Histological analysis of scaffolds implanted into rats was performed at 2, 4, 8, and 16 weeks. The fabricated PCLA tubular scaffolds had well-defined structures and uniform pore size. Also, we found that the fluorescence intensity of PCLA-FITC tubular scaffolds was declined over time and molecular weight of PCLA tubular scaffolds implanted into rats was decreased. In histological analysis, inflammatory responses of the scaffolds at 16 weeks were decreased compared to that at 2 weeks. In the present study, we successfully developed PCLA tubular scaffolds with biodegradability and biocompatibility. Biocompatible PCLA tubular scaffolds fabricated by using 3D bioprinting technique will be useful in tissue engineering and regenerative medicine fields.

### Gingiva Equivalent Derived from Human TERT Immortalized Keratinocytes and Fibroblasts for Investigation of Oral Wound Healing

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**Introduction:** The oral mucosa forms the protective barrier of the oral cavity. Physiologically relevant human *in vitro* models are preferred to animal models for investigating wound healing, cell biology and ultimately for identifying novel drug targets. Unfortunately the supply of human oral mucosa tissue is scarce. Therefore, in this study we developed a full thickness tissue engineered oral mucosa entirely from immortalized cell lines to investigate the mechanisms of oral wound closure.

**Methods:** Gingiva equivalents (GE) were constructed from human TERT immortalized gingiva keratinocytes and fibroblasts, and compared with native gingiva and primary GE. Characterization was assessed by immunohistochemical (IHC) stainings with markers for fibroblasts and epithelial proliferation and differentiation. To test the wound healing response of the human cell line GE, freeze wounds were applied, followed by measurement of re-epithelialization and determination of secreted wound-healing mediators (ELISA).

**Results:** The human cell line GE consists of a fully-differentiated epithelium and a fibroblast-populated lamina propria comparable to native gingiva and primary GE. The epithelium shows proliferating keratinocytes in the basal layer, with several suprabasal layers. IHC shows normal expression of involucrin, K10 and K13. The GE is able to completely re-epithelialize within 7 days after wounding and secrete cytokines typically related to wound healing.

**Conclusion:** We were able to construct GE entirely from immortalized human gingiva cells. We conclude that this model has the potential to replace the limited available constructs based on primary cells. The GE will be a valuable tool for *in vitro* wound-healing studies (e.g. testing novel therapeutics).

### Engineering a Functional Three-dimensional Organ with Biologic Scaffolds Composed of Liver Extracellular Matrix

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The concept of whole organ engineering has emerged as a potential solution for patients with end-stage liver failure. This approach involves the decellularization of an allogeneic or xenogeneic liver followed by recellularization of the resultant three dimensional biologic scaffold with autologous cells. Current limitations of this approach include: 1) the re-establishment of a non-thrombotic microvasculature and, 2) an effective method for delivering parenchymal cells to their native locations within the 3-dimensional scaffold. The objectives of the present work were to systematically investigate key variables associated with reconstructing a functional hepatic vascular network via endothelial cell seeding, and to develop a preferred method of delivering hepatocytes to achieve effective and viable cell engraftment, anatomically appropriate spatial location, and functionality. Metabolic activity and cell viability of the engrafted hepatocytes was evaluated by quantification of albumin and urea production, engrafted cell morphology, and expression of hepatic specific genes. Results showed that within three days of delivery, seeded endothelial cells attached to the native vascular network, displayed a normal flattened appearance, and formed within a venous, arterial, and sinusoidal network. Perfusion pressures of 5–10 mmHg (venous) and 80–100 mmHg (arterial) were maintained throughout the culture period. Results also showed high hepatocyte viability (> 80%), excellent cell morphology, albumin and urea production, and hepatocyte specific gene expression. This systematic approach represents notable steps toward clinical translation or whole organ engineering.

### Tri-Culture of Vascular Cells from Cellularised Collagen-based Tubular Scaffolds for Vascular Tissue Engineering

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Collagen gel is a commonly used scaffold in vascular tissue engineering due to its biological properties including a high potential for supporting and guiding vascular cells in the regeneration process. The approach we privileged consists in first reproducing the media, which provide the high elastic properties of the vessel wall, thus making it an essential and effective component for blood and nutrients transportation.

Starting from an original method, previously reported and aimed to process collagen and smooth muscle cells (SMCs), the overall goal of this project was to design and develop an endothelialised two layers collagen cell-based tube scaffold. The external layer is composed of fibroblasts (FBs) and SMCs seeded within collagen. The middle layer is composed of SMCs seeded within collagen and endothelial cells (ECs) are on the lumen of the construct. The construct is expected to provide vascular tissue remodeling due to cells/cells and cells/matrix interactions and to produce an engineered tissue with hierarchical structure close to that of blood vessel walls. It is also expected to provide a valid *in vitro* model for further studies of vascular patho-physiology.

The middle and external layer were mold around a mandrel, directly in the bioreactor chamber. Then, the mandrel was removed and an ECs solution was perfused inside the lumen. The interaction between cells enhanced the matrix remodeling and the properties of the arterial construct resulted were improved. This experiment shows that vascular cells tri-culture using collagen gel scaffold is a valid strategy for the regeneration of the vascular tissue.

### From Blood to Bone: Engineering Bone with Induced Pluripotent Stem Cells and Hollow Nanofibrous Microspheres

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Bone tissue engineering addresses a critical clinical problem currently dependent on auto- and allografts. While many osteoblast/scaffolding structures have been engineered, they can be bulky and often require a surgery, exposing a patient to the risk of co-morbidity. An injectable platform would reduce such risks by eliminating the need for surgery, but many are based on hydrogel platforms which do not allow for free cell movement and can have difficulty integrating with native tissue to promote healing. In addition, while many bone tissue engineering platforms use mesenchymal stem cells, these lines show limited *ex vivo* proliferation and can be difficult to harvest. On the other hand, induced pluripotent stem cells can proliferate indefinitely and can be obtained from the patient. We synthesized poly(L-lactic acid) nanofibrous hollow microspheres and attached induced pluripotent stem cells differentiated down an osteogenic lineage. Nanofibrous materials have been previously shown to promote osteogenic behavior in other cell lines, and cells grown on them easily interact with each other and native tissue. iPS cells were generated from human patient blood via non-integrating plasmids, and further differentiated via embryoid body formation into a mesenchymal stem cell line. As the blood was drawn using a syringe, our platform is non-invasive, from the initial patient cell harvest to the treatment injection. We characterized their behavior on 2D PLLA nanofibrous films and 3D nanofibrous microspheres, and we demonstrate that osteogenesis is enhanced when iPSC-derived mesenchymal stem cells are cultured on nanofibrous topologies.

#### Stem Cell Based Renal Tissue Engineering: Kidney De- and Recellularization in a Perfused System

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Decellularized extracellular matrix (ECM) scaffolds of whole organs hold great potential for whole organ tissue engineering. These native scaffolds retain the organ specific composition, ultrastructure and 3D architecture and thereby provide a microenvironment to tissue specific cells that supports their attachment and organization.

Whether these microenvironmental clues are sufficient to promote organ specific differentiation of renal precursor cells was investigated.

Therefore, we optimized the decellularization of rat kidneys in a perfusion system and established a protocol for recellularization with human iPSC-derived renal precursor cells. The detergents sodium dodecyl sulfate/TritonX-100 and sodium deoxycholate were compared at different temperatures in a standardized perfusion system that automatically controls pressure and temperature. Characterization of the ECM by histology, immunofluorescence and composition analysis revealed the optimal decellularization protocols. Moreover, we tested these decellularized ECM scaffolds by recellularization in perfusion culture. The vitality, localization, identity and tissue specific integration of the cells were determined by immunohistochemistry.

Our data provide the evidence that decellularized kidneys provide a suitable platform for stem cell based renal tissue engineering and are the basis for further investigations of the potency of these scaffolds to promote and direct terminal differentiation of renal precursor cells.

#### Controlling Retinal Cell Fate using Nanotopography and Neurotrophic Factors

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Bioscaffolds, supporting survival and guiding axonal growth, holds great promise for the advancement of cell-based therapies for retinal neurodegeneration. Increased knowledge is required on the effect of

nanotopographies, extracellular matrix (ECM) proteins and neurotrophic factors on retinal cell survival, cell fate and axonal guidance.

Hence, we investigated the influence of nanotopography, laminin (ECM protein) and neurotrophic factors on the behavior of retinal neuronal- and glial cells.

We cultured mouse post-natal retinal cells for 7 or 18 days *in vitro*, at either Poly-L-Lysine-coated chamber slides (control) or electrospun polycaprolactone (PCL) fiber substrates with random or aligned orientation and substrates were laminin-coated or non-coated. Either basic (DMEM-F12, B27 supplement) or the enriched Full-SATO (Neurobasal, CNTF, BDNF, Forskolin, Insulin) medium was used.

Excellent overall cell survival was found up to 18 DIV on all three substrates utilizing either medium. A significant increase in numbers of retinal ganglion cells (RGC; markers: RBPMS, NeuN,  $\beta$ -tubulin III), photoreceptors (PR; marker: rhodopsin) and glial cells (marker: GFAP) was found using Full-SATO medium. Nanotopography per se significantly affected neuronal morphological formation; with mainly uni- and bipolar profiles at aligned fibers and more multipolar profiles at random fibers and the control surface. Addition of laminin and use of Full-SATO medium in all three substrates clearly promoted both RGC- and PR maturation, demonstrated by complex neuronal morphologies and extensive neurite outgrowth. A remarkable 90° switch of neurite orientation was found after coating with laminin - a crucial finding for future tissue engineering applications within ophthalmology.

#### Heparin Containing Polyelectrolyte Scaffolds as Wound Dressings

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Chronic wounds and extensive burns are always under the risk of microorganism attacks leading to infection which cause discomfort and even morbidity and mortality in extreme cases. Wound dressings having both antibacterial efficiencies to prevent infections and bioactivity to promote healing of the wound by regeneration of the dermal and epidermal tissues are preferable for the treatment of this kind of wounds. In this study, cationic, anionic and sulfated polysaccharides, chitosan (CH), alginate (Alg) and heparin (Hep), were selected to prepare electrolyte type wound dressing scaffolds. CH-Alg scaffolds having different heparin (0.5, 2.5 and 5% w/v) contents were prepared, and stabilities were optimized by CaCl<sub>2</sub> crosslinking. Presence of functional groups and morphological structures were examined by FTIR, XPS and SEM. Addition of heparin increased the pore size and porosity which enhance gas permeability and tissue growth in the scaffolds. Antibacterial activities against *Staphylococcus epidermidis* were investigated under *in vitro* conditions. For control group of *S. epidermidis*, the log colony forming unit (log CFU) was obtained as 6.5 log CFU/mL. Treatment of the bacteria suspension with the samples CH-Alg and CH-Alg-Hep-0.5, the log CFU values decreased to 4.5 and 3.6, respectively. Increasing concentration of heparin demonstrated increasing antibacterial activity and no living bacteria was observed after 24 h of incubation of CH-Alg-Hep-2.5 and CH-Alg-Hep-5.0 with *S. epidermidis* (p<0.05). The results demonstrated that heparin/chitosan/alginate polyelectrolyte scaffolds have very high potential to be used as therapeutic wound dressings.

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Aksoy EA. *et al.* Heparin/Chitosan/Alginate Complex Scaffolds as Wound Dressings, *J.Biomater. Tiss. Eng.* 5, 1–10, 2015.

### Engineering Vascularized Tissue Constructs using an Injectable Cell-laden Collagen Hydrogel

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Introducing cells or tissues grafts, native to the injured tissues, to promote the regenerative process is the ideal strategy in tissue engineering. Thus, somatic cell-based therapeutic studies can be considered an important tool in regenerative medicine. In this study, we reported a method for preparing autologous extracellular matrix scaffolds, murine collagen-Ph hydrogels and demonstrated the suitability of injectable and enzymatically crosslinkable collagen-Ph hydrogels for use in supporting human progenitor cell-based formation of three-dimensional (3D) vascular networks *in vitro* and *in vivo*. We also demonstrated that the biodegradability, swelling properties and stiffness of collagen-Ph hydrogels controlled by altering the degree of crosslinking could be used to tune not only the extent of vascular network, but also adipose and mineralized tissue formation *in vivo*. This study emphasizes the importance of extracellular matrix (ECM) in providing appropriate signals for endothelial-mediated vascular formation, which is critical for the earliest stages of organogenesis to engineer cell-based 3D tissue construct. Moreover, these address some current clinical problems associated with cell-based therapies and may contribute to the design and optimization of clinically-compatible ECMs for human blood-derived endothelial colony-forming cells (ECFCs) and bone marrow-derived mesenchymal stem cells (MSC). Based on these data, we propose the use of collagen-Ph hydrogels in regenerative engineering applications, including the engineering of 3D thick tissues and organs that require an adequate vascular supply to guarantee their survival and function.

### Short-Term Delivery of Fibrin-Bound VEGF Protein in Osteogenic Grafts ensures both Increased Vascularization and Efficient Bone Formation

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Spontaneous vascularization of large osteogenic constructs based on bone marrow-derived mesenchymal stem cells (BMSC) is too slow for progenitor survival *in vivo*. We found that sustained over-expression of vascular endothelial growth factor-A (VEGF) by genetically modified human BMSC effectively improved osteogenic graft vascularization, but also impaired bone formation through excessive osteoclast recruitment. Here we hypothesized that short-term delivery of VEGF protein, immobilized in fibrin gels, may improve graft vascularization without impairing bone formation.

Recombinant VEGF was engineered with a transglutaminase substrate sequence (TG-VEGF) allowing covalent cross-linking into fibrin hydrogels. Human BMSC were embedded in the fibrin gels and seeded on apatite granules. Bone formation and vascularization were assessed 1, 4 and 8 weeks after subcutaneous ectopic implantation in nude mice. Retrovirally transduced BMSC stably expressing VEGF were used as control.

At all times, constructs containing fibrin-bound TG-VEGF with naive BMSC or VEGF-expressing BMSC displayed increased vascularization compared to the controls with naive BMSC only. After 4 weeks fibrin gels were completely degraded in all conditions. However, while bone formation at 8 weeks was severely impaired

with VEGF-expressing BMSC as expected, fibrin-bound recombinant TG-VEGF allowed the formation of bone tissue as efficiently as naive BMSC alone. Interestingly, TG-VEGF improved the bone formation kinetics, as TG-VEGF constructs contained more bone than even naive controls after 4 weeks.

In conclusion, VEGF effects on promoting vascularization and bone resorption can be uncoupled by short-term delivery of recombinant VEGF protein, providing an attractive and clinically applicable strategy to ensure both robust vascularization and bone formation.

### Rapid Release of Growth Factors Regenerates Force Output in VML Injuries

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A significant challenge in the design and development of biomaterials is to incorporate mechanical and biochemical cues to direct organized tissue regeneration. These considerations are essential for volumetric muscle loss (VML) defects, where the regenerative template that repairs skeletal muscle is destroyed or removed as a result of traumatic injuries such as those caused by improvised explosive devices or tumor resections. In this study, we developed novel scaffolds with morphologic properties comparable to native muscle and biochemical factors that direct endogenous regeneration. Specifically, we investigated the effect of hepatocyte growth factor (HGF) loaded, crosslinked fibrin (EDCn-HGF) microthread scaffolds on muscle regeneration in a murine VML model.

The rapid, sustained release of HGF significantly enhanced the force production of muscle tissue 60 days after injury, recovering more than 200% of the force output relative to measurements recorded immediately after injury. HGF delivery increased the number of differentiating myoblasts 14 days after injury, and supported an enhanced angiogenic response. The architectural morphology of microthread scaffolds supported the ingrowth of nascent myofibers into the wound site, in contrast to fibrin gel implants which did not support functional regeneration. Together, these data suggest that EDCn-HGF microthreads recapitulate several of the regenerative cues lost in VML injuries, promote remodeling of functional muscle tissue, and enhance the functional regeneration of skeletal muscle. Further, by strategically incorporating specific biochemical factors and precisely tuning the structural and mechanical properties of fibrin microthreads, we have developed a powerful platform technology that may enhance regeneration in other axially aligned tissues.

### Osteoinductive Effects of ProBMP-2 Compared to BMP-2 on Bone Regeneration *in vivo*

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For the reconstruction of critical size bone defects, autologous bone is used but availability is limited and morbidity of donor site may occur. Alternatively, bone substitutes are used combined with osteoinductive growth factors like bone morphogenetic protein 2 (BMP-2). Because of side effects on implantation site like edema, initial high BMP-2 release appears to be problematic. The application of proBMP-2 as an inactive protein precursor may retard the release of active BMP-2. This study aims at analyzing both the osteoinductive effect of proBMP-2 compared to BMP-2 on bone regeneration in an ectopic rat model and the occurrence of side effects.

Three experimental groups (control, BMP-2, proBMP-2) were tested up to 12 weeks. Bone substitute blocks (BioOss<sup>®</sup>, 1 cm<sup>3</sup>) were coated with 57 µg BMP-2 or 200 µg proBMP-2 and subcutaneously implanted into rats. Each specimen was histologically evaluated by

Hematoxylin-Eosin staining/Masson-Goldner trichrome staining and histomorphometrically analyzed. The area of newly formed bone in proBMP-2 and BMP-2 groups increased from  $0.29 \pm 0.06 \text{ mm}^2$  and  $0.42 \pm 0.04 \text{ mm}^2$  at 3 weeks up to  $1.00 \pm 0.04 \text{ mm}^2$  and  $1.33 \pm 0.27 \text{ mm}^2$  at 12 weeks, respectively. No significant differences between these two groups were found. Formation of local edema on implantation site was observed in the proBMP-2 (5%) as well the BMP-2 group (16%) compared to no edema formation in the control group.

In conclusion, proBMP-2 is able to induce comparable bone formation as BMP-2 in an ectopic rat model and causes significantly less side effects. These promising findings indicate a potential clinical relevance of proBMP-2, but further investigations are needed.

### 3D Tissue Growth *in vivo* under Geometrical Constraints

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Substrate curvature influences cell behavior and tissue formation *in vitro* [1, 2]. We propose that the mean curvature of the scaffold surface guides the organization of collagen fibers and following mineralized tissue growth *in vivo*.

Scaffolds produced by rapid-prototyping were implanted in critical-sized defects in sheep tibiae. Scaffolds before implantation and bone growth after 12 months were visualized with microCT. The mean curvature map of the scaffold surface was used as input for a curvature-driven 3D tissue growth model [2]. Simulation of tissue growth around the scaffold was compared with *in vivo* data. Histology, backscattered electron imaging, second-harmonic generation (SHG) imaging and synchrotron small angle X-ray scattering (sSAXS) were used to characterize the distribution of cells, collagen fibers, mineral particle thickness, orientation and degree of alignment, respectively.

Simulated tissue growth initiated in regions of high mean curvature and developed into cylindrical pores. In agreement with the simulation, *in vivo* collagenous tissue formation evolved towards cylindrical pores. Highly aligned collagen fibers developed parallel to the long bone axis in the core of the cylindrical pores. Mineralized tissue grew in a cone-shaped fashion inside the pores, apparently following the central fibers. Microscopic analysis of longitudinal and transversal sections of the cone-shaped bone structures using SHG and sSAXS allowed quantitative evaluation of this geometry-driven *in vivo* tissue formation. This knowledge can be used to optimize scaffold design.

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### Designer Cell-laden Polyacrylamide-alginate Gels for Stem Cell Delivery

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Cell-laden hydrogels is an intriguing option to site-specific stem cell delivery into human body by exploiting degradation and mass transportation process of its hydrophilic polymer network in response to external stimulus. In this study, we designed and investigated the impact of polyacrylamide/alginate (PAM/Algi) hydrogels for encapsulation and growth of human bone marrow-derived mesenchymal stem cells (hBMSCs) with five different ratios (1:1, 1:2, 1:3, 1:4

and 1:5). The gels were characterized for their physicochemical properties. Swelling behaviour of the gels was also studied as it facilitates mass transfer of nutrients, oxygen and waste removal by diffusion, which in turn regulates the cell fate and function. Based on physicochemical characteristics, PAM/Algi hydrogel of 1:5 ratio has been selected for cell culture *in vitro*. For comparison of the cell culture systems (2D Vs 3D), hBMSCs were cultured under a defined condition using three different culture systems, such as on the tissue culture plate (TcP 2D system), on the gel (OnG 3D system) and in the gel (InG 3D system). The results of TcP 2D and OnG 3D systems were found to be comparable in terms of cell attachment, viability and proliferation. In contrast, InG 3D system showed slightly less viability than the other systems but supported a significant proliferation in 7 days. The cells cultured in InG 3D system showed morphology and cellular behaviour quite similar to native tissue-like growth. The overall results suggest that cell-laden gels based on PAM/Algi can be custom designed and used as a carrier for stem cell delivery.

### Universal Cell Labelling of Living Cells with Magnetic Nanoparticles for Applications of Cell Patterning and Stem Cell Tracking

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Magnetic labelling of cells raised up increasing interest due to the various biological or medical applications involving magnetism in living organisms. Magnetic forces are widely used to separate cells *in vitro* [1], but also to manipulate or attract cells by an external stimulus [1], cell patterning and stem cell tracking for tissue engineering [1] and magnetically-assisted cell delivery. Here we describe a non-specific labelling method based on positively charged magnetic nanoparticles (MNPs). These nanoparticles adsorb electrostatically to the outer membrane of the cells, by exploiting the ionic bond between the positive charge of the nanoparticles and the negative charge of cell plasma membrane. This work shows a method to functionalize several cell lines with magnetic nanoparticles via a single-step biocompatible process by mean of electrostatic interactions. The nanoparticles were characterized with TEM and FESEM. The interaction between cells and nanoparticles were characterized concerning cytocompatibility. In addition, cells were functionalized by immersion of sterile MNPs suspension in saline solution and after separating MNP-functionalized cells using a permanent magnet. Magnetic nanoparticles were localized on the cellular membranes and do not penetrate cross the plasma membrane. The magnetically responsive cells were viable and able to colonize and grow on culture plates. In summary magnetically facilitated positioning and migration of functionalized cells into viable living clusters was demonstrated. Magnet-facilitated spatial distribution on the culture plates could be potentially extended to controlled deposition and magnetic-field-oriented growth of functionalized cells.

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### Renal Ecm Scaffolds from Discarded Kidneys are Bioactive, Pro-tolerogenic, Cyto-Compatible and Feature an Intact, Patent and Resilient Vascular Feature an Intact, Patent and Resilient Vasculature

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In order to identify a new source of transplantable kidney, we used human renal ECM scaffolds (hrECMs) obtained by decellularization of discarded kidneys. This study aimed at: 1) assessing the status of the innate vasculature post Dec; 2) quantifying the growth factors (GFs) stored in the matrix; 3) studying the behavior of seeded multipotent cells; 4) evaluate the immunogenicity of hrECMs. hrECMs were compared with intact kidneys. We studied the morphometry of hrECMs' native vasculature with resin casting at SEM and pulse-wave measurements. We determined the 40 critical GFs post Dec with ELISA array and *in vitro* immunofluorescence. We established a 3D *in vitro* model where stem cells (SC) were seeded on hrECMs and studied with histology, analysis of secretomes, and PCR to assess gene expression. Finally immunological studies were performed by CD4 isolation, *in vitro* cultures and flow cytometry assays. Data showed that hrECMs preserved the microvascular morphology and morphometry, and physiological function. GFs were retained within hrECMs. SC attached, migrated, proliferated, remodeled the matrix, expressed genes of kidney development, mounted an impressive inflammatory response and induced angiogenesis. Our scaffolds were able to weaken the immune response *in vitro*. hrECMs featured a well preserved and resilient innate vasculature, which is a critical finding in view on *in vivo* implantation. Moreover, they maintained GFs, were cytocompatible and could determine organ-specific phenotype. hrECMs showed also impressive immunomodulatory properties.

#### Single-Photon Emission Computed Tomography versus Bioluminescence for Noninvasive Imaging of Mesenchymal Stem Cells within Rat Critical-sized Bone Defects

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Noninvasive imaging techniques can improve preclinical evaluation of mesenchymal stem cell (MSC)-based therapies for large bone defects, allowing longitudinal assessment of cell activity. Single-photon emission computed tomography (SPECT) has been used with high sensitivity to detect cells expressing the sodium iodide symporter (NIS) for non-skeletal applications. The purpose of this study was to compare SPECT and bioluminescence imaging for detection of MSCs implanted within rat critical-sized bone defects. Low-passage human and rat bone marrow MSCs were modified with lentiviral vectors to constitutively express human NIS or firefly luciferase. *In vitro* characterization of NIS expression was performed in cell monolayers and within fibrin gels by qPCR or by equilibrium uptake of [<sup>99m</sup>Tc]-pertechnetate ([<sup>99m</sup>Tc]O<sub>4</sub>), scanning cells on a NanoSPECT/CT system. For *in vivo* studies, mid-femoral, critical-sized defects were made in F344 rats, and fibrin gels containing MSCs implanted. Rats were imaged for up to 4 weeks post-implantation following intraperitoneal injection of 6 mCi [<sup>99m</sup>Tc]O<sub>4</sub> or 150 mg/kg D-luciferin. MSCs dose-dependently expressed NIS, as determined by qPCR and SPECT/CT. When pre-labeling 1e6 MSCs with [<sup>99m</sup>Tc]O<sub>4</sub> just before implantation in femoral defects or subcutaneously, cells in both sites could be detected immediately by SPECT. However, when delivering [<sup>99m</sup>Tc]O<sub>4</sub> by intraperitoneal injection, specific signal could only be detected from the subcutaneous site. In contrast, bioluminescence signal could be detected from the femoral defects for up to 2 weeks after implantation of 1e6 cells. We are currently assessing whether environmental conditions within the early defect (hypoxia, low pH, inflammation) influence NIS expression or activity.

#### Determining Characteristic Differences in Early Fracture Hematoma

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Hematoma formed at the fracture site has great significance in the way fractures heal. Surprisingly, there are no studies that have characterized the difference in quality of hematoma formed between the healing of fractures and large bone defects. The aim of this study is to determine structural properties of formed hematomas in rat models.

**Methods:** Bone defects of 1 mm and 3 mm were created in the medial femoral condyles in groups (n=8) of Fisher 344 rats. The animals were sacrificed at day 1, 4, and 7 postoperatively in order to evaluate structural properties of the hematomas. The structural properties of hematomas at the various time points were assessed using scanning electron microscopy (SEM). Enzyme-linked immunosorbent assays (ELISA) and qPCR is used to evaluate the concentration of various pro-inflammatory cytokines.

**Results:** There were discernible structural differences between blood clots formed in the smaller compared the larger bone defects. For example, the clot from the 1 mm defect had a more porous and thicker fibrin fibres compared to one from the 3 mm defect. Additionally, fibre number was less in 1 mm defects than 3 mm defects. Moreover, there were lower expressions of proinflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in 1 mm defects compared to 3 mm defects by qPCR and ELISA (P<0.01).

**Conclusion:** The data suggest that there is a distinguishable structural and biological difference in quality of hematoma formed between natural healing vs. delayed bone healing. These differences in the haematoma might be related to the poor bone formation in the large bone defects.

#### Osteogenic Differentiation and Angiogenic Potential of Alkaline Phosphatase Functionalized Bioactive Glass Scaffolds

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Bioactive glasses (BG) are widely investigated as promising materials for bone regeneration. In the broad family of BGs, boron-doped BGs continue to attract the attention of researchers. We report the fabrication of three-dimensional, porous boron-doped BG scaffolds using foam replication technique. Functionalizing the surfaces of these scaffolds with biologically active enzymes or molecules is a suitable strategy to enhance both the inorganic and biological responses of the biomaterial. Alkaline phosphatase (ALP) is a metalloenzyme involved significantly in osteogenesis and it is highly expressed in mineralized tissue cells. The release of B ions and Si species from the functionalized scaffolds soaked in RPMI cell culture medium over a period of 14 days was measured using Inductively Coupled Plasma-Optical emission analyses. X-ray photoelectron spectroscopy and enzymatic activity tests confirmed the presence of ALP on the surface of the scaffolds. We investigated the ability of the boron-doped BG scaffolds, with and without ALP grafting, to stimulate the expression and secretion of angiogenic growth factor, vascular endothelial growth factor (VEGF) from ST-2 cells (mouse bone marrow stromal cells). VEGF secretion was measured quantitatively using the VEGF ELISA Kit. *In vitro* osteogenic differentiation capability of ST-2 cells on ALP functionalized boron-doped BG scaffolds was investigated. The cell proliferation was determined using the BrdU (calorimetric) assay and cell morphology was observed after H&E staining. The influence of an enzyme (ALP), in terms of ion release kinetics, directing better cell-matrix interactions, osteogenic differentiation and angiogenic potential was established, to select suitable BG compositions for bone regeneration applications.

#### Primary Normal Human Dermal Fibroblasts with Impaired Migration for *In Vitro* Models of Aged Wound Healing

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In a healthy wound healing response inflammation, new tissue formation and finally remodelling are orchestrated stages leading to tissue repair. Ageing significantly affects wound healing in the geriatric population, who is prone to chronic wounds, which only in the UK cost £4 billion annually. Unfortunately, current treatments for chronic wounds are limited as the cellular and molecular mechanisms behind them have not been elucidated yet, which limits the development of new therapies. *In vitro* models that mimicked the *in vivo* scenario would be advantageous to both elucidate these mechanisms and test new therapies. The development of an *in vitro* model of aged wound healing would require to generate a population of aged dermal fibroblasts, which show deficits in cell migration due to a significant reduction in  $\alpha 2\beta 1$  integrin function. Using Arg-Gly-Asp peptides, which bind to integrins, and cost-effective, simple techniques and equipment we generated primary human dermal fibroblasts (pHDFs) with an impaired migration capacity. A first scan of conditions was tested by alamarBlue<sup>®</sup> on 3 different surfaces: uncoated, fibrinogen and gelatin coated culture plates. Morphology by phase-contrast light microscopy and a scratch assay to test migration were performed on the chosen conditions. A second experiment was carried out to further investigate the chosen conditions in terms of cell number, viability, attachment and morphology (trypan blue assay and immunocytochemistry with confocal microscopy). Results showed that pHDFs treated at the chosen conditions had significantly reduced migration ( $p=0.039$ ). Such population of cells would be useful for *in vitro* models of aged wound healing.

#### Adipose Stem Cell-Enhanced Fat Grafting for Breast Reconstitution

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**Introduction:** Autologous fat grafting is increasingly used for breast reconstruction in oncological patients cured by lumpectomy or mastectomy combined with radiotherapy. The aim of ASC-enhancement of graft was to improve local repair of radiation-treated tissues, reduce apoptosis of grafted adipocytes and reduce time-dependent resorption of transplanted fat. Study was approved by the Bioethical Committee of Polish Ministry of Health.

**Materials and Methods:** 94 ASC-enhanced fat grafted patients, and 27 control, fat-grafted patients were enrolled into study. 150–200 mL of autologous fat was collected using Coleman technique, 50% v/v of collection was immediately processed for ASC isolation, the other part was used for breast reconstruction. Cells were isolated and purified using collagenase/gradient density technique optimized for clinical purposes. Stromal vascular fraction (SVF), containing  $5-8 \times 10^{-7}$  ASC, was suspended in salt solution and injected into reconstituted breast 4 h after fat collection with the long needle. The follow-up observation, lasting 1–3 years, included the search for adverse results, control of transient inflammatory episodes, lymphatic system status, and the rate of fat resorption.

**Results:** No adverse effects of ASC implantation, and no incidents of cancer recurrence were observed. ASC treatment resulted in non-significant reduction of fat resorption and the reduction of inflammatory episodes. In selected patients, treated with ASC in regions of radiation wounds and lymphatic edemas, statistically non-significant improvements have been observed. It may be concluded, that ASC-enhancement of fat graft for breast reconstruction is safe treatment, however, longer observation time is needed for statistical analysis of clinical data.

#### Biodegradable 3D Bone Substitutes Incorporating PRGF for Tissue Regeneration

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Preparation rich in growth factors is an autologous cocktail of proteins obtained from platelet-rich plasma and ready to be used in tissue engineering as it creates an environment enriched in growth factors that participate in several healing stages<sup>1</sup>. Biodegradable polymers and growth factors derived from platelets are being combined using a wide range of methods such as electrospinning or gas foaming, in order to produce biomimetic tissue engineering scaffolds. The aim is to direct tissue regeneration into the implant as it is degraded over time. Biocompatibility, stimulation of proliferation and pore interconnectivity are some of the main requirements that scaffolds must fulfil for tissue regeneration purposes<sup>2</sup>. In this work, porous synthetic scaffolds with an architecture similar to the bone matrix were designed using compressed and supercritical CO<sub>2</sub> green technology. A preparation rich in growth factors (PRGF) was included in order to stimulate bone regeneration. Solvent-free scaffolds with an open structure and interconnected pores were produced through a process that avoids harsh conditions which could degrade or remove the growth factors contained in the PRGF. Fluorescent staining confirmed the homogeneous distribution of the PRGF in the biodegradable scaffolds. Bulk degradation and sustained growth factors release revealed the potential of the scaffolds for tissue regeneration purposes. *In vitro* cell culture showed the positive influence of PRGF in attachment and cell growth, confirming that growth factors maintained their activity after processing.

#### A Perfusable, Hierarchical, and Three-Dimensional Vascular Network in a Collagen Gel

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Vascularization of hydrogel materials remains an unsolved problem in the field of tissue engineering. The lack of a perfusable channel network within a three-dimensional, large-scale tissue engineered construct, which would rapidly transfer nutrients, oxygen, and waste products, leads to necrotic core formation. Thus the field would benefit from a technique that allows a vascular network to be incorporated into hydrogel structures. We report a method for producing a perfusable, hierarchical, and three-dimensional vasculature in a collagen gel. We have used three-dimensional printing to generate a large-scale, perfusable vascular network, and melt spinning to form intermediary to capillary-sized channels. When used in conjunction with co-culture of endothelial and osteoblast cell types, a hierarchy of channels is formed. Detection of angiogenesis can be used as an indicator of a functional vascular system. We have used fluorescent immuno-histochemistry to detect tight junctions between the endothelial cells and sprouting from the templated channels.

#### Coupling Thedes - Therapeutic Deep Eutectic Solvents And Supercritical Fluid Technology for the Development of Controlled Delivery Systems

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Deep eutectic solvents (DES) can be formed by bioactive compounds or pharmaceutical ingredients. A therapeutic DES (THEDES) based on ibuprofen, a non-steroidal anti-inflammatory drug, and menthol was synthesized. A controlled drug delivery system was developed by impregnating a starch:poly-ε-caprolactone polymeric blend (SPCL 30:70) with the menthol:ibuprofen THEDES in different ratios (10



and 20 wt%), by supercritical fluid sintering at 20 MPa and 50 °C. The morphological characterization of SPCL matrices impregnated with THEDES was performed by scanning electron microscopy (SEM) and micro-computed tomography (micro-CT) and drug release studies were carried out at 37°C, in phosphate buffered saline (PBS). The results obtained have shown that the solubility profiles of ibuprofen in PBS are similar in powder form and in THEDES. Nonetheless, when incorporated in a polymeric matrix, ibuprofen in the THEDES form has a faster release profile. The drug release in this system is mostly governed by diffusion, as expected due to the hydrophobic nature of the polymer used as carrier. The outcomes of this work demonstrate the improved performance of THEDES based pharmaceutical formulations and the feasibility to couple with green production technologies to develop enhanced biomaterials for therapeutic applications.

### Biomaterial-Mediated Control over Macrophage Behavior in Bone Regeneration

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The inflammatory response plays a central role in bone repair. Macrophages, the primary cells of the inflammatory response, are recognized as crucial regulators of healing. We have shown that M1 (classically activated) macrophages are required at early stages of healing to initiate angiogenesis, while M2 (alternatively activated) macrophages are needed later to facilitate anastomosis and tissue maturation. Similarly, a rapid - but temporary - period of increased inflammation has been shown to enhance bone regeneration. Recently, Roohani and Zreiqat engineered novel ceramic-based scaffolds using Baghdadite (Ca<sub>3</sub>ZrSi<sub>2</sub>O<sub>9</sub>) and Strontium-Hardystonite-Gahnite (Sr-HT Gahnite, Ca<sub>2</sub>ZnSi<sub>2</sub>O<sub>7</sub>-ZnAl<sub>2</sub>O<sub>4</sub>), and demonstrated enhanced ability to regenerate large bone defects under load compared to clinically utilized tricalcium phosphate-hydroxyapatite (TCP-HA) scaffolds. We hypothesized that interactions with macrophages contribute to the success of these scaffolds to promote tissue regeneration.

We evaluated the behavior of primary human monocyte-derived macrophages on these ceramic scaffolds *in vitro* in terms of gene expression for multiple markers indicative of the M1, M2a, and M2c phenotypes. Interestingly, while TCP-HA scaffolds induced increasing expression of M1 markers over time, Baghdadite promoted a mixed M1/M2 phenotype early and M2-like behavior later, consistent with the M1-to-M2 transition observed in normal healing. Sr-HT Gahnite promoted M2 behavior early and suppressed M1 behavior later. These results suggest that part of the success of these scaffolds may be due to modulation of macrophage behavior, while TCP-HA scaffolds promote chronic inflammation. Improved understanding of the interactions between novel scaffolds and cells of the inflammatory response will aid in designing biomaterials that facilitate bone regeneration.

### Surface Modified Polyurethane Discs Promote Early Angiogenesis in Skin Defects

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**Introduction:** In skin tissue regeneration, the success of three-dimensional scaffolds depends on various qualities such as material, growth factors, and pore size, as they are critical in ensuring efficient revascularization, and cell proliferation. In this study, the biomaterial polyurethane (PU) has been heparin surface modified and coated

with the angiogenic growth factor vascular endothelial growth factor (VEGF) to improve this process.

**Methods:** PU discs (5.4 mm diameter, 300 µm thickness, pore size 150 µm) were coated with heparin and implanted into male C57BL/6J mice via the modified dorsal skin chamber (MDSC). The influence of heparin and VEGF<sub>165</sub> on revascularization was assessed by intravital microscopy (IVM) over a period of 21 days in four groups (n=5), containing the following surface coatings: (I) plain surface (uncoated), (II) low concentration heparin surface (15.1 mg/g), (III) high concentration heparin (84.9 mg/g), (IV) high concentration heparin and VEGF<sub>165</sub> (250 µg/ml). Additionally, immunohistological analysis was performed to test for signs of inflammation.

**Results:** High concentration heparin surface modified PU discs loaded with VEGF<sub>165</sub> showed a marked increase in angiogenesis and healing in comparison to the other experimental groups. Not only does revascularization start as early as day 4, the process is completed earlier with faster wound closure and full integration of PU into the tissue.

**Conclusion:** High concentration heparin coated PU discs loaded with VEGF<sub>165</sub> displayed faster angiogenesis in addition to earlier wound healing and vessel normalization. The use of VEGF<sub>165</sub> may be useful in applications where a rapid revascularisation is required.

### Hyaluronic Acid-Based Materials for Bone Tissue Engineering

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Hyaluronan (HA) is a naturally occurring glycosaminoglycan and it is one of the major components of extracellular matrix and connective tissues. HA plays an important role in biological processes, such as morphogenesis, cell migration and differentiation. One of the promising applications of HA is its use in tissue engineering as a material for a hydrogel and derived types of scaffolds.

The aim of this work is to develop HA hydrogel-based materials for support of bone defects healing. For this purpose, a derivative of hyaluronic acid is used, which can undergo noncytotoxic cross-linking reaction initiated by an addition of H<sub>2</sub>O<sub>2</sub> and mediated by horseradish peroxidase. This system is a non-cytotoxic and besides a possibility of the cells incorporation into the hydrogel structure also enable an *in situ* crosslinking reaction and therefore it allows the possibility of injection application and hydrogel forming into the defect. For a closer approximation to the natural structure of a native bone tissue, calcium phosphate particles are incorporated into the hydrogel structure.

The results achieved up to now show that materials could reach suitable mechanical and viscoelastic properties as a filler for non-loadable bone defects. Also, the results obtained from *in vitro* cultivation of mesenchymal stem cells implemented into these materials indicate that our materials provide an appropriate environment for their proliferation and producing corresponding extracellular matrix. All these results suggest that our new design hydrogel based materials are promising candidates for tissue engineering in the field of bone tissue regeneration.

### Therapeutic Deep Eutectic Solvents as Solubility Enhancers for Different Active Pharmaceutical Ingredients

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In recent years, deep eutectic solvents (DES) have emerged as a sustainable alternative to ionic liquids mainly due to their lower production costs and higher biodegradability/ biocompatibility. DES are defined as a mixture of organic compounds with a melting point significantly lower than that of either individual component. DES constituted by bioactive substances or active pharmaceutical ingredients (APIs), which we define as therapeutic deep eutectic solvents

(THEDES), are a promising source for several pharmaceutical and biomedical applications due to their increased ability to dissolve model drugs by increasing their solubility, permeation and absorption. In this work, we synthesized several THEDES based in choline chloride (ChCl) or menthol conjugated with three different APIs: acetylsalicylic acid (AA), benzoic acid (BA) and phenylacetic acid (PA) with different molar ratios (ChCl:AA 1:1, ChCl:PA 1:1, Menthol:AA 3:1, Menthol:BA 3:1, Menthol:PA 2:1, Menthol:PA 3:1). All THEDES produced were characterized by differential scanning calorimetry and polarized optical microscopy. Dissolution studies performed in PBS for 24 hours shown that THEDES increased significantly the dissolution of all APIs studied compared to the control (APIs only). This increased solubility was even more noticeable for the cases of THEDES with menthol in their composition. Antibacterial activity was assessed using *E. coli* and *B. subtilis* as gram negative and gram positive model organisms. Results show that all the THEDES had antibacterial activity. In overall, our results suggest the great promise of using THEDES as solubility enhancers in the development of novel and more effective drug delivery systems.

### Study of the Effect of Collagenous Microcarriers on the Vasculogenic Potential of Endothelial Cells Obtained from Different Sources

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The development of an efficient vascular network in tissue engineering (TE) is still a challenge, since in most cases the blood vessels formed are not fully functional (no lumen or leaking of material). In consequence, there is low rate of graft survival after implantation, caused by poor supply of oxygen and nutrients to the cells. This supply is dependent on the vessel structures, mainly formed by endothelial cells (ECs). In their role of building and remodelling blood vessels, ECs have the capability to proliferate, migrate, adhere, sprout and form vessel-like structures. In this study we want to standardize the isolation of ECs from different sources, to further determine their suitability as vascular cell type for TE, by studying the aforementioned properties when cultured on collagenous microcarriers. Two EC sources are studied: skin (human dermal microvascular endothelial cells, HDMECs) and peripheral blood (endothelial progenitor cells, EPCs). The isolation protocols are focused on obtaining a characteristic, viable and large population of cells with minimal invasion procedures to the patient. The ECs obtained are cultured in 2D conditions and then transferred to dynamic conditions on collagenous microcarriers. After 7 days of culture, sprouting assay in type I collagen is performed. Growth factors such as VEGF, FGF and Ang1 are used as pro-angiogenic factors. Additionally, these colonized microcarriers are used to re-seed the lumen of the biomatrix BioVaSc<sup>®</sup> in order to provide vascular support to any cell type used for differentiation into tissue-like substitutes (e.g. hMSCS for bone substitutes).

### Tuning Architecture and Dimensionality of the ECM Microenvironment *in vitro* unlocks Brown Adipogenic Potential in Adult Human Bone Marrow-derived MSCs

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Brown adipose tissue, comprising classical brown and beige adipocytes, holds therapeutic and diagnostic promise for treatment of diabetes and metabolic syndrome. Key to realizing this potential is the ability to generate functional human brown adipocytes from a renewable, easily accessible and safe tissue source of progenitor cells, and an efficacious *in vitro* differentiation protocol. We demonstrate the generation of a functional brown phenotype from adult human bone marrow-derived mesenchymal stem cells (bmMSCs) under macromolecular crowding (MMC), a biophysical principle applied to cell culture in order to mimic a more crowded *in vivo* environment by sterically excluding volume and speeding up the rate of extracellular matrix formation. Adipogenic differentiation of bmMSCs yielded substantial UCP1 expression only under MMC. The MMC-generated adipocytes, stimulated by forskolin, underwent mitochondrial membrane depolarisation, uncoupled respiration and increased oxygen consumption. Adipocytes generated under MMC showed increased deposition of collagen IV and heparan sulphate proteoglycans, indicative of a richer pro-adipogenic matrix, which through cell-matrix reciprocity would drive adipogenesis of bmMSCs into a more mature phenotype. In addition, MMC also induced "browning" in bmMSC-derived white adipocytes. Mechanistically, MMC creates a 3D extracellular matrix architecture encasing the maturing adipocytes in a collagen IV cocoon that is reminiscent of what is observed *in vivo*. This basement membrane structure allows for increased cell-matrix engagement which in turn leads to an enhanced downstream phosphorylation of ATF2, a key transcription factor in UCP1 regulation. Thus, tuning the dimensionality of the microenvironment *in vitro* can unlock a strong brown potential dormant in bone marrow.

### Hypoxia-Mimicking Bioactive Glass/Collagen Glycosaminoglycan Composite Scaffolds to Enhance Angiogenesis and Bone Repair

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One of the biggest challenges in tissue engineering is promoting vascularisation of scaffolds. One approach to overcome this targets the Hypoxia Inducible Factor (HIF-1 $\alpha$ ) pathway, which responds to low oxygen concentration (hypoxia) by activating pro-angiogenic genes including vascular endothelial growth factor (VEGF). Cobalt ions mimic hypoxia by stabilising HIF-1 $\alpha$ . The aim was to incorporate cobalt bioactive glass into CG scaffolds developed for bone tissue regeneration to enhance the initial angiogenic step vital for bone regeneration. Resorbable bioactive glass particles (38  $\mu$ m and 100  $\mu$ m) with cobalt ions within the glass network were used to create bioactive glass/collagen-glycosaminoglycan scaffolds by a lyophilisation method. Inclusion of the bioactive glass improved the compressive modulus of the scaffolds while maintaining high degrees of porosity (>97%). *In vitro* analysis demonstrated that incorporation of cobalt bioactive glass (100  $\mu$ m sized particles) significantly enhanced the production and expression of VEGF in endothelial cells, and conditioned media from this scaffold promoted enhanced tubule formation as measured using enzyme-linked immunosorbent assay, RT-PCR and Matrigel assays respectively. Furthermore, our results prove the ability of these scaffolds to support osteoblast cell proliferation and osteogenesis in all bioactive glass/collagen-glycosaminoglycan scaffolds irrespective of the particle size as measured by alkaline phosphatase, calcium assay and alizarin red staining of scaffolds. In summary, we have developed a hypoxia-mimicking tissue-engineered scaffold with pro-angiogenic

and pro-osteogenic capabilities that may encourage bone tissue regeneration and overcome the problem of inadequate vascularisation of grafts commonly seen in this field.

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### Involvement of Macrophages Reverses the Osteogenic Effects of Cobalt Incorporated $\beta$ -tricalcium Phosphate: The Role of Osteoimmunomodulation When Evaluating and Developing Bone Substitute Materials

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Osteoblast lineage cells are direct effectors of osteogenesis and commonly used to evaluate the *in vitro* osteogenic capacity of bone substitute materials. This strategy has achieved a degree of success when developing bone biomaterials; however, inconsistent results between *in vitro* and *in vivo* studies are common, suggesting the mechanisms that govern the material's capacity to mediate osteogenesis is still not well understood. The emerging field of osteoimmunology and immunomodulation in osteogenesis has informed a paradigm shift in our view of bone biomaterials from one of an inert to an osteoimmunomodulatory material-highlighting the importance of immune cells in the material-mediated osteogenesis. Here we evaluated a potential bone substitute material cobalt incorporated  $\beta$ -tricalcium phosphate (CCP) using both a traditional and a novel approach to assess osteogenesis, the latter including the use of immune cells. It was found that CCP extract by itself was sufficient to enhance osteogenic differentiation of bone marrow stem cells (BMSCs), whereas this effect was attenuated when macrophages were involved. In response to CCP, macrophages switched M1 phenotype extreme, releasing pro-inflammatory cytokines and bone destructive factors. When the CCP materials were implanted into a rat femur condyle defect model, there was an increase of inflammatory markers and bone destruction, coupled with fibrous encapsulation rather than new bone formation. These findings demonstrated that the inclusion of immune cells (macrophages) in the *in vitro* assessment matched the *in vivo* tissue response, and provides a more accurate indication of the essential role of immune cells when assessing material-stimulated osteogenesis *in vitro*.

### Using Electrospun Scaffolds to Promote Macrophage Phenotypic Modulation and Support Wound Healing

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Macrophages (MACs) play a critical role in inflammation. MACs have been found to possess the potential to be activated by external factors from their M0 inactive state to an M1 pro-inflammatory or M2 regenerative state; and to date, researchers are still trying to elucidate the impact of these factors on MAC phenotype. This study provides a comprehensive evaluation of MAC phenotype in response to electrospun scaffolds of varying material (silk fibroin, gelatin, and polycaprolactone), fiber/pore diameter, fiber stiffness, and +/- inclusion of platelet-rich plasma (PRP).

Scaffolds were electrospun at varying concentrations, to a range of fiber/pore sizes with or without PRP. Initial atomic force microscopy data showed that these materials also possessed a wide range of individual fiber elastic moduli. Cell proliferation data found that silk scaffolds incorporated with PRP promoted the highest proliferation. At the time of submission, MAC chemotaxis, cytokine release profiles, immunohistochemistry, and angiogenesis assay data were being evaluated to correlate MAC phenotype with scaffold properties.

Previous studies have shown that a higher pore size/fiber diameter leads to the differentiation of M2 like MACs. Additionally, fiber stiffness may induce phenotypic modification. Preliminary data has found that the addition of PRP led to increased pore size and silk

scaffolds doped with PRP created fibers with lower moduli. Further testing will optimize scaffold composition and air-impedance electrospinning will be utilized to further increase scaffold porosity to M2 phenotype. This data may be used to create an idealized scaffold with the potential to modulate MAC phenotype to promote wound healing.

### Bottom up Approach in Modular Tissue Engineering of a Vascularized Tissue

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Mass transfer is considered as the most important culprit for the failure of an engineered tissue construct post implantation *in vivo*. *In vitro* for tissues (> 500  $\mu$ m), mass transfer is met via bioreactors, whereas *in vivo*, due to lack of a functionalized vasculature, hypoxia driven central necrosis causes construct failure. Top down approach to vascularize a tissue construct has limited success, due to lack of rapid, extensive, anastomosed and perfused vasculature as seen *in vivo*. Modular tissue engineering is emerging to address these limitations by fabricating constructs from the bottom up. In the present study we have synthesized HUVEC microtissues by standard hanging drop method. These microtissues were embedded in goat tendon collagen type I-fibrin hydrogel and were studied for angiogenic morphometric parameters viz., vessel volume, branching points, inter and intra connectivity, vascular orientation, average segment length and diameter. Microtissue size, gel composition, strength and media conditions affected the morphometric parameters. Microscopic analysis demonstrated endothelial cell sprouting within 12 h, where stack cells were guided by tip cell. Tube extension was highest between 24-48 hour. Tubes were stable for three days without pericytes and regressed thereafter. The prevascularized hydrogel incubated for 48 h can be co-cultured with microtissues of desired cells and can be implanted *in vivo*. The rudimentary blood vessels from the prevascularized hydrogel then can anastomose with the host vasculature that can further attain stability by recruiting host pericytes. This procedure may result in an engineered tissue construct of clinical relevance that may circumvent mass transfer issue.

### Polymer-Bioglass Scaffolds to Augment Bone Regeneration

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Bioactive glasses are regarded as an important bone regeneration material owing to their resorbable and osteoinductive properties, in addition to an established bone bonding capability. Substitution of the bioglass structure with a range of inorganic ions implicated in bone metabolism including magnesium (Mg) and strontium (Sr) has been investigated to augment the bioactive potential. In the present study, ionic substitution of the bioglass (SiO<sub>2</sub>-P<sub>2</sub>O<sub>5</sub>-Na<sub>2</sub>O-CaO) structure was undertaken through incorporation of Sr or a combination of cobalt (Co), zinc (Zn) and Mg. Porous organic-inorganic, cylindrical composite scaffolds were prepared by gas-foaming PLG (85:15) polymer with the substituted bioglasses. The morphology, bioglass distribution and ion release were evaluated using SEM-EDS and ICP-AES. The impact on the viability and cell response of a clonally derived murine MSC line, D1, was investigated using WST-1 assay, cell proliferation counts and biochemical analysis. *In vivo* evaluation of hMSC seeded scaffolds was undertaken using a subcutaneous rat model at 3 and 6 weeks. The cell viability and metabolic activity of D1 cells cultured with Sr conditioned media was similar to the control but cell proliferation was lower in cells supplemented with the CoMgZn dissolution products. Gene expression analysis of Sr scaffolds demonstrated an increase in the expression of osteogenic markers (bone sialoprotein, osteonectin and collagen type I) compared to controls. Strontium-containing scaffolds also reached peak alkaline phosphatase levels at earlier time points compared to PLG controls or scaffolds containing the growth factor

BMP-2 highlighting the role of the ionic dissolution products in eliciting bone growth.

#### Evaluation of Osteoblast-like Cell Behavior in Core-shell Alginate - Gelatin Microcapsules

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Modified alginate hydrogels are the subject of intense interest for their use as cell carriers in bone tissue engineering. The object of this study is utilizing a simple one-stage technique which offers numerous attractive features for manufacturing applicable gelatin-alginate core-shell microcapsule by improved microenvironment for superior cell expansion and functional activity. For this purpose, co-axial nozzle was applied to produce uniform core-shell microcapsule by electro spraying technique. MG63 osteoblast-like cells was examined as a model for evaluation microcapsule application in bone tissue engineering. Cell proliferation and activity were considered by hemocytometer cell counting method and MTT essay respectively. The functional activity of the MG63 cells was examined by measuring the ALP activity and calcium deposition. The effect of various core materials (blends of gelatin to alginate ratio: 2.5, 5, 10, 20, 40 w/w) and initial cell density ( $1.5-4.5 \times 10^6$  cell/ml) were considered on cell proliferation and functional activity. Alginate 1% w/v was chosen as shell. The results showed that a significantly higher proliferation about 18.7-fold after 4 weeks by initial cell density  $3 \times 10^6$  cell/ml. Moreover increasing gelatin/Alginate enhanced cell proliferation and ECM deposition in the core. Alizarin red staining and ALP assay results identified these results noticeably. In conclusion, uniform cell distribution, high cellularity, high density and dense ECM formation could be achieved in the core. The present study demonstrated that core-shell microcapsules have high potential for using in bone tissue engineering bottom-up approach with cell-laden microcarriers.

#### Differentiated Induced Pluripotent Stem Cells Grown on Xenogenic Decellularized Scaffold as a Possible Approach to Circumvent Xenograft Rejection

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**Introduction:** The shortage of donors for organ failure patients highlights the need for alternatives. Xenotransplantation provide an option, yet a number of hurdles needs to be addressed. Here we investigate the interaction of decellularized rat heart with human vascular endothelial cells and human induced pluripotent stem cells in 3D culture, and the scaffold ability to support cellular growth.

**Material and Methods:** Decellularization was achieved by retrograde coronary perfusion through the aorta with 1% SDS, 1% Triton-X 100 and PBS.

**Recellularization:** In 3D culture, scaffold was seeded with Human vascular endothelial cell through the intramural injections into the left ventricle, of human induced pluripotent stem (iPS) cell-derived cardiomyocytes. The construct was in the bioreactor for more than 3 weeks after. 2D culture was carried in Parallel as well.

**Histology and immunohistochemistry:** Sections were tested using H&E, collagen, and elastin staining, along with Endothelial and cardiac cells markers detection.

**Results:** Our results showed an intact scaffold, free of cells or nuclei. The scaffold integrity and ability to support cellular growth was confirmed in re-endothelialization experiments using (HUVECs). In addition the growth and contractility of differentiated human cardiac myocytes derived from IPS cells for more than 3 weeks.

**Discussion:** Our preliminary results indicate the possibility of using human cardiac derived iPS cells to populate xenogeneic scaffold, and studying the xenogenic interactions in the absence of donor derived cells.

#### Development and Characterization of a Decellularized Mitral Valve Scaffold

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Mitral valve regurgitation is the second most common cause for heart valve surgery. Replacement options include mechanical valves, which are durable but require lifelong anticoagulation therapy; and bioprosthetic valves, which don't require lifelong anticoagulation but are less durable. Neither option offers the opportunity for growth and remodeling. Tissue engineering is a potential solution for these problems. The aim of this study was to develop and characterize a decellularized mitral valve scaffold for whole mitral valve replacement.

Porcine mitral valves were aseptically dissected within 4 hours of slaughter. The valves were disinfected with antibiotics and placed in hypotonic buffer, followed by a 0.5% SDS and 0.5% sodium deoxycholate solution for 36 h, nucleic acid digestion, and extensive washing steps. Finally, they were sterilized in 0.1% peracetic acid. The valves were characterized by histological staining (H&E, DAPI, Masson's trichrome, van Gieson's and Alcian blue PAS), immunohistochemistry (anti-collagen IV, and anti-alpha gal), DNA quantification, biochemical assays (collagen, denatured collagen, and sulfated glycosaminoglycan quantification), and uniaxial tensile tests.

Results indicate a conserved hstoarchitecture devoid of cell nuclei, with conserved collagen and elastin fiber hstoarchitecture. Glycosaminoglycans and collagen IV were lost after decellularization and there was a significant reduction in DNA content. Alpha-gal was reduced but still present. The tissue biomechanical properties were not significantly affected.

The protocol effectively decellularized the mitral valve, and produced a good quality scaffold that could potentially be used for mitral valve replacement using human allografts.

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#### Engineering Bioartificial Lymph Nodes using Polycaprolactone Scaffolds

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**Objective:** Treatment of chronic lymphedema is still a significant medical and social problem. In recent years microvascular lymph node transplantation gains more and more popularity. We would like to introduce a novel approach of bioengineered lymph nodes.

**Methods:** Lymph nodes from human beings were used which were mechanically shredded. The tissue was placed on polycaprolactone scaffolds fixed by Matrigel. We implanted them into the groin region nearby the femoral artery of 14 immunodeficient nude mice. The first group was explanted after 8 weeks and the second group after 16 weeks. All tissue samples were histologically examined using H&E staining and LYVE1 antibodies for detection of lymph vessels. We also performed a digital imaging *in vivo* for the second group using MSOT.

**Results:** The bioartificial lymph nodes showed a good vascularisation compared to the control site and were accepted by the recipient organism without any complications. Mechanical shredding of the lymph nodes did not significantly effect the survival of

lymphatic cells. *In vitro* studies indicate excellent cell attachment, proliferation and migration on PCL-scaffolds.

**Conclusion:** The microvascular lymph node transplantation is a time consuming procedure. Therefore we developed bioartificial lymph nodes as a new approach for the treatment of lymphedema in a short and less risky way. Things still to clear out are the optimal fragment size and effective cultivation of lymph node tissue for the increase of bioartificial lymph nodes using just 1 or 2 lymph nodes to minimize donor site morbidity and simultaneously maximize the amount of bioartificial lymph nodes.

#### Basal Osterix Expression Designates MSC Populations Capable of *In Vitro* Osteogenesis

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Mesenchymal stem cells (MSC) are, by definition, capable of tri-lineage differentiation; however, MSCs isolated from different sources demonstrate clear distinctions in osteogenic capacity. This study compared osteogenic capacities of MSCs from bone marrow (BM), synovium (SYN) and adipose tissue (FAT), to identify the molecular basis for differential osteogenic capacity in these populations.

Bone marrow, adipose tissue and synovial cells were collected from six adult horses and expanded through two passages. Third passage cells remained in basal medium or were transferred to osteogenic medium. Osteogenesis was assessed after 7 and 14 days, by Alizarin Red (AR) staining, alkaline phosphatase (ALP) up-regulation and osteogenic transcript (Runx2 and Osterix) up-regulation. Responses were assessed by two-way repeated measures ANOVA and Holms-Sidak post hoc tests.

Under basal conditions, only Osterix mRNA was elevated in BM cells, compared to SYN and FAT cells. BM monolayers developed aggregates that stained strongly with AR by day 7. Staining was delayed and less intense in SYN and FAT cultures. BM cells increased ALP activity, along with ALP, Runx2 and Osterix mRNAs. ALP activity was increased in FAT cultures, but no other osteogenic marker increased in FAT or SYN cultures during the 14 days in culture.

BM cells were significantly more responsive to osteogenic conditions than SYN or FAT cells. Elevated 'basal' Osterix expression in undifferentiated BM-MSCs likely 'primes' these cells for osteogenesis, while the inability of SYN and FAT cells to up-regulate Runx2 and Osterix mRNA expression under osteogenic conditions signifies a molecular constraint to osteogenesis in these populations.

#### Composition and Dynamic Development of Extracellular Matrix during Adipogenic Differentiation of Adipose-Derived Stem Cells in a Three-Dimensional Environment

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For the successful engineering of adipose tissue, a fundamental understanding of adipose tissue development is a prerequisite. Recently, it has been increasingly acknowledged that the extracellular matrix (ECM) is an important modulating factor in the process of adipogenesis and the function of mature adipocytes. To get an insight in ECM and tissue development in a more *in vivo*-like context, the dynamics of major ECM components during adipogenesis of human adipose-derived stem cells (hASCs) in different 3D environments (hydrogel-based constructs and cell carrier-free spheroids) were investigated in comparison to conventional 2D monolayer culture. By immunohistochemical analysis, dynamic changes in expression and structure of major ECM components during adipogenesis were revealed under 2D and 3D conditions. Whereas the development of col

IV was found to be highly linked to adipogenesis regardless of culture dimensionality, distinct differences in the expression of col I and laminin emerged during adipogenic differentiation between 2D and 3D culture. This differential ECM expression coincided with a distinct adipogenic differentiation behavior of the cells depending on the culture system, suggesting a specific role of these ECM components in the process of adipogenesis. Especially laminin is in the focus of current investigations in order to elucidate its possible role in the adipogenic differentiation of hASC. Altogether, the results of this work contribute to a better understanding of the impact of specific ECM components on the differentiation process and may also aid in the rational engineering of adipose tissue implants in the long term.

#### Treatment of Chronic Non-union in a Critically-sized Rat Femoral Defect Model

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Over 600,000 bone grafting procedures are performed annually in the US, costing over \$5 billion. Despite improvements in recent years, delayed union and non-union are still common, often resulting in multiple revision surgeries involving more grafts and/or BMP delivery. Yet, the pathophysiology of non-union remains poorly understood and optimal treatments for these cases are unclear and may even require considerations different from treatment of primary injuries.

Our goal was to better understand the healing of bone defects originally fated for non-union, but subsequently subjected to a revision surgery and delayed treatment with BMP-2 - a clinically relevant therapeutic route. We have previously shown that immediate delivery of 2.5 µg BMP-2 to a critically-sized femoral bone defect was sufficient to heal the defect. We hypothesized that this minimum healing dose would not be adequate to heal a defect that has progressed 3 weeks towards non-union. Preliminary results suggest that even delayed delivery of BMP-2 in our hybrid delivery system results in functional regeneration. After 12 weeks, new mineral levels were comparable to historical data of healed defects, and torsional testing of the femurs revealed mechanical properties that were within the range of native bone. Forthcoming work will test even longer term establishment of non-union (8 weeks), characterize early gene expression differences between healing and non-union defects, and examine the effect of delayed BMP-2 treatment. This study in a preclinical animal model of chronic non-union will lead to better understanding and improved regenerative strategies for a significant, currently underserved patient population.

#### Effects of Platelet Rich Plasma on Bone Marrow Derived Mesenchymal Stem Cells and Adipose Stem Cells for Cartilage Repair

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Post-traumatic and focal degenerative cartilage defects of the knee affect over 3 million Americans annually. Focal cartilage repair procedures, such as matrix assisted autologous chondrocyte implantation (MACI), involve the introduction of autologous cells with extracellular matrix to the defect site. However, MACI is complicated by the need for *ex vivo* cell expansion and iatrogenic damage to host cartilage caused by autologous chondrocyte harvesting. Chondrogenic stem cells are thus considered a promising cell type for cartilage repair. This research aims to test the use of platelet-rich plasma (PRP), which has shown ability in promoting stem cell proliferation and tissue healing, to enhance chondrogenic differentiation of human mesenchymal stem cells derived from bone marrow (BM-MSCs) and infrapatellar fat pad (IFP-ASCs).

BM-MSCs and IFP-ASCs were placed in high density pellet cultures and maintained in serum-free, TGFβ chondrogenic medium supplemented PRP at different concentrations (1, 5, 10, and 20%) for

different durations (1-, 3-, 7-, and 21-day pulses starting at the beginning of culture period). After culture day 21, RT-PCR and histological staining showed that short, 1- and 3-day pulses of low concentration (1%) of PRP enhanced cell proliferation and TGF $\beta$ -stimulated chondrogenesis. On the other hand, high PRP concentrations delivered over 7–21 days decreased proteoglycan deposition as compared to TGF $\beta$ +PRP- controls, and increased catabolic gene expression (MMP13 and collagen type X). These *in vitro* findings suggest that for the purpose of enhancing MSC chondrogenesis, treatment with PRP must be at low concentrations and for short durations. (Support: US Department of Defense - W81XWH-14-2-0003)

#### Comparison of the Viability Indicators of Xenon Clathrate Preserved Skin and Other Preservation Methods on the Animal Engraftment Model

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At present, work on the applicability of the xenon clathrate preservation protocol has been explored for the live tissue storage processing. The custom-made gas chamber with temperature and gas pressure regulation was used for modeling and keeping up the adjusted conditions. Skin grafts were obtained from the 28 albino rats tails and separated into four groups. The experimental groups were the following: 10% DMSO in RPMI-media with deep-freezing (195K), Xenon hyperbaric (30–60 psi, 277K) and Xenon clathrate (90–105 psi, 277K). Skin samples were auto-transplanted after 7 days preservation on the new freshly-made tail places. As a control measure, the unpreserved fresh skin samples were auto-transplanted immediately. We immediately assessed the viability of the graft's sections after one week preservation by 5 microscopic criteria in specimen stained by hematoxylin eosin. The engraftment was assessed 14 days after transplantation by 3 macroscopic criteria. Each criterion consisted of 2 scores, and the total sum of criterion scores was calculated in each preservation group. Then groups were compared with each other. In the Xe clathrate preservation group, we observed significantly better engraftment and microstructure of dermal and epithelial layers that were comparable with unpreserved fresh skin. Contrariwise, the usual method with DMSO as a main preservation agent and Xe hyperbaric protocol showed more destruction in all skin layers and the lowest engraftment. Apparently, using the Xe clathrate storage processing in accordance with the described conditions, it is able to provide opportunity for saving the viability of any types of tissues, engineered or donor grafts.

#### Combination Regenerative Modalities in the Reconstruction of Traumatic Soft Tissue Defects: Application of Spray Skin Technology with Dermal Regenerate Templates

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**Purpose:** Full thickness soft tissue (FTST) defects require approaches to restore the functional properties of skin. The reconstruction of FTST deficits using a dermal regenerate template (DRT) with a spray-on epidermal regenerate in comparison to DRT with traditional skin grafting (STSG) will be compared.

**Methods:** A retrospective review of two patients treated with a DRT (Integra<sup>®</sup> bilaminar DRT) in combination with spray-on skin technology (ReCell<sup>®</sup>) and two patients treated with the same DRT with traditional STSG was performed. Recipient and donor site de-

fect sizes, time to re-epithelialization, outcomes, and complications were reviewed.

**Results:** The four patients were males between 21–30 years-old, three suffered blast injuries with the other suffering a motorcycle crash injury. Our DRT/ReCell cohorts had an average recipient wound defect of 895 cm<sup>2</sup> versus 1378 cm<sup>2</sup> for our DRT/STSG cohort. The average skin donor site size for the DRT/ReCell cohort was 301 cm<sup>2</sup> versus 1438 cm<sup>2</sup> for the traditional cohort. The recipient sites were healed on average 2 weeks earlier and had re-pigmentation of the recipient sites which were better matched to the patient's native skin color within the DRT/ReCell cohort as compared with the traditional cohort.

**Conclusion:** Staged use of dermal regenerate matrices with spray skin epidermal regenerates may offer safe, effective treatment for FTST defects while decreasing donor skin grafting morbidity. This technique has shown to provide functional skin coverage, a greater expansion ratio, may minimize donor site morbidity, affords improved re-pigmentation strategies, and may enable faster recipient site healing.

#### Zone-Specific Collagen Alignment in Porous Polymer Scaffolds for Articular Cartilage Tissue Engineering

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Articular cartilage is organized into distinct functional layers characterized by varying stiffnesses, collagen alignments, and chondrocyte phenotypes. We have designed a poly( $\epsilon$ -caprolactone) (PCL) scaffold, which incorporates the structural and mechanical anisotropy of native tissue. Our scaffold design integrates a fibrous superficial zone that provides tensile strength, an isotropic foam intermediate zone, and a stiff vertically aligned deep zone, while maintaining full interconnectivity, mechanical fusion, and >90% porosity.

Scaffolds exhibited intermediate and deep zone Young's moduli of 38 $\pm$ 6 kPa and 2.0 $\pm$ 0.7 MPa, respectively, at 10% strain, that mimic stiffnesses within the physiological range and result in a bulk modulus of 640 $\pm$ 40 kPa. Scaffolds were validated *in vitro* with bovine chondrocytes up to 12 weeks, exhibiting full cellular penetration from a single seeding injection. Furthermore, chondrocyte-specific gene expression, and glycosaminoglycan and collagen II production sufficiently filled the scaffold pores. The ECM produced *in vitro* was analyzed by multi-photon microscopy and found to possess differential zonal alignment, with more mature collagen fibrils with parallel alignment at the superficial zone, random alignment in the intermediate zone, and perpendicular alignment to the articulating surface in the deep zone.

To our knowledge this is the first multi-layered porous polymer cartilage scaffold to possess an interconnected gradient of physiologically-relevant stiffnesses and induce zonal collagen alignment comparable to the native tissue *in vitro*. A preliminary *in vivo* validation of acellular scaffolds in a porcine osteochondral defect model indicates full tissue penetration at 3 months, additional *in vivo* investigations are ongoing.

#### Engineering Tubulous Cartilage Tissue Utilizing Eclotropun Gelatin-polycaprolactone Membranes for the Repair of Trachea

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**Objective:** Electrospinning materials have attracted increasing attentions and have been widely researched in field like tissue engineering in recent decades, of which, gelatin-polycaprolactone nanofiber membranes got much consideration. This study was aimed to investigate the feasibility of utilizing this biodegradable electrospun gelatin-polycaprolactone membranes accompanying with chondrocytes to construct tubulous cartilage with the assistance from inner supporters.

**Methods:** The chondrocytes were isolated from auricular cartilage of neonatal swines and applied to construct tubulous cartilage after expansion to second generation *in vitro*. The biocompatible and biodegradable scaffolds were fabricated via electrospinning with gelatin and polycaprolactone (GT:PCL=50:50). The membranes were tailored into rectangle-shape and rolled onto the inner supporters after chondrocytes seeding and diffusing on it. The tubulous cartilage was finally harvested after days of culture *in vitro* and weeks of subcutaneous incubation in nude mice. To evaluate the tubulous cartilage engineered, gross observation, histological and immunohistological staining, glycosaminoglycan (GAG) analyses, and Young's modulus measurement were performed.

**Results:** Not only the gross observation but also the histological and immunohistological identifications demonstrated the formation of tubulous cartilage tissue assembling native tracheal cartilage tissue. GAG analyses proved well matrix-secretion of chondrocytes and Young's modulus analysis further showed favorable mechanical properties. Moreover, the engineered tubulous cartilage performed even much better with the extension of time *in vivo*.

**Conclusions:** Electrospun Gelatin-Polycaprolactone nanofiber membrane was a promising candidate for engineering tubulous cartilage tissue. And the histological components as well as mechanical properties of the engineered tubulous cartilage were also qualified for trachea defect repair.

#### Responsiveness of Monocytes to Micropatterns under Biochemical Stimulation

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Macrophages are an important part of host response to implanted materials. They are persistent on the surface of implanted materials and their phenotype and related cytokine release profile are crucial for development of a pro- or anti-inflammatory microenvironment around implants. THP-1 monocytic cell line has been widely used for macrophage biology, since with PMA treatment a phenotype in THP-1 cells similar to that of monocyte derived macrophages can be obtained. As surface topography is an important modulator of cell behavior (1); we studied the effect of gelatin-based micropatterned (microgrooves of 2, 5, 10, 20 and 40  $\mu\text{m}$  width) surfaces with respect to their ability to drive THP-1 cell attachment on the surfaces as a model of monocyte interaction with biomaterial surfaces. In order to induce specific cytokine microenvironments, M1 phenotype and M2 phenotype inducing media were used and the macrophage behavior was quantified with time lapse microscopy, image analysis and cytokine level detection (ELISA). Cells in 20  $\mu\text{m}$  grooves conformed to the patterns and micropatterned surfaces in-

duced more cell attachment and directed cell movement; both in M1 and M2 inducing conditions. For the cytokine release, the microenvironment was the dominating factor for the release pattern of IL-1 $\beta$ , IL-1RA, TNF- $\alpha$ , IL-4, IL-10, IL-12 and CCL-18. Concomitant use of cytokine delivery and micropatterns can control the attachment of macrophages and their phenotypes in order to induce a remodeling microenvironment around implants.

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#### Intercalated 3-D Micro Blood and Lymphatic Vascular Plexuses for Organ Tissue Engineering

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Organ tissue engineering, including cardiovascular tissues, has been an area of intense investigation. The major challenge to these approaches has been the inability to vascularize and perfuse the *in vitro* engineered tissue constructs. Attempts to provide oxygen and nutrients to the cells contained in the biomaterial constructs have had varying degrees of success. The aim of this current study is to develop a three-dimensional (3-D) model of vascularized cardiac tissue to study the concurrent temporal and spatial regulation of cardiomyogenesis in the context of postnatal de novo vasculogenesis during stem cell cardiac regeneration. In order to achieve the above aims, we have developed an *in vitro* 3-D functioning vascularized cardiac muscle construct using ventricular embryonic cardiac myocytes (ECMs) and mesenchymal stem cells (MSCs). Firstly, to generate the prevascularized scaffold, human cardiac microvascular endothelial cells (HMVEC-C) and MSCs were co-cultured on a 3-D collagen cell carrier (CCC) for 7 days under vasculogenic culture conditions, HMVEC-C/MSCs underwent maturation and differentiation characteristic of micro vessel morphogenesis and formed extensive plexuses of vascular networks. Next, the ECMs and MSCs were co-cultured onto this generated prevascularized CCCs for further 7 or 14 days in myogenic culture conditions. Following these co-culture conditions, the vascular and cardiac phenotypic inductions were analyzed at the morphological, immunological, biochemical, molecular and functional levels. Expression analyses of the differentiated cells revealed neo-cardiomyogenesis, neo-angiogenesis and/or neo-lymphangiogenesis. Thus, our unique 3-D co-culture system provided us the apt *in vitro* functioning prevascularized 3-D cardiac patch that can be utilized for myocardial repair and/or regeneration.

#### Wound Healing, Skin Regeneration and Tendon Repair with MSC-sourced Exosomes: Functional Evaluation *In Vitro* and *In Vivo* and Methods of Manufacture

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Mesenchymal stem cells (MSCs) are characterized by their self-renewing capacity and their ability to differentiate into chondrocytes, adipocytes, and osteocytes. It has been suggested that much of the observed benefit of these stem cell injections arises from stem cell-secreted factors carried in discrete microvesicles called exosomes. These small vesicles contain bioactive components related to wound healing and present a potential new allogeneic therapy for dermal repair and regeneration. Here, we present our findings on the physical and functional characteristics of MSC exosomes relevant to wound healing and tissue regeneration. Data is presented on the miRNA profile of these exosomes, their ability to mediate cell migration and incorporate into the recipient cell membrane, and their ability to down-regulate STAT3 phosphorylation. Scalable production of exosomes was accomplished using a hollow-fiber bioreactor, whereby the total bioreactor yield was approximately 10-fold more than T25 flask

controls based on exosome number and at a 10-fold higher concentration as well. We evaluate the application of exosome preparations sourced from MSC and other stem and progenitor cell populations in rodent models of wound healing and skin repair; preliminary, proof-of-concept data will be presented. Having successfully developed scalable exosome production, isolation procedures, and *in vitro* and *in vivo* assays to functionally characterize these particles which are secreted by the cultured MSCs and other cell populations, our goal is to leverage the regenerative and healing properties of adult stem cells by developing exosomes as a non-surgical and non-cellular therapeutic or cosmeceutical agent for skin repair.

#### Effects of Low Level Laser Therapy in the Expression of Genes Related to Bone Repair Process

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The aim of this study was to investigate the process of bone healing and the expression of genes after low level laser therapy (LLLT) application in an experimental model of tibial bone defects. A total of 20 male Wistar rats were randomly distributed into 2 groups (n=10): bone defect control group (CG) and laser irradiated group (LG). Laser irradiation (830 nm, 100 mW, 120 J/cm<sup>2</sup>) was initiated immediately after surgery and was performed every 48 h for 15 days. Bone defects were surgically performed on both tibias. The histological analyses showed, in the laser treated group, a higher amount of newly formed bone and a better tissue organization compared to CG. Microarrays analysis demonstrated that LLLT produced an up-regulation of several genes related to bone repair process, such as transforming growth factor alpha (Tgfa), angiopoietin 4 (Angpt4), growth differentiation factor 5 (Gdf5), prostaglandin E receptor 2 (Ptger2), caspase 3 (Casp3) and bifunctional apoptosis regulator (Bfar). Our results indicate that LLLT improves bone repair process as a result of increasing bone formation probably by modulation of genes related to bone healing in a model of tibial bone defect in rats.

#### Assessment of Hybrid Dressing of Pu Foam and Pva with Adsc on Wound Healing

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Although adipose-derived stem cells (ADSCs) have the potentials to be used for damaged tissues, few researches have dealt with effects of ADSCs on wound healing. And polyurethane (PU) is frequently used wound dressings. We investigated the effect of ADSC seeded on Poly vinyl alcohol (PVA) nano web fiber and newly developed high absorbent PU foam hybrid dressing on wound healing.

**Methodology/Principal Findings:** Surgical wounds were created on the dorsum of rat and were assigned into 4 groups: Group A, as the control group, with sterilized gauze, Group B with PVA nano web fiber-PU foam hybrid dressing, Group C with ADSCs seeded PVA nano web fiber-PU foam hybrid dressing, Group D with Medifoam<sup>TM</sup>. Group C showed statistically higher wound healing rate. In a histological aspect, more thick and dense epithelial layer was observed in Group C. In Western blot, on 14th day after wound formation, the expression of TGF- $\beta$ 1, VEGF and Laminin  $\alpha$ -3 was detected to be relatively strong in Group C.

**Conclusion and Significance:** This study suggest that ADSC seeded on PU dressing foam which has highly absorbent degree induces stable reepithelization, angiogenesis and collagen synthesis in wound healing. So, it is considered as an effective biological dressing material.

#### Maturation of Decellularized Esophagus Previously Seeded or Not With Sheet of Human Adipose Derived Stem Cells in Epiploon of Nude Rats for Organ Substitution

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After an extensive review of the literature showing failure of the strategies involving synthetic materials and non-seeded allogenic tissues [1], we chose to address the challenge of esophagus reconstruction by an "all biologic" approach: a decellularized esophagus used as a scaffold (with appropriate mechanical and biological properties) for seeding of Adipose-derived Stem Cells (AdSC) cultured in sheets with the Tissue Self-Assembly (TESA) technique. Furthermore, the epiploon could be used as an *in vivo* bioreactor to mature the scaffold to obtain vascularization and/or recellularization and primary survival of the whole construct after further grafting [2]. In the present work, we studied the role of the maturation process in the epiploon of 30 nude rats. We implanted patches of two swine decellularized matrices (DM) obtained by a dynamic process (Bose system), with or without sheets of human AdSC obtained by the TESA technique after 21 days of culture with ascorbic acid. The DM was enrolled (extemporaneously) or not in the AdSC sheet before implantation. The follow up was 2, 4 or 8 weeks (n=10). We studied the key issues of inflammatory reaction and neovascularization by MRI imaging and DM fate, AdSC survival and integration by histological (HES and Masson trichrome staining) and immunohistochemical techniques. Results are in progress.

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#### Engineered Human Liver Organoid with Near Physiological Metabolic Function

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Stable and functional human liver tissue has been established *ex vivo* by seeding human fetal liver cells into rodent biomatrix scaffolds. Liver biomatrix scaffolds were prepared using gentle delipidation strategies followed by extraction with a buffer with salt concentrations at levels sufficient to keep insoluble all of the tissue's collagens and all matrix components and other factors bound to those collagens. Human fetal liver cells seeded into the scaffolds attached within hours, with full attachment of all cells by 36 hours; demonstrated differentiated functions; and liver histology and ultrastructure, including cell-cell contact and bile canaliculi, within less than a week. They remained functional for weeks thereafter by perfusion with a serum free, hormonally defined medium tailored for optimization of liver functions. Functions were monitored for 14 days by qRT-PCR, ELISAs, and nuclear magnetic resonance spectroscopy (NMRS). Differentiation was evident by gene expression showing decreased fetal markers (e.g. alpha-fetoprotein, AFP) and an increase and then maintenance of mature hepatic markers (e.g. albumin, urea



and P450). Protein analysis presented a decrease in AFP secretion, an increase in albumin and, in parallel, a steady production of urea. NMRs data indicated the organoids stabilized metabolically within the first few days in culture and were able to convert glucose to lactate through the TCA cycle. The strategies resulted in rapid establishment of functional human liver tissue with results not observed by any other recellularized scaffold preparation and ones not requiring transplantation into a host to achieve functionality.

### ***In Vivo* Myc Gene Delivery to Adult Mouse Inner Ear Stimulates Proliferation of Post mitotic Supporting Cells Leading to New Hair Cell Formation**

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The loss of inner ear hair cells causes disabling hearing and balance impairments. When significant numbers of hair cells die, the adult mammalian inner ear fails to replace them, resulting in permanent deficits of sensory function. MYC genes control both proliferation and differentiation. The goal of our research is to overexpress MYC genes to enhance the regenerative capacity of inner ear supporting cells *in-vivo*, thereby permitting them to replace lost hair cells. A canalostomy was used to deliver adenovirus to the mouse inner ear through posterior semicircular canal. Adenoviruses encoding a mutant form of c-Myc containing green fluorescent protein (c-MycT58A-2A-GFP) was amplified and purified. GFP expression was used as a measure of the viral infection efficiency. The levels of proliferation in treated and control inner ear utricles were measured. *In-vivo* 5-Ethylene-2'-Deoxyuridine (EdU) labeling was used to follow proliferating cells. Aminoglycoside gentamicin was injected in adult mice to induce *in-vivo* hair cell ablation in utricle. *In-vivo* delivery of adenoviruses encoding GFP resulted in moderate GFP expression in mice semicircular canals and utricles. In addition, *in-vivo* gene delivery of c-MycT58A into the inner ear stimulated the proliferation of supporting cells of the utricles of adult mice leading to formation of new hair cells not only in intact adult mice utricle but also in *in-vivo* gentamicin damaged adult mice utricle. This is the first evidence of *in-vivo* gene delivery into the inner ear which stimulated proliferation of inner ear supporting cells leading to new hair cell formation in adult mammal.

### **A 3D Printed, PCL-Based Scaffold for Potential Use in Meniscal Regeneration**

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Meniscus is a wedge-shaped, semicircular fibrocartilaginous tissue located between the tibia and femur. Its functions are load bearing and transmission, shock absorption, and joint stability and lubrication. Because of its low regeneration capacity, the meniscus tissue should be treated surgically when damaged.

There are various treatment methods for meniscal injuries; however, each has its own disadvantages. Tissue engineering may be an alternative method for the treatment of serious meniscal injuries. In this study, PCL-based scaffolds produced in the shape of a meniscus via 3D printing method were used. Some of the scaffolds were coated with methacrylated gelatin (Gel-MA) containing human meniscus cells (hMCs) and crosslinked upon exposure to UV (PCL/Gel-MA group). The untreated PCL scaffolds were seeded with hMCs (PCL group). The cells cultured on the scaffolds were analyzed in terms of adhesion, proliferation, and morphology.

The results showed that cells adhered strongly and proliferated rapidly on PCL scaffolds, whereas adhered less strongly and proliferated slower on PCL/Gel-MA scaffolds. Besides, cells spread well on PCL scaffolds, while assumed more rounded morphology on PCL/Gel-MA. These results indicated that introduction of a hydrogel system might induce the chondrogenic phenotype of the cells.

### **A Multifactorial Approach Towards Enhanced Extracellular Matrix Deposition and Maintenance of Mesenchymal Stem Cell Phenotype using Macromolecular Crowding and Low Oxygen Tension**

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Cell-based tissue engineering strategies have limited clinical applicability due to delayed extracellular matrix (ECM) deposition and prolonged production time. Scaffold-free tissue production *in vitro* can be enhanced by macromolecular crowding (MMC), a biophysical phenomenon that governs the intra- and extra-cellular milieu of multicellular organisms<sup>1</sup>. Although MMC has been proven to be effective in enhancing ECM deposition in permanently differentiated cells<sup>1</sup>, its effectiveness in mesenchymal stem cell (MSC) culture has still to be fully assessed. It is hypothesised that MSCs cultured under MMC and low oxygen tension<sup>3</sup> will maintain their phenotype and function, resulting in multipotent and ECM-rich sheets. Human MSCs were cultured for 7 and 14 days with 100 µg/ml of carrageenan at 20% and 2% oxygen. Collagen I deposition was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Phenotypic assessment was performed by flow cytometry for the expression of CD90, CD105, CD73, CD44, CD34, CD11b, CD19, CD45 and HLA-DR. Under MMC conditions, collagen I deposition was significantly increased at 20% and 2% oxygen tension, as compared to non-crowded controls and expression of positive cell surface markers was not affected, indicating maintenance of the multipotent phenotype. MMC and low oxygen tension can be used to enhance ECM deposition in human MSC culture, without detrimental effect on cell phenotype and function.

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### **Vascularization of Bioengineered Tissues: Additive Effects of Physical and Biological Cues**

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Three-dimensional bioengineered tissues offer promise for the replacement and regeneration of organs damaged through injury or disease, but the size of these bioengineered constructs is generally limited to several hundred micrometres due to restrictions in exchange of nutrients, gases and waste in larger constructs. In the human body, tissue homeostasis is maintained via an extensive network of blood vessels, which deliver oxygen and nutrients to every cell in the body and take away harmful waste products. Lack of this vascular network has been a major limiting factor to translating advances in bioartificial tissue development to the clinic.

We utilised silk biomaterials as a platform to study the effects of physical and biological cues on oxygen and nutrient transport, cell viability and biomaterial vascularisation *in vitro* and *in vivo*.

Vascular-like hollow channels engineered into 3D, porous silk scaffolds significantly enhanced cell infiltration and solute transport in silk scaffolds, and promoted enhanced host tissue integration (cell infiltration & matrix deposition) and vascularisation *in vivo*. Interestingly, hollow channels were more effective at promoting biomaterial vascularisation that *in vitro* pre-vascularisation with endothelial cells. However, a combination of hollow channels and pre-vascularisation had an additive effect, driving biomaterial vascularisation and anastomoses of *in vitro* formed rudimentary blood vessels with the host vascular system. This points to the importance of both physical and biological cues in tissue vascularisation strategies.

#### A Comparative Study of the Osteogenic Potentials Of rhBMP-2 and Tauroursodeoxycholic Acid (TUDCA) in a Murine Spine Fusion Model

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A non-union of spinal fusion occurs at high rate in case of spine surgery. Recombinant human bone morphogenetic protein-2 (rhBMP-2) is widely used as osteoinductive molecule to promote bone fusion in spinal surgery; however its safety has been questioned in recent studies. Herein, we compared the bone formation ability of rhBMP-2 and tauroursodeoxycholic acid (TUDCA), an amphiphilic bile acid using posterolateral lumbar murine spinal fusion model. Twenty four female ICR mice were allocated into four groups (n=6 in each group). Group I served as SHAM, Group II served as control and received collagen sponge+PBS, Group III received collagen sponge+rhBMP-2 and Group IV received collagen sponge+TUDCA. The microstructure of posterolateral spinal fusion mass at L4-L6 was evaluated using micro-computed tomography scanning at 2 and 4 weeks after surgery. Animals were sacrificed at 4 weeks after surgery for histological analysis. Treatment with either rhBMP-2 or TUDCA resulted in significant increase in bone volume and other microstructural parameters at 2 and 4 weeks compared with control group. In the histological results, the fusion site of the rhBMP-2 group showed over-growth of bone as compared with the TUDCA group. Our results suggest that TUDCA also possess osteoinductive capabilities to promote bone fusion in spinal surgery comparable with rhBMP-2 with no safety issues. Our data indicates that TUDCA could be used as an alternative to rhBMP-2 and has potential clinical application.

#### Development of a Versatile Gene-activated Scaffold for Orthopaedic Tissue Regeneration

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Gene-activated scaffolds have recently shown potential in localised gene delivery resulting in tissue regeneration (1, 2). The objective of this study was to explore the potential of a series of collagen-based scaffolds (3–5), developed in our laboratory, as gene-activated scaffolds suitable for bone or cartilage tissue engineering. The use of a number of different osteogenic and chondrogenic genes, and combinations thereof, was also assessed to find the optimal gene-activated scaffold formulation for each application. Chitosan, a biocompatible material derived from the shells of crustaceans, was developed as a gene delivery vector for use specifically with mesenchymal stem cells (MSCs). A transfection efficiency of >45% is reported which is similar to what is achieved with polyethyleneimine (PEI), a non-viral gold standard vector, without causing cytotoxic side effects. When incorporated into a series of collagen-based scaffolds, transgene expression by MSCs was maintained for up to 28 days. The osteogenic potential of the gene-activated scaffolds was assessed by culturing MSCs on the scaffolds for 28 days. Alizarin red staining and calcium quantification showed that the combination of bone morphogenetic protein-2 (BMP-2) and vascular endothelial

growth factor (VEGF) caused a significantly higher amount of calcium deposition than other groups. The chondrogenic potential of this system is now being assessed with the triple combination of Sox-5, 6 and 9 showing the most potential. These promising results demonstrate that by simply varying the scaffold composition and the gene (or combinations thereof) chosen, the system has potential for a myriad of therapeutic applications.

#### Glucose Delivery System Based-Hydrogel Composite Scaffold for enhancing MSC survival

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Constructs developed for tissue engineering present limited potential due to massive death of transplanted cells after engraftment. This issue can be overcome by *in situ* supplying glucose that acts as the main metabolic fuel for Mesenchymal Stem Cells in severe hypoxia. In this study, a composite construct is engineered to provide glucose to MSCs and to enhance their survival when transplanted. Two combined strategies are developed. The glucose leakage outside the tissue construct is delayed by increasing the intrinsic viscosity of the fibrin hydrogels by supplementation with starch. The starch polysaccharide serves as the main source of glucose. The long-term supply of glucose is achieved adding  $\alpha$ -amylglucosidase (AMG)-containing poly(lactic-co-glycolic acid) nanoparticles. Sustained delivery of  $\alpha$ -amylglucosidase into the starch-loaded fibrin gel provides continuous production of glucose *in situ* via the enzymatic hydrolysis of starch. Hydrogels containing fibrin, starch and AMG are self-supported and present a storage moduli superior to 1 kPa. Enzymes containing-nanoparticles are able to produce glucose over a month and allow sustained glucose delivery inside hydrogels. Experiments show that MSCs loaded in these hydrogels and placed either *in vitro* under hypoxia or ectopically implanted in nude mice have a survival rate significantly higher than MSCs in fibrin gels over 14 days. This work is a proof of concept that an innovative-engineered construct, based on a starch hydrogel containing  $\alpha$ -amylglucosidase is able to deliver glucose versus time. In the context of regenerative medicine, it represents a highly pertinent system to enhance Human Mesenchymal Stem Cells survival in cell constructs.

#### Treatment of Non-Healing Complex Wounds in Autoimmune Disorders via Bone Marrow Aspirate Concentrate

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**Purpose:** Development in immunology has marked an era in medicine over the last decades. While mechanisms of autoimmune diseases (AD) interest the immunology, systemic symptoms enter to occupational areas of other disciplines. Behcet's Disease (BD), Antiphospholipid Antibody Syndrome (APPLAS) and non-specific vasculitis (NSV) are some of the examples to AD that have skin manifestations. Theories behind the pathogenesis currently suggest autoimmune etiologies, but not clearly. The aim of the present study was to assess the efficiency of bone marrow aspirate concentrate (BMAC) on non-healing complex wounds (NHCW) in AD.

**Methods:** A total of 3 patients having NHCW located in lower extremities due to different etiologies were enrolled in the study. The patients were consulted as follows; a 25-years-old man with BD which has been grafted for 22 times and has received colchicine (1 mg/day) and methylprednisolone (80 mg/day), a 10-years-old girl with APPLAS which has been grafted for 5 times and has received azathioprine therapy (0.5–1 mg/day), a 60-years-old woman with

NSV. 60 cc of BMAC was harvested from the anterior iliac crest and processed using the SmartPreP BMAC<sup>®</sup> system (Harvest Technologies Corp.). BMAC was applied after effective debridements. Skin grafts were performed and V.A.C. VeraFlo™ Therapy (KCI Corp.) was used for closure.

**Results:** Average follow-up period was two years. Average operation number per patient was 3. The wounds healed totally and no recurrence occurred during the two-year follow up.

**Conclusions:** BMAC therapy has an active role on NHCW by a mechanism that can not be fully explained. If this mechanism is solved, there will be more effective methods for wound therapy.

### Effects of Titanium Surfaces and Simvastatin in Estrogen-deprived Cell Culture

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**Objective:** The study aimed to investigate effects of titanium surface topography and simvastatin on growth and osteogenic differentiation potential of human bone marrow stromal cells (hBMSCs) in estrogen-deprived (ED) cell culture.

**Methods:** Under permission from an ethic committee of Songnarin Hospital and patients' informed consent, hBMSCs were seeded on titanium (Ti) disks, smooth and SLA Ti surfaces (Straumann, Switzerland) and cell culture plates. After that they were cultured in regular (FBS)- and ED-growth or osteogenic (OS) media for 21 days and ED-OS was supplemented with 1 μM β-estradiol or 100 nM Simvastatin for 7 days. Cell attachment and morphology were examined under scanning electron microscope (SEM). Osteoblastic differentiation was determined by measuring levels of OS-markers, alkaline phosphatase (ALP) activity, calcium contents and expression levels of osteoblast-related genes, runx2 and bone sialoprotein (IBSP) on culture-days 7 and 21 (n=3-5, Mean ± SD).

**Results:** Estrogen-deprived cell culture decreased cell attachment, growth and osteogenic differentiation potential of hBMSCs. Levels of OS-markers on every surface in ED-OS were significantly lower than FBS-OS media (p<0.05), excepting for expression levels of IBSP that was significantly increased on SLA titanium surface (p<0.05). Beta-estradiol and simvastatin increased levels of OS-differentiation markers in ED-OS medium, particularly on SLA titanium surface (p<0.05).

**Conclusion:** Estrogen was essential factor enabling promoting effects of titanium surface topography on osteogenic differentiation. Titanium surface modification alone might be.

### Gellan Gum-Hyaluronate Spongy-like Hydrogels Promote Angiogenesis in Hindlimb Ischemia

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Gellan gum (GG) spongy-like hydrogels are highly attractive materials for Tissue Engineering applications once they present hydrogel main properties and are capable to promote cell adhesion. Considering the inefficient vascularization of thick 3D constructs, we hypothesized that GG spongy-like hydrogels containing hyaluronate (HA) would promote the recruitment of endothelial cells upon degradation, due to the angiogenic nature of the released HA fragments.

GGHA spongy-like hydrogels (1% and 2%, w/v) were prepared as previously described 1, 2. The susceptibility of the materials to hyaluronidase degradation was demonstrated *in vitro* by the increased amount of reducing sugars (DNS assay) and low molecular weight HA fragments released up to 28 days (GPC). After implantation in an ischemia hindlimb mouse model, increased blood perfusion, in comparison to the control, was observed in the presence of GGHA materials. Furthermore, blood vessels density was higher in the presence of the materials, 1% (5.5 ± 1.5) and 2% (6.0 ± 1.6), relatively to the control (4.5 ± 1.5). To understand vascular network maturation and stability, α-SMA positive vessels were quantified and

showed a higher percentage in the presence of GGHA 1%. Based on the degradation results these differences seem to be related to the faster GGHA 1% degradation rate.

GGHA spongy-like hydrogels, combining specific intrinsic features with the biorecognition of HA, are potential materials to be used in acellular and cellular approaches to improve neotissue vascularization.

1. da Silva LP *et al.* WO2014167513, 2013.

2. da Silva LP *et al.* Acta Biomater 10, 4787, 2014.

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### Development of an Artificial Oesophagus Engineered with Mesoangioblasts in a Peristaltic 3d Dynamic Culture

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The aim of the project is to build a functional muscularis externa of artificial oesophageal constructs combining human mesoangioblasts with a decellularized matrix in a dynamic 3D culture system in order to provide optimal nutrient exchange, oxygenation and pulsatile stimulation. Human mesoangioblasts, isolated from paediatric muscle biopsies, were delivered into the muscular layer of decellularized rat oesophagi. Seeded matrices were cultured in dynamic bioreactor conditions using a chamber for luminal and extra-luminal media flow.

Delivery of mesoangioblasts and subsequent dynamic culture produced consistent cell engraftment and migration from the delivery sites along the scaffold thickness after nine days of culture. Dynamic culture conditions allowed better homogeneous distribution in the oesophageal scaffold wall when compared to static conditions, producing an overall growth of the tissue in culture. An optimized combination of media conditions produced cell expansion and subsequent differentiation towards smooth muscle. In particular, 40% of mesoangioblasts were positive for smooth muscle marker SM22, also maintaining a proliferating cellular pool (Ki67+ cells), with a significant increase in the scaffold's thickness.

In conclusion, we identified cell delivery method for the oesophageal muscle layer followed by dynamic culture as a successful method for optimal re-colonization of acellular oesophagi. Use of a bioreactor is a key factor in supporting and improving mesoangioblasts engraftment, proliferation, migration and differentiation, leading to *in vitro* tissue growth. Future work will include maximization of cell differentiation to obtain a functional fully engrafted scaffold suitable for *in vivo* transplantations.

### Fundamental Technology for the Creation of Whole Liver Engineering, and Functional Evaluation of Recellularized Liver

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Technology for regenerative medicine based on tissue engineering is desired earnestly as an effective medical treatment for serious organ diseases. Especially, liver is a central organ for metabolism in our body and is complicated structure. Therefore, liver tissue engineering is one of the most important and difficult themes. However, formation of tissue-like structure with the thickness more than 1 mm is still impossible, because oxygen consumption rate of hepatocytes is higher than the other organs' cells. Scale-up and easy process

development are required. For the realization, creation of whole liver engineering (WLE) consisting of cells, functional ECM and fine organ template will be indispensable.

Heparin-collagen conjugate and solubilized liver ECM were developed as growth factor-immobilizable materials. VEGF and HGF were immobilized on these functional materials (>90%). Hepatocytes on these materials well expressed various liver-specific functions *in vitro*. Hepatocytes or fetal liver cells (FLCs)-embedded functional gel was subcutaneously transplanted into rat. Angiogenesis and viability of hepatocytes were enhanced in the gel. Furthermore, transplanted FLCs form liver tissue-like structure with vascular network.

Organ-scale scaffold having a template of blood vessel network was obtained by decellularization with detergent. The fineness of the network was the same as original liver, evaluated by 3D-CT. Furthermore, endothelialization and expression of liver-specific function of hepatocytes were confirmed. Furthermore, recellularized liver well metabolize ammonia during blood circulation.

Based on the above-mentioned results, we expected that fundamental technology for the creation of WLE was developed.

### Development of Macroporous Ceramic Scaffolds for Bone Engineering Applications using Human Induced Pluripotent Stem Cells

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Treatment of bone defects remains a clinical challenge worldwide. Approaches of bone engineering using human induced pluripotent stem cells (iPSC) and compliant scaffolds open new opportunities for future applications. Calcium phosphate cements create a stable bond with the bone, are biocompatible, bioresorbable and osteoconductive, thus representing suitable biomaterials for the construction of porous scaffolds for optimal skeletal reconstructions. The objective of this study was to engineer bone cement scaffolds and study their potential to support attachment, proliferation and osteogenic differentiation of human iPSC-derived mesenchymal progenitors.

Macroporous synthetic cement scaffolds (8 mm  $\varnothing$  x 3 mm height) were fabricated using a dissolving phase approach by mixing monocalcium phosphate monohydrate and  $\beta$ -tricalcium phosphate with polyethylene glycol (PEG) particles of different size (100–600  $\mu$ m) in distilled water. Fabricated scaffolds were then characterized for chemical composition, PEG content, porosity and mechanical properties and used for seeding optimization and differentiation studies. Human iPSC were expanded and induced into the mesenchymal lineage for 7 days, and then cultured on bone cement scaffolds for 7 weeks. Decellularized bone scaffolds were used as control for all experiments. Cell viability and proliferation, and osteogenic differentiation and tissue formation were investigated along the experimental period.

Results demonstrate that biomimetic bone cement scaffolds with select porosity and mechanical properties can be manufactured and support cell attachment, proliferation and osteogenic differentiation.

The use of progenitor cells derived from iPSC lines in combination with clinically-compliant scaffolds allow engineering unlimited amount of bone tissue graft for basic and applied research.

### The Role of Sphingosine-1-phosphate Receptor 1 in Rat Periapical Lesions

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The bone remodelling process is highly regulated by the immune response. Recent studies have shown that Sphingosine-1-phosphate

receptor 1 (S1P1) is crucial in bone homeostasis, in the way of regulating immune cell migration and function, especially inducing the expression of RANKL, the key factor in osteoclastogenesis; while impeding the differentiation and function of immune suppressive cells—regulatory T cells (Treg cells). Periapical lesions (due to root canal infection), which cause pulp damage and lesion formation around the apex of the root, are accompanied by alveolar bone resorption, an immune response to pathogen invasion resulted by osteoclast activation and imbalanced bone remodelling. Our study aims to investigate the expression of S1P1 in rat periapical lesions and to unveil its possible role in the pathogenesis of periapical lesion. The results demonstrated that S1P1 expression was positively correlated with RANKL and negatively correlated with Treg cell infiltration in the periapical lesions in a rat model. Modulating S1P1 could down-regulate RANKL expression and attenuate the imbalance of RANKL/OPG, thereby suppressed the inflammatory bone destruction in periapical lesions. S1P1 is therefore a contributing factor in the pathogenesis of periapical lesions and could be a potential target for developing novel clinical treatment of the diseases.

### Division-Linked Phenotypic Changes and Chondrogenic Potential of Chondrocytes

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The production of engineered cartilage using primary chondrocytes typically requires an initial *in vitro* proliferation phase, during which cells lose their native phenotype and chondrogenic potential with cumulative population doublings. Despite numerous efforts, chondrocytes with superior regenerative capacities have not been identified so far, possibly because of heterogeneous growth rates causing a domination of the expanded population by rapidly proliferating cells. We hypothesized here that selecting chondrocytes based on their number of cell divisions reveals differences in the progression of de-differentiation and chondrogenic potential among subpopulations. Freshly isolated human chondrocytes were stained with the cell division tracking dye carboxyfluorescein succinimidyl ester (CFSE) and cultured in 2D polystyrene dishes with or without growth factors. At different time points, expression of cell surface markers was assessed by flow cytometry or cells were FACS sorted according to their prior divisions to assess gene expression and re-differentiation potential. CFSE labelling allowed the identification of distinct subpopulations of proliferating chondrocytes. Changes in the expression of CD54, CD90 and CD166 as well as of extracellular matrix genes collagen type I, collagen type II, aggrecan and versican clearly progressed with cell divisions. Different culture conditions influenced cell doubling rates but not the link to phenotypic alterations. Interestingly the highest chondrogenic potential was measured for chondrocytes that were growing slowly, as compared to non-dividing or rapidly dividing cells. The correlation between high cell proliferation rate and low re-differentiation capacity constitutes an important challenge for cartilage tissue engineering applications. Future studies will have to reveal the underlying molecular mechanisms.

### $\mu$ -Particle Image Velocimetry and Computational Fluid Dynamics Study of Perfusion Cell Seeding Within a 3d Porous Scaffold

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Cell seeding of 3D scaffolds is a critical step in tissue engineering since it precedes all steps in tissue formation. Homogeneous cell distribution and high seeding efficiency are desired. The combination of 3D interconnected porous structures with perfusion bioreactors can enhance such outcomes. This study aims to combine Computational Fluid Dynamics (CFD) simulations with micro Particle Image

Velocimetry ( $\mu$ PIV) experiments to predict seeding efficiency and optimise experimental parameters. Local velocity profiles at the pore level inside a 3D scaffold were extracted using  $\mu$ PIV system. A  $\mu$ CT-based CFD model was developed replicating scaffold perfusion conditions using Fluent Ansys. To analyse cell motion, labelled MG63 cells were tracked with  $\mu$ PIV and cell velocities were calculated in Image J. Cell motion was calculated using the CFD model where cells were simulated as a discrete phase of microsphere particles that move along the fluid volume in a Lagrangian frame. This study shows that cells reach all pores inside the scaffold mainly following streamlines with a higher number of cells passing by the center of the pores where fluid velocities are higher. Since cells do not intercept with scaffold substrate, low cell seeding efficiency was observed and compares well with the CFD model. Cell deposition is a complex mechanism sensitive to the hydrodynamic environment so new configurations should be developed to aid cells to collide with scaffold substrate. Herein an experimental approach was developed to investigate cell seeding inside a 3D scaffold and a computational model was able to predict local fluid dynamics and cell seeding efficiency.

### Piezoelectric Scaffolds for Cartilage Tissue Engineering

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Articular cartilage damage is irreversible and currently, no technology exists that can repair or regenerate functional cartilage tissue. In this study, we evaluated the phenomena of piezoelectricity in stimulating cartilage tissue formation. Piezoelectricity is when a material exhibits an electric charge in response to deformations. The synthetic material, poly(vinylidene fluoride-trifluoroethylene) (PVDF-TrFE) was used as a model piezoelectric material because of its proven biocompatibility and established piezoelectric activity. PVDF-TrFE was electrospun to form a fibrous scaffold and annealed to enhance its piezoelectric properties. This study evaluated the chondrogenic differentiation of human mesenchymal stem cells (MSCs) on as-spun and annealed PVDF-TrFE scaffolds in a compression bioreactor. Electrospun polycaprolactone (PCL) was used as a non-piezoelectric control. Both PVDF-TrFE scaffolds contained the piezoelectric  $\beta$ -phase as determined by FTIR and XRD, with a significantly higher  $\beta$ -phase content for annealed PVDF-TrFE. Also, higher voltage was detected for annealed PVDF-TrFE when subjected to compression. Using a compression bioreactor, MSCs laden scaffolds were evaluated for chondrogenic differentiation for up to 28 days by ELISA, qRT-PCR, and immunohistochemistry for cartilage tissue formation. Chondrogenic differentiation was enhanced for cells on as-spun PVDF-TrFE scaffolds, as demonstrated by significantly higher GAG, collagen type II production and collagen type XI expression compared to annealed PVDF-TrFE and PCL. Hypertrophic marker, collagen type X, was not up regulated for cells on as-pun compared to annealed PVDF-TrFE. Our findings demonstrate that piezoelectric scaffolds can provide additional electrical cues for MSC chondrogenesis, and there may be an optimal electrical stimulus needed to achieve hyaline cartilage tissue repair.

### Bone Tissue Engineering for Maxillofacial Area: A Sheet-like Substitute Based on Human Endothelial and Stem Cells Co-culture

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The future of regenerative medicine involves « tailor made » processes, strongly specific to each patient. We set up here a sheet-like bone substitute relevant for maxillofacial reconstruction: its shape would allow surgeons to adapt it to the specific geometry of each patient's injuries. After a first step with murine osteoblast cells [1], we went further with the co-culture of primary human mesenchymal stem cells (MSC) for osteoblastic differentiation and endothelial cells (EC) for vascularization.

MSC were seeded on a monolayer of calcium phosphate granules inside a parallelepiped chamber and cultured for one month with the addition of EC at day 14. Analysis was then performed, focusing on cell spreading and morphology (SEM), viability (fluorescence microscopy), differentiation (RT-qPCR, ELISA) and mineralization (histology). Cells were able to bound granules together until the formation of a cohesive sheet-like substitute. Compared to respective monocultures, the co-culture showed better results for all investigated properties. Bone differentiation, specific and non-specific markers production (such as RUNX2, Col1a2, ALP) were enhanced. Mechanical cohesion could be improved to guarantee an easy handling during surgery. Mature rebuilt tissues will be implanted in a murine model to confirm the results *in vivo*. Further studies will investigate new materials to assure a better mechanical stability of the biohybrid substitute.

### Reference:

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### Tracing of Transplanted Keratinocytes with CFSE

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Treatment of wounds with transplantation of autologous keratinocytes has been proven efficient. Delivering keratinocytes with the aid of gelatin microcarriers to wounds has been investigated but the contribution of the transplanted keratinocytes has been difficult to establish. We have investigated the use of 5(6) Carboxy-fluorescein N-hydroxysuccinimidyl ester (CFSE) as a traceable stain for human primary keratinocytes. In this *in vitro* study different CFSE concentrations and viability and migrational capacity of CFSE-stained keratinocytes have been evaluated. The tested CFSE-concentrations did not significantly affect the viability of the keratinocytes or their ability to migrate when compared to unstained control keratinocytes. CFSE-stained keratinocytes were cultured in spinner flasks to adhere to gelatin microcarriers followed by administration to *in vitro* wounds inflicted in viable human skin in culture. Tracing of transplanted keratinocytes in tissue sections with the aid of fluorescence microscopy revealed that keratinocytes retained the CFSE stain over three weeks of tissue culture and migration of keratinocytes from the microcarriers to the wound bed. We propose a novel use for CFSE as a passive and simple staining for tracing keratinocytes in tissue sections.

### Site-directed Immobilization of BMP-2 and Noggin Inhibitory Peptides onto Collagen Beads for Bone Regeneration

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Bone regeneration requires concomitant processes of osteogenesis and neovascularization. The use of new materials, cells and growth factors has been therefore in focus of bone tissue engineering. In this study, we want to covalently couple Bone Morphogenetic Protein 2

(BMP-2) variants onto collagen beads in a site directed manner and test their osteogenic potential *in vitro* and *in vivo*. Because of the problems observed in all the approaches developed to incorporate growth factors into biomaterials, we decided to immobilize the growth factor BMP-2 site-directly to collagen beads by the use of “click chemistry”. This method potentially overcomes the drawbacks connected with surface absorption and encapsulation. For this purpose, we created two BMP-2 variants through site directed mutagenesis, comprising one unique non-natural amino acid substitution in each chain of the mature polypeptide, allowing a site-specific coupling by a specified coupling chemistry.

Apart from the physical and chemical aspects connected to the immobilization, we focused our attention on improving the growth factor's bioactivity by a caption of the BMP antagonist Noggin. BMPs perform their pro-osteogenic effect by binding to and oligomerizing of membrane receptors. Noggin binds to BMPs and interferes with their ability to induce receptor dimerization. Here we identify peptide sequences, through phage display method and analyze them in terms on their Noggin-binding characteristics and their potential to inhibit BMP-2 mediated biological responses. Both tools, the covalently coupled BMP-2 and the peptides, which might act as Noggin “deflectors” might produce an innovative biomaterial with superior bone healing properties.

#### **Polyhydroxybutyrate-co-valerate/La-containing Apatite Composite for Tissue Engineering**

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Three-dimensional (3D) structures (scaffolds) have been widely studied for bone regeneration in tissue engineering, mainly polymeric scaffolds. Therefore, this study developed a bioactive-radiopaque polymer based on polyhydroxybutyrate-co-valerate (PHBV) and La-containing apatite (La<sub>20</sub>AP) (PHBV/La<sub>20</sub>Ap) for 3D printing. First, the composite was prepared in a twin screw extruder for producing standardized filaments. A technology similar to “fused deposition modeling”, 3D printer - Fab@CTI (open source, Fab@Home), was used for printing the 3D scaffolds. The samples were characterized by Scanning Electron Microscope (SEM), Digital Radiography, Differential Scanning Calorimetry (DSC) and Dynamic Mechanical Analysis (DMA). Cytotoxicity was evaluated using CHO-K1 cells. SEM images showed the presence of apatite particles homogeneously distributed throughout of the extruded material. Radiographic images revealed that the composite presents suitable radiopacity for diagnostic imaging. The DSC results showed that thermal properties of polymer are maintained after incorporation of La<sub>20</sub>Ap into the PHBV; moreover, PHBV/La<sub>20</sub>Ap composite revealed better mechanical properties than PHBV polymer. *In vitro* assays demonstrated no cytotoxic effects in CHO-K1 cells for PHBV/La<sub>20</sub>Ap composite. In conclusion, the PHBV/La<sub>20</sub>Ap composite produced by extrusion process might be a potential material for 3D printing and for application in tissue engineering.

#### **Generation of a Bone Organ by Human Adipose-derived Stromal Cells through Endochondral Ossification**

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Among mesenchymal progenitors (MSC), only skeletal MSC from bone marrow form ectopic bone through endochondral ossification (ECO). The goal of this study was to assess whether chondrogenically or hyperthrophically-primed human adipose derived stromal cells (ASC) were capable to form bone organ through ECO. Minimally-expanded ASC were either cultured as micromass-pellets or onto type-I-collagen scaffolds in chondrogenic medium containing TGF- $\beta$ 3 and BMP-6 for 4 weeks (early hypertrophic templates). A medium supplemented with  $\beta$ -glycerophosphate, L-thyroxin and IL1- $\beta$  was added for 2 additional weeks to further induce hypertrophy (late hypertrophic templates). Constructs were subcutaneously implanted in nude mice and analysed 8 weeks post-surgery.

*In vitro*, deposition of a cartilaginous matrix, positive for glycosaminoglycans, type II collagen and Indian Hedgehog was shown. Upon induction of hypertrophy, gene expression analysis showed up-regulation of type X collagen, BSP and MMP-13. RT-PCR data were confirmed at protein level. *In vivo*, both early and late hypertrophic constructs underwent cartilage remodelling evidenced by the presence of MMP-13 and TRAP positive cells and further developed into bone tissue, containing osteocyte-like cells embedded in a matrix rich of BSP. Newly-formed bone was vascularized and included a bone marrow cavity. *In situ* hybridization for human-specific sequences and staining with a human specific anti-CD146 antibody demonstrated a direct contribution of ASC not only to bone tissue formation but also to the bone marrow stroma. In conclusion, human ASC can undergo a developmental program of ECO. This specific bone regenerative potential should now be evaluated in an orthotopic model of bone repair.

#### **Effect of Elastic Moduli of Micro-Fiber/Collagen Composites on Ligament Development**

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Traditional hydrogels lack the topographical and mechanical cues to guide mesenchymal stem cell (MSC) differentiation into oriented tissues. Toward the development of a ligament-like tissue, we hypothesized that the incorporation of sparse electrospun fibers within a 3D collagen gel could confer both topographic cues and anisotropic mechanical properties to guide MSC alignment and differentiation. Further, we postulated that a low density of fibers would allow MSCs to proliferate and migrate freely, and to remodel the collagen gel without being impeded by the electrospun fiber network.

Toward this goal, sparse aligned fiber networks with elastic moduli of 5.6 (soft), 15.1 (moderate), and 31 (stiff) MPa were electrospun from blends of polycaprolactone and polyurethane. MSCs in collagen were introduced to the fibers to form seeded fiber/collagen composites, and cultured for 14 days. Confocal microscopy revealed that soft fibers induced orientation of cells both attached to the fibers and with the collagen bulk, while cells exposed to stiff fibers were primarily localized to the fibers with remaining cells in the collagen relatively unoriented. Additionally, expression of the ligament transcription factor scleraxis and the contractile fibroblast marker  $\alpha$ -smooth muscle actin were enhanced on the soft and moderate fiber composites, while collagen 1  $\alpha$ 1 expression was increased in stiff fiber composites. These results indicate that sparse fibers, regardless of stiffness, induce cell alignment, with softer fibers promoting ligament phenotype development. Moving forward, our ongoing effort is to guide MSCs toward a ligament phenotype in thicker composites suitable for implantation.

#### **Tissue Forming Capacity of Nasal and Articular Derived Chondrocytes for Intervertebral Disc Regeneration-Effect of Oxygen and TGF $\beta$ Stimulation**

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Autologous Disc Cell Transplantation (ADCT, CODON) and allogeneic juvenile chondrocytes (NuQu, ISTO Technologies) have demonstrated good outcomes in clinical trials. However in the former, concerns remain with limited tissue forming capacity of culture expanded nucleus pulposus (NP) cells derived from degenerated tissue, and in the latter, immunoreactivity. High growth factor requirements, difficulties with isolation and differentiation towards appropriate phenotype makes the use of stem cells challenging. Therefore, there is a need to identify alternative cell sources for the development and clinical translation of next generation therapies for intervertebral disc (IVD) regeneration. The objective of this work was to assess the potential of isolated nasal chondrocytes (NC) relative to conventional cell sources such as articular chondrocytes (AC) and NP cells in terms of cell yield, morphology, proliferative and functional capacity under environmental conditions of 20% and 5% O<sub>2</sub>, in the presence or absence of growth factor, TGF- $\beta$ 3.

NC exhibited the highest cell yield, robust proliferative capacity and secreted NP-like matrix in the absence of TGF $\beta$  in 5% O<sub>2</sub>. This is in contrast to NP cells which exhibited poor proliferative and synthetic capacity in the absence of growth factors even in hypoxia (5% O<sub>2</sub>). Similarly, AC was inferior to NC in this regard. Consequently, NC obtained from a minimally invasive nasal biopsy retained NP-like tissue forming capacity when cultured in a simulated disc microenvironment (low glucose and low oxygen) without exogenous growth factor supplementation. Hence, nasal derived chondrocytes may provide a potent and clinically feasible autologous cell source for IVD regeneration.

#### Immune Profiles of Particulate ECM Scaffolds in Volumetric Muscle Wounds

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The immune system plays a critical role in regeneration, and imbalances in immune polarization can lead to pathologies such as damaging inflammation and scarring. Secreted immune proteins such as cytokines can also interact with stem cells, altering their growth and differentiation. Therefore, there is a need for understanding the effect of scaffold microenvironment on immune polarization and subsequent tissue regeneration. Here we show that bone-derived and cardiac muscle-derived extracellular matrix (ECM) scaffolds applied to traumatic wounds can alter both local and systemic immune profiles. Overall cellular recruitment to traumatic muscle wounds, which is 45–50% immune cells, increases if treated with particulate ECM compared to saline control. Furthermore, using multicolor flow cytometry, we describe three distinct myeloid cell populations within subcutaneous and traumatic wounds that vary in polarization along the M1-M2 axis. All treatment groups showed adaptive immune cell recruitment, specifically CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggesting communication with myeloid cells within the wound microenvironment. Systemically, we noted gene expression changes in both proximal and distal lymph nodes with upregulation of *Il4* in scaffold treated wounds at both 1 and 3 weeks post-operation. These phenotypes, both local and systemic, depend upon scaffold tissue source and evolve over time.

Our results show that scaffolds recruit immune cells with a complex polarization profile in muscle wounds and a previously undescribed systemic response. These immune responses can be modulated with application of ECM scaffolds derived from different tissues. Future studies will apply understanding of immune microenvironments created by these scaffolds to biomaterial design.

#### Parthenolide Inhibits Osteoclastogenic Response and Promotes Osteogenesis of Human Periodontal Ligament Cells under Inflammatory Environment

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**Background:** Periodontitis is an inflammatory disease that causes osteolysis and tooth loss. In this study we investigated the effects of

parthenolide (PTL), a nuclear factor kappaB (NF- $\kappa$ B) inhibitor found in medical herbs, on the osteoclastogenic response and osteogenesis of human periodontal ligament-derived cells (hPDLCs) under the inflammatory situation.

**Methods:** The effect of PTL on inflammatory and osteoclastogenic cytokine expression in LPS-stimulated hPDLCs were detected by qRT-PCR. The effects of PTL on hPDLC-induced osteoclastogenesis in macrophages were determined using a Transwell co-culture technique. The effect of PTL on osteogenesis property of osteoblasts under TNF- $\alpha$  addition was detected. Furthermore, the NF- $\kappa$ B and ERK signaling pathways were detected to reveal the underlying molecular mechanisms of PTL.

**Results:** PTL significantly reduces the mRNA level of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , RANKL, OPG and M-CSF in hPDLCs stimulated by LPS. In the co-culture model PTL also suppressed the osteoclastogenic ability of LPS-stimulated hPDLCs. In addition, PTL enhances osteogenic differentiation in osteoblasts manifested by elevated ALP activity and osteogenic gene markers under TNF- $\alpha$  environment. Moreover, PTL decreased NF- $\kappa$ B activation, I- $\kappa$ B degradation and ERK activation.

**Conclusion:** PTL inhibits the production of inflammatory and osteoclastogenic cytokines in LPS-stimulated hPDLCs, suppress osteoclastogenic differentiation of macrophages induced by hPDLCs, as well as enhances osteogenic differentiation of osteoblasts. The mechanism could be via the inhibitory effect of PTL on the NF- $\kappa$ B and ERK signalling pathways. Our results suggest PTL may therefore have therapeutic value in periodontitis.

#### Vascularization of Large Bone Implants using Bioreactor Technology - A Pilot Study

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Vascularization of large tissue-engineered constructs denotes a big challenge. Within this project a vascularized bone implant was developed and evaluated *in vitro*.

Scaffolds made of  $\beta$ -TCP were seeded with MSCs and cultured statically for 7 days. At the same time tubular fibrin segments were generated in a high velocity rotating custom-made mold and seeded with EPCs. Thereafter, segments were placed in a pulsatile bioreactor and cultured for 7 days. Subsequently,  $\beta$ -TCP scaffolds and fibrin segments were combined and placed in a special-designed bioreactor enabling perfusion of the vascular component. The experiment was run with 3 groups: (1) scaffold without vessel, static cultivation in tube; (2) scaffold with vessel, static cultivation within bioreactor and (3) scaffold with vessel, perfusion of the vessel within bioreactor. After 14 days of cultivation O<sub>2</sub> concentration was measured at different positions within the  $\beta$ -TCP scaffolds. Cell viability and distribution was assessed by live/dead staining whereas cell differentiation and cell outgrowth was analyzed by CD31, vWF, eNOS and Col4 antibody staining.

Combination of  $\beta$ -TCP scaffold and artificial vessel following perfusion improved O<sub>2</sub> availability and consequently cell viability in the center of the scaffold. Cells expressing CD31 were only found on vessel-containing scaffolds cultured dynamically in the bioreactor suggesting that cells were grown out from the artificial vessel into the  $\beta$ -TCP scaffold. Histological analyses revealed enhanced eNOS, vWF and Col4 expression in perfused vessels.

Our investigations show, that it is possible to create a tissue-engineered vascularized bone implant *in vitro* which should be further evaluated *in vivo*.

#### Sintering of High Loading TCP:HAP Microsphere Particles

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We and others have shown that poly(lactic-co-glycolic acid) (PLGA) microsphere-based scaffolds loaded with osteogenic

materials enhance bone regeneration. Our previous studies, however, have been limited to concentrations of hydroxyapatite (HAp) and tricalcium phosphate (TCP) below 30% in the PLGA microspheres. The purpose of this study was to determine whether it would be possible to construct a scaffold exclusively of PLGA microspheres that were themselves predominantly composed of TCP and HAp. Although various microsphere sintering methods were ruled out, we are pleased to report that a methylene chloride vapor treatment was an efficient and effective means to sinter scaffolds at HAp:TCP (1:1) concentrations up to 70%. The analysis for the performance of the scaffolds will include scanning electron microscopy, mechanical stress, calcium release, and PCR after a two-week *in vitro* study. The results of this study will allow us to build upon the foundation of microsphere-based scaffolds for osteochondral regeneration, with a predominantly natural material composition in the bone phase, and a PLGA composition to allow for a continuous transition to the chondrogenic side of the scaffold in the future.

#### Reference

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#### Fluoride and Carbonate Co-incorporated Porcine Bone Derived Biological Apatite Stimulates Osteogenesis *in vitro* Through Wnt/ $\beta$ -catenin Pathway

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Biological apatite (BAP) is widely recognized as a favorable substitute in bone tissue engineering due to it being biodegradable, biocompatible, and bearing osteoconductive properties. Incorporation of trace ions into BAP was reported to contribute to the physicochemical and biological properties of the substitute. In this study, fluoride and carbonate, two elements that are generally accepted to play vital roles in early osteogenesis were incorporated into porcine bone derived biological apatite (pBAP) through chemical-thermal treatment. The prepared fluoride carbonate co-incorporated porcine bone derived biological apatite (fc-pBAP) exhibited significant changes in crystal shape, size and crystallinity compared with pBAP. It was also found that fc-pBAP could release fluorine, magnesium etc. into culture medium, thus, significantly stimulated the proliferation and osteogenic differentiation, while inhibited M-CSF and RANKL/RANK system of rat bone mesenchymal stem cells (rBMSCs) *in vitro*. This phenomenon was further demonstrated associated with the activation of Wnt/ $\beta$ -catenin signaling pathway, which was recently reported as a major signaling cascade in bone biology. As the extract of fc-pBAP can induce phosphorylation and inhibition of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), which resulted in nuclear accumulation of the  $\beta$ -catenin. Moreover, the effects of fc-pBAP on ALP activity and osteogenesis-related gene expressions of rBMSCs was abolished by DKK-1, a blocker of Wnt/ $\beta$ -catenin receptor. These findings suggest that incorporation of fluoride and carbonate into BAP can enhance osteogenesis *in vitro* by triggering Wnt/ $\beta$ -catenin signaling. Ions incorporation could be a valuable strategy for the development and modification of BAP for applications in bone regeneration.

#### Are Tendon-derived Stem Cells a Better Source for Bone Regeneration?

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Despite significant advancements in bone tissue engineering applications, the clinical impact of bone marrow stromal cells (BMSCs) for the treatment of large osseous defects remains limited. Therefore, other cell sources are under investigation for their osteogenic potential to repair bone. In this study tendon-derived stromal cells (TDSCs) were evaluated in comparison to BMSCs to support the functional repair of a 5 mm critical-sized, segmental defect in the rat femur.

Analysis of the trilineage differentiation capacity of TDSCs and BMSCs cultured on collagen sponges revealed an impaired osteogenic differentiation and mineral deposition of TDSCs *in vitro*, whereas chondrogenic and adipogenic differentiation was evident for both cell types. Radiographic assessment demonstrated that neither cell type significantly improved the healing rate of a challenging 5 mm segmental femoral defect *in vivo*. Both, transplanted TDSCs and BMSCs led to the formation of only small amounts of bone in the defect area and histological evaluation revealed non-mineralized, collagen-rich scar tissue to be present within the defect area. Newly formed lamellar bone was restricted to the defect margins resulting in closure of the medullary cavity. Interestingly, in comparison to BMSCs, significantly more TDSC-derived cells were present at the osteotomy gap up to 8 weeks after transplantation and were also found to be located within newly formed lamellar bone, suggesting their capacity to directly contribute to de novo bone formation. To our knowledge this is the first study investigating the *in vivo* capacity of TDSCs to regenerate a critical-sized defect in the rat femur.

#### Bioengineering Brain Matrix Composition to Establish *in vitro* 3d Physiological Brain Cultures

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Adult human brain ECM constituting about 20% of the brain volume is highly organized and unique amongst the ECM of other tissues. The heterogeneity in the molecular composition of the brain ECM enables its functional role in integration and differentiation of stem cells in the embryonic brain or the neurogenic niche in the adult brain. Furthermore, this matrix helps maintain soluble growth factors and in the organization of axonal and pre/post-synaptic proteins. The goal of this research is to investigate how the brain extracellular matrix (ECM) changes over developmental stages and to incorporate that knowledge to establish 3D physiological cultures that more closely approximate the structural, functional and biochemical properties of complex brain tissue. The approach is based on biomaterial scaffold designs gained from development of the brain. Additionally, astrocyte-secreted matricellular proteins are included due to their role in the ECM. Initial results indicate a role for native brain peptides and matricellular proteins towards axonal outgrowth of primary neurons in *in vitro* 3D tissue models of the brain. The measured parameters include axonal network density, axonal caliber, synaptogenesis and electrophysiological recordings. This study will also help elucidate previously unrecognized ECM components that are critical for neuroregeneration. Moreover, greater differentiation of neural progenitor cells into specific neural lineages is expected due to the biochemical cues provided by the native ECM components incorporated within the 3D cultures.

#### A Unit Cell Approach for Scaffold Design

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In the last decade, tissue engineering (TE) has benefited from the development of additive manufacturing (AM) techniques. A so far



unsolved problem of these approaches is the generation of tissue specific mechanical properties with the available biomaterials and techniques. Along with the diffusion of AM, computer-aided TE has started to transform the design process. Here, we aim to establish a computer-aided scaffold design platform to enable bioengineers and scientists to design scaffolds with predictable mechanical properties using a unit cell-based approach. Firstly, a variety of unit cells with TPMS-based and spring-like architecture were designed. Following that, a cuboidal scaffold was virtually filled with repeating units of the designed unit cells and printed by means of additive manufacturing, producing porous scaffolds with different internal microarchitecture. Theoretical mechanical properties of the scaffold obtained by Finite Element Analysis (FEA) using ANSYS were validated with the experimental mechanical properties obtained by subjecting the fabricated scaffolds to compression and torsional testing. Indeed it was found that the mechanical properties of the scaffold were largely affected by the internal unit cell based microarchitecture. Spring-like unit cells led to increased overall elasticity (even while using a stiff polymer) whereas TPMS-based unit cells generally led to increased stiffness. With this computer-aided approach for scaffold design, bioengineers and scientists can avoid the trial-and-error approach and can easily manipulate the scaffold's internal architecture as desired, to obtain scaffolds with the required mechanical properties.

#### **An Osteoinductive Acellular Bone Scaffold Produced from Aged Donors Enhances Osteogenic Activity of Seeded MSCs**

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Tissue engineering using allograft bone enables utilization of bones' innate osteogenic abilities. However, to improve safety, sterilization techniques are required. The group has previously demonstrated the efficacy of a novel bone-wash to generate an acellular and osteoinductive scaffold<sup>1</sup>; however, given that mesenchymal stem cell (MSC) functionality and bone structure differ with age, this study aims to assess the effect of cell and bone donor age on MSC osteogenic differentiation. Therefore, washed bone cubes (1 cm<sup>3</sup>) from donors <50 and >70 yrs were seeded with 5x10<sup>5</sup> MSCs from donors <50 and >70 yrs, and cultured in osteogenic medium for 28 days. At 0, 14 and 28 days, cell metabolic activity (Alamar-Blue), gene expression (QRT-PCR), and alkaline phosphatase (ALP) activity were assessed, as well as H&E and ALP staining at day 28.

Seeded MSCs displayed significant increases in expression of osteogenic genes; runx2, osteopontin and osteocalcin, as well as metabolic activity after 14 and 28 days; expression in young donor cells was statistically higher than older donor cells. Interestingly, old bone induced significantly greater osteogenic gene expression compared to young bone. ALP staining, and ALP enzyme activity was present in all seeded bone samples.

These results demonstrate the influence of cell and tissue donor age on osteogenic ability, with younger cells better able to osteogenically differentiate on the acellular bone, and with older bone better able to promote osteogenic activity. This suggests that, if autologous MSCs are to be used from aged patients, old donor allograft bone will better promote their osteogenic function.

#### **Synergistic Effect of Macrophage Recruitment Agent and Growth factors Release on Bone Regeneration**

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Macrophages function as an osteoclast precursor which involved the process of bone resorption. In addition, studies demonstrate that macrophages play an important role in not only the modulation of inflammation, but also physiological contribution to bone formation. Herein, we focused on macrophages recruitment by SEW2871 of

a sphingosine-1 phosphate 1 receptor agonist. Platelet-rich plasma (PRP) and bone morphogenetic protein (BMP)-2 were used as the growth factors to induce bone regeneration. The objective of this study is to evaluate the effect of macrophages recruitment on bone regeneration by the growth factors release. The water-insoluble SEW2871 was solubilized in water through the formation with L-lactic acid grafted gelatin, and then the SEW2871 micelles were incorporated together with PRP or BMP-2 into gelatin hydrogels for their controlled release. To investigate their effect on bone formation, a critical bone defect of rat ulna was prepared. SEW2871 and PRP or BMP-2 were released from the gelatin hydrogels in a controlled fashion both *in vitro* and *in vivo*. *In vitro* culture with the growth factor enabled macrophages to increase the production of anti-inflammatory cytokines, in contrast to the growth factor-free culture. When applied to the bone defect, the hydrogels incorporating SEW2871 and PRP or BMP-2 recruited a higher number of macrophages and induced the cytokine production, resulting in significantly enhanced bone regeneration compared with hydrogel incorporating either SEW2871 or a growth factor. Taken together, dual release of a macrophage recruitment agent and growth factors modulated inflammatory responses and consequently promoted bone regeneration.

#### **Enhanced Angiogenic Response on Chitosan-graft-poly(ε-Caprolactone) by Wharton's Jelly Mesenchymal Stromal Cells Secretome**

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Enhanced vascularization is critical for the treatment of ischemic tissues and engineering new tissues and organs. Paracrine factors from Wharton's Jelly-derived Mesenchymal Stromal cells (WJ-MSCs) and ECM-mimicking copolymeric matrices could act as a stimulant for the vascular formation. Here, we focus on the effect of secretome, derived from WJ-MSCs, on the angiogenic response using a chitosan-based copolymeric biomaterial. Our long-term goal is to develop a complete inductive scaffold to promote blood vessel regeneration. Chitosan-graft-poly(ε-caprolactone) (CS-g-PCL) copolymer was synthesized by grafting the modified PCL-COOH onto a chitosan backbone [1]. We investigate the cellular adhesion and viability of human umbilical vein endothelial cells (HUVECs) on the copolymer by confocal fluorescence and scanning electron microscopy, and the induction of "tip cell" phenotype by ELISA and microscopy. Expression of the angiogenesis-related genes DLL4, ANGPT2, Jagged 1, HB-EGF, SPROUTY2, PDGFBB and MMP2 in mRNA levels was determined by semi-quantitative RT-PCR. Successful seeding of HUVECs on CS-g-PCL copolymer led to strong cell adhesion and cell proliferation increase over time. Simultaneous treatment of the cells with secretome from WJ-MSCs results in the induction of "tip cell" phenotype and initiation of tubular formation supported by the copolymer. Semi-quantitative RT-PCR reveals significant increase of angiogenesis-related genes. Our results demonstrate the potential of this biomaterial-secretome combination as an inductive system for blood vessel regeneration and vascularized tissue.

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#### **The Effect of Intervention of Wnt and Notch Signaling Pathways on Wound Healing by Targeting Genes C-myc and Hes1**

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**Objective:** To detect the biological effects of Wnt and Notch signaling pathways during cutaneous tissue repair.

**Methods:** We employed a self-controlled model (Sprague-Dawley rats with full-thickness skin wounds) to observe the action and effect of Wnt/ $\beta$ -catenin and Notch signaling *in vivo*. The quality of wound repair relevant to the gain/loss-of-function Wnt/ $\beta$ -catenin and Notch activation was estimated by hematoxylin and eosin (H&E) and Masson staining. Immunofluorescence analysis and western blot analysis were used to elucidate the underlying mechanism of the regulation of Wnt and Notch signaling pathways in wound healing. Meanwhile, ESCs were cultured in keratinocyte serum-free medium (K-SFM) with Jagged1, or DAPT to investigate whether the interruption of Notch signaling contributes to the expression of Wnt/ $\beta$ -Catenin signaling.

**Results:** The results showed that *in vivo* the gain-of-function Wnt/ $\beta$ -catenin and Notch activation extended the ability to promote wound closure. We further determined that activation or inhibition of Wnt signaling and Notch signaling can affect the proliferation of ESCs, the differentiation and migration of keratinocytes and follicles regeneration by targeting c-Myc and Hes1, which ultimately lead to enhanced or delayed wound healing. Furthermore, western blot analysis suggested that the two pathway might interact each other by Jagged1 *in vivo* and *in vitro*.

**Conclusions:** These results suggest that Wnt and Notch signaling play important roles in cutaneous repair by targeting c-Myc and Hes1, and targeting Jagged1 might provide a novel strategy in regulation of action of the two pathways for treatment of wounds.

#### A Human Plasma-Extracellular Matrix Composite for Improved Skin Regeneration

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**Introduction:** Current skin substitutes to treat large deep partial and full thickness burns have limited success due to poor revascularization that leads to poor integration into the debrided wound bed. To address this problem we have developed a vascularized dermal composite scaffold using decellularized porcine adipose tissue extracellular matrix (AECM) in combination with PEGylated platelet free plasma (PEGylated PFP) and adipose derived stem cells (ASCs) (PEGylated PFP-AECM-ASCs).

**Methods:** Composite scaffolds were developed by applying PEGylated-PFP+ASCs onto 1.5 mm thick AECM, and gelled *in situ* with thrombin. Genotypic and phenotypic changes of ASCs within the composite scaffold were determined using RT-PCR and immunofluorescence technique respectively. The efficiency of composite scaffolds was evaluated *in vivo* using a full thickness athymic rat excision wound model. Human blood and abdominoplasty samples were obtained from IRB approved protocols (H-10-023 & H-11-003).

**Results:** *In vitro* results indicate that ASCs within the composite scaffold proliferated and differentiated into two distinct phenotypes: a tubular network-like structure within the gel and striated spindle shape cells associated with the AECM. ASCs within the composite scaffold expressed  $\alpha$ -SMA, F-Actin and NG2 markers associated with vasculogenesis. Histological and immunohistological analysis of wounds treated with composite scaffolds  $\pm$  ASCs showed increased presence of CD31 and retention of human mitochondrial protein + cells by day 8. By day 16, the majority of the composite scaffold remodeled with a well-defined dermal and epithelial layer.

**Conclusions:** From this study we show that a PEGylated PFP-AECM-ASCs composite can be used as a vascularized dermal substitute for improved skin regeneration.

#### Human Adipose Tissue-Derived Stem Cells: Analysis of Biological Properties During Serial Passaging

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In this study we investigated the effect of serial passaging (10 passages) on the biological characteristics of human adipose tissue-derived stem cells (ATSCs). ATSCs were isolated from lipoaspirates of healthy females (n=5, mean age 28.3 years) according to standard protocol and cultured in DMEM/F12 containing 10% FBS and antibiotics up to 10th passage. The morphology was investigated by inverted microscope and TEM. The proliferation was assessed by CEDEX XS. The expression of selected antigens and course of cell cycle was analyzed by flow cytometry. Cytogenetic stability was assessed by karyotype analysis. Moreover, multilineage differentiation experiments were performed. Obtained data showed that ATSCs display fibroblast-like morphology; we recorded only slight changes in last three passages - cells became bigger and contain increased amount of lamellar bodies in cytoplasm. Growth curves provide evidence that ATSCs up to 7th passage had similar course of proliferation kinetics; then growth kinetics slightly decreased. ATSCs were positive for CD73, CD90 and CD105; and negative for CD14, CD20, CD34 and CD45. The levels of these markers did not change significantly during experiment. ATSCs also shared course of cell cycle and we did not record any change in karyotypes. ATSCs underwent chondrogenic, osteogenic and adipogenic differentiation *in vitro*. However, chondrogenic and adipogenic differentiation slightly decreased after 6th passage. On the other hand osteogenesis was slightly elevated in higher passages. In summary, our data indicates the safety of ATSCs in respect to clinical use during first 10 passages.

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#### Adipose Endothelial Cells as an Alternative to Dermal Endothelial Cells for Skin Tissue Engineering

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**Background:** Regenerative medicine strategies are being developed to treat patients with difficult to heal wounds, such as deep burns. For large wounds, donor skin can be limiting. Therefore, tissue-engineered skin substitutes are being developed. Quick vascularization is essential for graft take. However, isolation and culture of sufficient amounts of dermis-derived endothelial cells (Dermal-EC) is a relevant limiting factor. The abundantly present adipose tissue may provide an excellent source for EC (Adipose-EC) for vascularization. Here we studied their characteristics and angiogenic potential and compared them to Dermal-EC.

**Methods:** Surface marker and angiogenic receptor expression on Dermal-EC and Adipose-EC was determined. The ability of EC to proliferate, migrate and sprout was investigated, both at basal level and after stimulation with pro-angiogenic factors bFGF or VEGF. Secretion of angiogenic cytokines was determined at basal level and after TNF $\alpha$  activation.

**Results:** Surface marker and angiogenic receptor expression was similar in both cell populations. Proliferation in response to both bFGF and VEGF was only 1.8 fold higher (p<0.01 or p<0.001) for Dermal-EC. Nevertheless, proliferation under standard culture conditions was equal. Both EC populations showed similar migration rate upon bFGF stimulation. Dermal-EC showed 1.5 fold more sprouting compared to Adipose-EC in fibrin matrices upon bFGF (p<0.05), but only 1.2 fold after VEGF (p=0.06) stimulation. The secretion of angiogenic cytokines of Dermal- and Adipose-EC was similar.

**Conclusion:** Dermal-EC and Adipose-EC behave very comparable as shown by their proliferative, migratory and angiogenic capacity. Therefore, adipose tissue provides an excellent alternative source of endothelial cells for skin tissue engineering.

### Rat *In Vivo* Incubator for Tissue Engineering

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**Background:** The microenvironment of a skin wound plays a major role in healing. Understanding the roles of different factors in a wound and tailoring the treatment accordingly creates a potential for individualized and more effective wound care. We have developed an *in vivo* incubator for tissue engineering. The incubator is molded from a single piece of titanium and can be placed subcutaneously on the dorsum of a rat. This model provides a mean for controlled monitoring and modification of the wound environment *in vivo* with minimal foreign body reaction.

**Material and Methods:** In this study we investigated the effects of topical glucose, pH and oncotic pressure on primary skin cells' behavior. In addition the same factors were studied in a rat *in vivo* incubator. Full-thickness wounds were created on the dorsum of a rat. After wound creation a titanium chamber was placed subdermally on the wound. The chamber encloses the wound creating an isolated, well-controlled environment and importantly prevents the wound contraction from the wound edges. The glucose concentration, pH level and oncotic pressure inside the chambers were modified and wound healing was followed over time. Tissue biopsies and wound fluid samples were collected on days 4 and 8 postoperatively.

**Results:** Our *in vitro* results have shown that all these factors have a great impact on primary cell's migration, proliferation and viability. The *in vivo* incubator has shown to inhibit the wound contraction efficiently allowing us to study the impact of glucose, pH and oncotic pressure on wound re-epithelialization.

### Development of a Structurally Reinforced, Load Bearing Tissue-Engineered Bone Scaffold

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With over 500,000 bone grafting procedures performed annually in the United States, the advancement of bone tissue regeneration research has become a medical necessity. A major challenge for tissue-engineered approaches has been the development of a load-bearing design that exhibits the mechanical properties of native bone. In this study, we investigate the use of sintered hydroxyapatite (HAp) nanopowder to structurally reinforce our previously reported polymeric-based trabecular and cortical biomimetic bone scaffold. HAp is widely studied for its similarity to the naturally occurring calcium phosphate crystals found in the inorganic matrix of bone. HAp nanopowder was packed into a cylindrical framework and sintered at different temperatures for several time periods. The post-sintering morphology and mechanical properties of the columns were investigated; a 5 hour sintering time at 1200°C was selected. Degradation properties of the columns were characterized over 6 weeks and demonstrated no significant loss in mechanical properties over the time period. Finite element analysis (FEA) was used to establish the Von Mises stress criterion and determine the final size and arrangement of HAp within the scaffold. Scaffolds incorporated with HAp columns were mineralized using electro-deposition; preliminary results have shown compressive properties at least matching that of native whole bone. Ongoing work with murine preosteoblastic cell line MC3T3-E1 has confirmed the columns' biocompatibility and osteoconductivity. The integration of sintered HAp columns is an encouraging step towards the development of an effective load-bearing scaffold that exhibits the architecture of native bone and promotes osteoblastic and vascular differentiation.

### First Clinical Trial of Gene-activated Bone Graft: from Basic Studies to Opening Clinical Cases

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Gene-activated bone grafts (GABG) are a promising alternative to tissue-engineered and protein-activated materials for bone tissue repair, but do not have their shortcomings. However, so far no GABG have been evaluated in the clinical studies.

The first GABG we developed consisted of collagen-hydroxyapatite scaffold and plasmid DNA with gene encoding vascular endothelial growth factor (VEGF), which is an active substance of the "Neovasculgen". We showed the safety and efficacy of the GABG *in vitro* and *in vivo*: angiogenic effect of the gene constructions determined osteoinductivity of the material. The preclinical results have been reported in details on the TERMIS 2012–2014.

In 2014, we initiated the open label clinical trial of above-mentioned GABG (NCT02293031), which became the first in this category of bone substitutes. 12 patients with congenital and acquired maxillofacial bone defects (sockets of extracted teeth, bone defects after injuries, surgeries, excision of benign neoplasms and pseudotumors, etc.) or alveolar bone atrophy will be enrolled in the study. A few patients have been already included.

In the most complicated clinical case the patient was suffering from non-unions in the mandible previously reconstructed with non-vascularized bone autograft. We performed excision of the non-unions and bone grafting with the GABG. No adverse events were observed. According to CT, the newly formed tissues in the implantation areas had an average density more than 250 HU 2, 5 months after surgery, which corresponded to the density of cancellous bone.

Thus, there are the first clinical data showing the safety and efficacy of the GABG.

### Effect of Interleukin-1 Beta on Structural Properties of Fibrin Clots

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Fracture hematoma formation occurs within minutes of a bone injury and includes a cascade of biological events that engage not only activated coagulation factors in the haematological system, but also a number of molecular factors, such as pro-inflammatory cytokines. In our previous studies we found discernible differences in the levels of interleukin-1 beta (IL-1 $\beta$ ) between blood clots formed in the smaller compared the larger defects. Based on these findings here we tested if IL-1 $\beta$  could influence the structure of haematomas.

**Methods:** The effects of IL-1 $\beta$  on fibrin polymerization process were investigated using turbidity measurements at different concentrations (0, 10, and 50 ng/mL). We then assessed, the morphological differences in fibrin formation of clots using scanning electron microscopy (SEM). The rigidity of clots was analysed by compaction studies to evaluate the mechanical integrity.

**Results:** The lag time of protofibril formation was significantly longer in the control group (143.5 $\pm$ 43.0, s) compared to IL-1 $\beta$  groups. In contrast,  $V_{max}$  and maximal turbidity were higher in the control group. Under SEM observation, the fibre diameters were increased in the control group compared to IL-1 $\beta$  groups. In addition, the density of fibre formation was thicker in the control group compared to 10 and 50 ng/mL IL-1 $\beta$  groups, respectively. Clot rigidity analysed by compaction study revealed that 50 ng/mL IL-1 $\beta$  can produce clots with thinner and denser fibres.

**Conclusions:** In summary, we can conclude that IL-1 $\beta$  can considerably reduce fiber diameter and increase fiber density, which may provide a promising target for fibrin scaffold fabrication.

#### Bioinspired Material Design to Tune Inflammation towards Tissue Restoration

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In the past twenty years, biomaterial's design for tissue regeneration has continued to evolve as an exciting and multidisciplinary field aiming to restore defective tissues. The classic approach meant to develop a biomaterial able to target and positively stimulate the stem cell niche towards proper healing. However, recently, the host immune response has been identified as a key player to consider in the development of implantable biomaterials. In fact, the ability to control the fate of immune infiltrating cells would help reducing chronic inflammation and result in a better therapeutic outcome (healing, tissue restoration). Here we show that chondroitin sulfate (CS)-functionalized collagen scaffolds are capable of mimicking a blastema-like niche *in vivo* through the early recruitment, *in situ* retention, and sustained induction of anti-inflammatory macrophages. CS is one of the major components of natural extracellular matrix of many tissues and has proved immunomodulatory properties. We fully investigated the physiological phases of healing triggered by the implantation of CS-functionalized collagen scaffolds, from immediate clotting to long term tissue restoration. We performed integrated gene analysis, proteins array, flow cytometry and immunochemistry and provided a full picture of the entire process in a spatiotemporal fashion: from gene expression to final tissue organization, from hours to weeks. This study support a new slant in biomaterial design, expanding the target from stem to inflammatory cells.

#### Microfluidics for Monitoring of Stem Cell Differentiation Dynamics

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Despite the large number of recent studies on stem cell differentiation, many questions remain unanswered and often conflicting results are reported. In order to gain a better understanding of the differentiation process, we developed a microfluidic cell culture platform that allows to control a number of parameters including surface properties, nutrient supply, cytokine exposure, oxygen concentration and mechanical stimulation. The newly developed device enables a systematic evaluation of the combined effects of hypoxic conditions and shear stress, which cannot be achieved by conventional approaches. Another novel aspect of the system is the integration of microfluidic pumps. Micropumps allow to expose stem cells to dissolved chemical stimuli such as the cytokine IL-1 $\beta$ ; for defined and reproducible durations, obtaining a time resolution in the range of seconds. In order to adequately detect short-term cell responses, an improved analytical approach was implemented: impedance electrodes integrated into the cell cultivation chambers allow label-free, continuous, non-invasive and quantitative cell measurements. While existing approaches often rely on end-point staining, the present system enables tracing of dynamic cellular changes. We successfully established and monitored long-term cell cultures in the microfluidic system. Impedance curves allowed for detection of adipogenic and osteogenic

differentiation within 3 days after induction and dynamics of the biological processes could be traced. The investigation of dynamic cell behavior in response to well defined and combined stimuli will help to deepen our understanding of the complex biological processes involved in stem cell differentiation and eventually lead to optimized protocols for tissue engineering.

#### Effects of Decellularization on the Collagen Crosslinking of Aortic Valve Leaflets

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Decellularized heart valves offer potential as a scaffold for the tissue engineered heart valve (TEHV) due to their excellent hemodynamic properties and natural composition. However, before decellularized heart valves are used as TEHV scaffolds, the effects of the decellularization process should be fully understood. This is increasingly important as there is currently no standard decellularization method. Previous studies show that decellularization affects the mechanical properties of heart valves [Converse, Acta Biomaterialia, 2012], though decellularization has no effect on the concentration of collagen, the primary structural protein [VeDepo, SFB Conference, 2015]. This suggests that decellularization may cause structural changes to the collagen fibers. The purpose of this study was to investigate collagen crosslinking in native and decellularized leaflets and correlate those findings to the mechanical properties. Ovine aortic heart valves were decellularized using a multi-detergent, endonuclease, osmotic shock protocol. The mechanical behavior of heart valve leaflets was determined under uniaxial loading, which indicated increased stretch and decreased stiffness following decellularization. An increase in the enthalpy of collagen denaturation was observed, using differential scanning calorimetry. Preliminary HPLC/MS data also indicates increased concentrations of the collagen crosslinking compounds pyridinoline and deoxypyridinoline in the decellularized tissue, suggesting that changes in the mechanical behavior of the leaflet following decellularization are dominated by the removal of cells, not changes in collagen crosslinking. This study provides valuable insight into the effects of decellularization on the heart valve leaflet, helping to more fully characterize this potential scaffold for the TEHV.

#### Myogenic Phenotype Expression of Human Mesenchymal Stem Cells in an Engineered Anisotropic Tubular Tissue

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Human mesenchymal stem cells (hMSC) are highly multipotent, regenerative, and capable of self-renewal. An anisotropic and hierarchical hMSC tissue construct with myogenic phenotype holds great promise for engineering mechanically strong and functional vascular and skeletal muscle tissues. We have recently developed a technology to engineer a tubular construct in a rotating wall vessel (RWV) bioreactor using a fibroblast-derived extracellular matrix (ECM) scaffold and hMSCs. The dynamic culture environment in the bioreactor provides efficient exchange of nutrients and waste to maintain a construct with high cell density. In addition, the mechanical stress generated in the bioreactor stimulated the formation of thick aligned ECM fiber bundles and organized groove-like structure formed in the outer surface, which resulted in enhanced anisotropic property and mechanical strength in the direction of nanofibers. The tubular construct was cultured in myogenic induction medium either in the bioreactor or after bioreactor maturation. The smooth muscle cell-related markers  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), calponin, smooth muscle myosin heavy chain, and desmin were positive on all samples. The outer layer of the tube showed even stronger signals. The mechanical stress slightly increased expression of  $\alpha$ -SMA and desmin, but significantly decreased calponin expression. The myogenic

induction of tubes during bioreactor maturation resulted in much higher myogenic marker expression than those with induction performed after bioreactor maturation. The anisotropic hMSC tissue construct with myogenic phenotype shows great potential for mimicking organization and cellular characteristics of muscle layers, which are essential to the mechanical strength and functional performance of vascular and skeletal muscles.

### Clinical Application of Autologous Adipose Stem Cells in Patients with Multiple Sclerosis - Preliminary Results

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**Introduction:** Multiple sclerosis (MS) disease is caused by the autoimmune reaction against the self-myelin antigens of central nervous system. As a result, the irreversible neurodegradation and axons damage causes stepwise limb paralysis, vision and sphincter problem, leading to disability and resulting death. There is no cure for MS, the existing therapies aim for the reduction of relapse rates and decrease of disease progress. The study was aimed on the local immunomodulation of patient's immune system by intrathecal transplantation of autologous ASCs.

**Materials and Methods:** 16 patients with relapsing-remitting MS form (11 patients) and secondary progressive MS form (5 patients) were enrolled into study under the approval of Bioethical Committee. Adipose tissue was collected according to the Coleman's technique, cells were isolated using collagenase/density gradient technique modified for the clinical purposes. ADSs were injected intrathecally ( $12 \times 10^{-6}$  cells/dose) upon the enrollment, and at 3rd and 6th month the injection was repeated using cryopreserved ASCs, the follow-up observation time varied from 12 to 16 months, the efficacy parameters (EDSS, MS Functional Scale scores, relapse incidents, MRI lesion burden, and whole brain and gray matter atrophy rates) were monitored for 12–16 months period. The study is continued.

**Results and Conclusions:** No adverse effects were seen in 16 patients who received intrathecal infusions of autologous ASCs, some patients manifested improvement of EDSS scores and other efficacy measures. Results suggest that ADS intrathecal transplantations slow down the progress of disease. The longer observation time and patients number increase will allow for more concluding analysis.

### Development of an Automated Bioreactor for *In Vitro* Full Skin Expansion

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Full skin autografts are required for reconstruction of skin burns and trauma scars. However, currently available clinical approaches such as sheet skin graft, mesh skin grafts, artificial skin graft, and *in vivo* skin expansion have limitations due to their potential danger for secondary damage and scar formation at the donor site, and discomfort during skin expansion.

We developed a new bioreactor and evaluated its function in skin expansion using porcine full skin. The reactor is designed as a pneumatic cylinder type, is programmed to adjust the pressure and the operating time. The system is composed of culture chamber unit,

environmental control unit, and monitoring unit. Skins were expanded at various conditions and the expanded skins were analyzed by immunohistochemistry, histology, and RT-PCR.

The results showed that the bioreactor expanded skins to 160% in 4 hours. Histological analysis of the expanded skins showed that epidermal cells and dermal fibroblasts were viable and remained integrity. This study demonstrates that this skin bioreactor enabled to obtain large sized full skin in a short time.

### Reference

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### Multi-functional PLGA Scaffolds For Vascularization In Bone Tissue Constructs

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In our previous work, a new technique in which electrospun nanofibers were rolled into multilayered-rod-like 3D scaffolds has been used in rabbit radius regeneration *in vivo*. This has been highly successful in bone formation. However these scaffolds have no or low capacity to form blood vessels. With the presence of blood vessels, nutrition and oxygen can be delivered to cells in the implanted scaffolds, therefore enhancing the viability of the implants. In this study, we fabricated new multi-functional PLGA scaffolds with porous and nanofibers (by electrospinning) components enabling vascularization along bone formation. PLGA porous component was generated by leaching patterned gelatin particles, which was obtained by microfluidic method. To achieve sustained release and better spatial configuration of VEGF from the scaffolds, VEGF has been immobilized to the PLGA scaffolds through specific binding affinity to heparin which was grafted on the surface of the scaffold covalently. MSCs and HUVECs were cultured on scaffolds to investigate the effects of surface topography, chemistry property, and cell types on cells attachment, viability, and the formation of blood vessel and bone. The results showed that VEGF grafted surface enhanced vascularization greatly on the new PLGA scaffolds in the presence of MSCs or HUVECs or co-culture of both cell types, whilst co-culture showed more organized CD31-positive lumens. Amino group on scaffolds surface combined with co-culture method enhanced osteogenesis of MSCs. Thus, the composite PLGA scaffolds grafted with VEGF can enhance both bone and blood vessel formation, and potentially become a candidate scaffold for bone tissue engineering.

### Regulation of Neurotransmitter Expression During *In Vitro* Neural Differentiation

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One challenge in designing effective stem cell therapies is to ensure their accurate functionality. For neuronal therapies, this means controlling neurotransmitter expression and, ultimately, the electrochemical signaling needed to conduct action potentials. We used bioinformatics and modeling tools to identify the molecular regulatory networks controlling the onset of expression for neurotransmitters in differentiating stem cells. We focused on those associated with neurodegenerative diseases including dopamine, acetylcholine, and GABA. These molecular interaction networks indicate that the expression and interaction of a subset of transcription factors influences specification of particular neuron subtypes. Signaling regulators of embryonic development including growth

factors and retinoic acid are also associated with neurotransmitter fate choice. These models highlight molecules that together enable switches in fate choice between two or more neurotransmitter phenotypes. Finally, this metadata analysis revealed substantial shared signaling between circuits responsible for different neurotransmitter fates. Ongoing work is focused on testing the predictions of the metadata analysis and bioinformatics model using an *in vitro* embryonic stem cell-based system for neuronal differentiation. Understanding and manipulating the regulatory logic controlling specific neurotransmitter profiles provides a critical enabling technology for directed differentiation and assessment of stem cell-based neural therapies.

### Priming Stem Cell Aggregates for Cartilage Tissue Engineering

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Cell-based techniques for articular cartilage regeneration, in particular, bone marrow mesenchymal stem cells (BMSC), have gained prominence over the last several decades, enabling several treatment strategies for osteochondral regeneration. To overcome some of the limitations posed by 2D cell encapsulation, we fabricated rat BMSC aggregates by the hanging drop technique. The aggregates were transferred to hyaluronic acid (HA) (0.01 mg/mL) coated 96 well plates and cultured under different chondrogenic medium compositions. Experimental groups and control groups were tested for differentiation by cytotoxicity and biochemical assays, gene expression and histological staining. The priming medium included (a) rBMSC media ( $\alpha$ MEM with 10% FBS), (b) 5 ng/mL TGF- $\beta$ 3 in high glucose DMEM, (c) 100 ng/mL IGF in high glucose DMEM, (d) 40 ng/mL chondroitin sulfate dissolved in high glucose DMEM (e) 40 ng/mL aggrecan dissolved in high glucose DMEM. An additional group with TGF- $\beta$ 3 media and changeover to IGF at the midway time point. Data revealed that among the different aggregate groups, hyaluronic acid coated plated TGF- $\beta$ 3 media group displayed a statistically non-significant 3% increase in glycosaminoglycan (GAG) content compared to rBMSC media and 5% increase compared to non-coated aggregates. The 3D TGF group aggregates stained more intensely with Safranin-O, revealing an increased collagen content in these groups compared to the non-primed aggregates. These findings suggest that priming stem cell aggregates under defined conditions may result in a superior construct capable of high potential for cartilage repair, given a promising biomaterial delivery vehicle. Funding source: Kansas Rising Star Award and NIH R01 AR056347.

### Dispersive Raman Spectroscopy to Assess Initial Protein Content and Cellular Remodeling of Vascular Constructs

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Tissue engineered vascular grafts (TEVGs) are a promising alternative to surgery for the treatment of thrombotic occlusion of small diameter arteries. The cellular remodeling response of the TEVG will dictate its viability. While initial tissue deposition is needed, continued proliferation of smooth muscle cells (SMCs) and extracellular matrix (ECM) deposition can lead to intimal hyperplasia and occlusion. Real-time monitoring of remodeling would allow for efficient screening of scaffold materials. Thus, we are developing a modified dispersive Raman technique to verify the initial incorporation of natural materials within fibrous scaffolds. We are also determining scaffold remodeling by SMCs *in vitro*. Raman has been used previously to characterize ECM components, but its use to analyze porous, 3-D scaffolds provides several challenges that we are addressing in this study. We electrospun meshes with 100% PCL, 90% PCL/collagen, and 90% PCL/fibrinogen for initial Raman assessment. We identified protein peaks for incorporated collagen and fibrinogen (e.g., 740, 1050 cm<sup>-1</sup>) that were higher than 100% PCL. For example, quantification of peak area at 1050 for electrospun PCL meshes show a significant increase compared to PCL/Collagen ( $p=0.0003$ ) and PCL/Fibrinogen ( $p<0.0001$ ) meshes. Preliminary

results for the 740 cm<sup>-1</sup> peak show an increase after 21 days of culture ( $p<0.04$  for PCL/collagen and PCL/fibrinogen). In addition, PCR and IF analysis for cellular markers (e.g., calponin) demonstrate differences in cell phenotype. Further analysis is ongoing, including tensile testing before and after culture. Overall, this study demonstrates the feasibility of using non-destructive Raman techniques to continuously monitor remodeling with different graft materials.

### Keratinocytes And Fibroblast Culture Onto Radiosterilized Human Amnios as a Biological Skin Substitute

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**Introduction:** In Mexico, 62% of the population have had an accident and 5.7% of this were by burns. In the Centro Nacional de Investigación y Atención a Quemados (National Center of Investigation and attention for the burn patient; CENIAQ) we are working to develop a dermal-epidermal substitute in order to repair damaged tissue in the third degree burn patients.

**Methodology:** We isolated human fibroblasts (dermal layer) with collagenase and human keratinocytes (epidermal layer) with trypsin, from skin biopsies obtained from abdominoplasty surgeries, after informed patient consent. The isolated keratinocytes were cultivated on a feeder layer for primary culture with DMEM F12 medium. The isolated fibroblast were cultivated on a culture dishes with DMEM F12 medium. After reaching confluency, cells were cultures independently onto a radiosterilized human amnion, RHA (scaffold).

**Results:** We performed viability and immunofluorescence assays for keratinocytes-RHA and fibroblast-RHA. For viability assay we obtained a green fluorescent signal in RHA constructs, and according to LIVE/DEAD<sup>®</sup> Cell Viability Assays protocol that indicates we obtained viable cells onto RHA. For immunofluorescence assay we obtained CK5 and CK10 keratinocytes antibodies positive and for fibroblasts, we obtained fibroblast surface antigen positive.

**Conclusion:** We were able to obtain viable constructs, this can be used in the future as a biological skin substitute.

### Prevention of Cartilage Calcification by Controlled Release of Dorsomorphin

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Ectopic calcification, i.e., the biomineralization of soft tissues that normally do not ossify, affects a variety of tissues, and may be an issue in regenerative medicine. Specifically, in cartilage repair procedures the formation of calcified cartilage or osteophytes may be a significant issue requiring follow-up surgery.

Current surgical procedures to treat local cartilage damage (focal lesions) often require reoperation because of calcification of the repair tissue, occurring in 49% of microfracture (MFX) and in 23% of autologous chondrocyte implantation (ACI), whereas nearly all mesenchymal stem cell (MSC) therapies exhibit some extent of chondrocyte hypertrophy, the initial stage of calcification).

Chondrocyte hypertrophy, and calcification in general, involve the canonical BMP signaling pathway of SMAD1/5/8 phosphorylation. Recently, a small synthetic molecule, dorsomorphin, has been found to interfere with SMAD1/5/8 phosphorylation, thus inhibiting chondrocyte terminal differentiation. In considering the application of dorsomorphin to prevent calcification in the repair of a focal lesion, a prolonged and local exposure to dorsomorphin would be required.

We show here that incorporation of dorsomorphin in a micro-particle system allows its release in daily amounts over an extended time period, relevant to *in vivo* calcification. Moreover, we developed a method to induce chondrocyte hypertrophy, as an *ex vivo* calcification model. Finally, we evaluated the positive effects of dorsomorphin both on hypertrophic chondrocytes in culture, and on human cartilage and bone *ex vivo*.

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### Skin Graft Mincing and 100-fold Expansion in the Or - An Alternative to *In Vitro* Cultures in the Treatment of Large Burns

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**Introduction:** Split-thickness skin graft (STSG) is the gold standard for treatment of major skin loss from major trauma or burn injuries. However, this technique is limited by the donor site availability in large burn injuries. Several techniques have been developed in the past to overcome this limitation but have been unable to achieve clinical relevance. With skin mincing technique, we have already successfully demonstrated an expansion ratio of 1:100 and wound healing comparable to STSG [1].

**Methods:** In this study, we explore the feasibility of the next-generation technique, pixelgrafting, which minces donor skin to small pieces measuring  $0.3 \times 0.3$  mm. Wound healing was studied in a full-thickness wound porcine model. After wound creation and pixelgraft transplantation a polyurethane device was placed to cover the wound. This device serves as a temporary skin replacement and creates an ideal environment for regeneration. Multiple wound healing parameters were used to study pixelgrafting technique.

**Results:** The pixelgraft transplanted wounds demonstrated faster re-epithelialization rate, decreased wound contraction, superior skin integrity and increased mechanical stability when compared to control wounds.

**Conclusions:** Pixelgrafting technique provides a promising advancement in the field of wound healing. This technique would help in addressing the most commonly encountered limitations of the STSG with the possibility of large expansion ratio with improved quality of healing.

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### Creating a Human Capillary Network in a Wide-pore Polyurethane Scaffold *In Vitro*, for *In Vivo* Wound Applications

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**Background:** Skin wound healing relies on the capacity to mount an angiogenic response. Certain ischemic pathologies demonstrate limited angiogenic capacity leading to delayed healing and tissue ulceration. In a preliminary study, we attempted to form an instantaneous capillary network in a wide-pore scaffold *in vitro* for subsequent transfer *in vivo* for potential chronic wound healing treatments.

**Methods:** *In vitro*: 400,000 primary human microvascular endothelial cells (hMEC) with/without 200,000 human vascular smooth muscle cells (hSMCs) were mixed with human fibrinogen, and ab-

sorbed with thrombin into a porous (300–600 micron wide pores) polyurethane scaffold (6 mm  $\times$  1.2 mm; PolyNovo Biomaterials, Pty. Ltd. Australia). The scaffolds were cultured for 1 or 3 days and assessed morphologically. Alternatively, 300,000–500,000 human iPSC derived ECs were seeded under similar conditions into the scaffold. *In vivo*: One and three day co-cultured hMEC +/-hSMC scaffolds were inserted under the dorsal skin of nude rats (immunosuppressed) and human capillary formation examined at 7 days.

**Results:** *In vitro*: Within the fibrin, tube-like capillaries (human CD31+ve) were evident at 1 (hMEC or iPSC ECs), and 3 days (hMEC) forming interconnected networks. When hSMC were co-cultured with hMEC, hSMC attached abluminally to the capillaries. *In vivo*: After 7 days hMEC scaffolds demonstrated survival of human CD31+ve capillaries, many showing evidence of perfusing rat blood. Preliminary (N=3) morphometric data indicated a doubling in human percent vascular volume when hSMCs were co-cultured in the scaffold.

**Conclusion:** Pre-vascularization of scaffolds with human capillaries could potentially be used in avascular wounds to promote wound healing.

### Recellularization of Whole Decellularized Porcine Hearts with Human Cardiac Fibroblasts and Endothelial Cells

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Cardiac tissue engineering may be the answer to the growing need of more effective therapies for patients with heart disease. A cardiac patch that can restore the diminished function of an infarcted heart or whole functional recellularized hearts could save thousands of lives per year [1]. In this study whole porcine hearts were decellularized in an automated perfusion apparatus that controls pressure and temperature. Decellularization was completed in 24 hours with 6 hours of sodium dodecyl sulfate (SDS) and 2 hours of Triton X-100 exposure. A hemolysis assay with human blood was used to optimize the process to preserve more natural ECM structural contents and minimize cytotoxicity due to residual SDS. The right ventricles of the hearts were dissected, cannulated through the main right coronary artery and any large open blood vessels were closed using cyanoacrylate tissue adhesive (Vetbond<sup>®</sup>). Sections of the hearts were placed in sealed, agitated and oxygenated bioreactors and sterilized with a protocol optimized for being less destructive and enhancing cell attachment to the ECM. Sections were then perfused with growth medium for 24 hours and reseeded with  $1 \times 10^8$  fluorescence-labeled human umbilical vein endothelial cells (HUVECs) and human cardiac fibroblasts (HCFs). The HUVECs were introduced through vasculature perfusion and HCFs by needle injection into the ECM at multiple times. After at least 7 days of growth, histological images showed HUVECs dispersed through the right ventricle, covering the vasculature and HCFs present in the myocardium. These studies demonstrate the feasibility of recellularizing whole decellularized porcine heart tissue.

### Synthesis of Mesoporous BG-CS Nanoparticles for Enhanced Gene Transfection

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Gene therapy has been proved to be a promising approach to treat numerous genetic diseases. However, safe and efficient gene delivery carries remain the most challenging aspects of gene therapy. Mesoporous bioactive glasses (BG) can be used as carriers for biomolecule delivery with improved functions. In this study, mesoporous bioactive glasses (rMBGs) were prepared by sol-gel process combined with a micro-emulsion method. To further improve the efficiency of gene transfection, modification of chitosan (CS) was decorated on the surface of MBG as a new nonviral gene vectors (MBG-CS). The chemical structure of MBG-CS was confirmed by the FT-IR

spectroscopy. pOGP containing the osteoblastic growth peptide and the green fluorescent protein fusion gene was incubated with MBG-CS. The diameter of MBG-CS/pOGP polyplexes was below 300 nm. The polyplexes exhibited suppressed nonspecific protein absorption, increased pDNA dissociation and enhanced resistance against DNase I degradation. MBG-CS/pOGP polyplexes performed excellent gene transfection efficiency, which displayed 5-fold higher than the unmodified MBG/pOGP in rMBGs. Additionally, the plasmid can be successfully expressed in cells transfected by rMBGs. Hence, these results suggest that MBG-CS nanoparticles can be used as a highly promising gene vector for gene therapy.

#### Assessment of Disinfection and Sterilization of Decellularized Pericardial Scaffolds

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Current approaches to tissue disinfection include the use of antibiotics; yet  $\approx 10\%$  are discarded due to contaminations. Decellularized xenogeneic scaffolds are a promising alternative, but the lack of optimized sterilization delays their clinical application. This study assessed the effectiveness of antibiotic/antimycotic (A&A) disinfection and peracetic acid (PAA) sterilization on decellularized pericardium.

Porcine and bovine pericardium were decellularized using Triton X-100, Cholic Acid and endonuclease, and assessed histologically and biochemically. Controlled contamination with Gram<sup>+</sup> and Gram<sup>-</sup> bacteria ( $\approx 10^8$  CFU/mL *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus*) for 2 h at 37°C was followed by A&A treatment for 24 h at 37°C and PAA treatment (0.1% v/v and 0.05% v/v) for 3 h at 27°C with agitation. Sterility was assessed according to European Pharmacopoeia guidelines. Microorganisms were identified by MALDI-TOF mass spectrometry and quantified with the most probable number method.

Histology and biochemistry revealed that decellularization removed all cell and xenoepitopes from pericardia, whilst maintaining their gross histoarchitecture. Decellularization resulted in sterile tissue. Following controlled contamination, A&A treatment reduced the bio-burden to  $\approx 10^3$  CFU/mL, while PAA in both concentrations eliminated all microorganisms and provided sterile scaffolds after 14 days.

These results demonstrate that PAA treatment completely eliminated microorganisms, while treatment with A&A alone was not sufficient. Further investigation will focus on the effect of disinfection/sterilization on the biomechanics and histoarchitecture of the decellularized pericardium.

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#### Development of an Artificial Ovary: Influence of Follicle Stage on Transplantation Outcome

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**Aim:** Evaluate the influence of the developmental stage on survival and growth of isolated murine preantral follicles after transplantation inside an artificial ovary.

**Methods:** Fifty isolated primordial-primary (PP) and 50 secondary (S) follicles were embedded in a fibrin matrix with 50,000 ovarian cells and grafted to immunodeficient mice for 2 and 7 days. Follicle diameter and viability were analyzed before grafting, while follicle survival (TUNEL), development (Ki67), ultrastructure (TEM), and graft vascularization (CD34) were evaluated after grafting.

**Results:** After isolation PP and S follicle diameter was 40.6  $\mu$ m and 90.9  $\mu$ m, and viability 67% and 66%, respectively. S follicles showed a significantly higher recovery rate (p value < 0.001) than PP follicles after both grafting periods. TUNEL assay evidenced live follicles in both groups (65% and 80% on day 2, and 100% and 82% on day 7, in the PP and S groups respectively), while Ki67 staining revealed a high proportion of growing follicles after transplantation (81.8% and 100% on day 2, and 100% and 96.7% on day 7). On day 2, new capillaries were found in tissue surrounding the fibrin clots. On day 7, capillaries were also observed inside the clots, with vessel area significantly increased in the S group (p value < 0.05). Preliminary TEM results showed follicles from both groups to have a normal ultrastructure.

**Conclusion:** Follicles from both groups were alive and growing after 7 days of grafting, but a higher recovery rate was observed for S follicles, possibly correlated with the physical properties of the fibrin matrix.

#### Single Walled Carbon Nanotubes and Gold Nanorods Facilitated Photoacoustic Stimulation Enhances Osteodifferentiation of Adipose-Derived Human Mesenchymal Stromal Cells

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The photoacoustic (PA) effect, which occurs when a material absorbs electromagnetic energy and creates acoustic waves, has recently shown to improve osteodifferentiation of MSCs *in vitro* [1, 2]. However, the wavelength of light in those studies had low penetration depth and is impractical for *in vivo* studies. In this study, we designed and tested a PA stimulation device with near-infrared laser diode (904 nm, 75 W, 60 ns pulse width, 500 Hz repetition rate). Furthermore, films made with single walled carbon nanotubes (SWCNT) and gold nanorods (AuNR) were used as absorbers to enhance the PA effect. Adipose-derived human MSCs (ADSCs) were stimulated 10 minutes daily for 4, 9 and 14 days. Osteodifferentiation was analyzed by cell number, alkaline phosphatase (ALP) activity and calcium content. No significant differences were found in ALP activity between the groups. Calcium amount was significantly higher in both SWCNT film and AuNR film groups compared to PA stimulated and non-stimulated control groups at day 14. No difference in calcium content was found between the AuNR film and SWCNT film groups. Results show that both SWCNT (90% absorbance) and AuNR (10% absorbance) enhance PA induced osteodifferentiation of ADSCs *in vitro*.

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#### Human-scale Whole-heart Decellularization - Do We Need Process Analytics?

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**Introduction:** Decellularization methods often lack an insight into the outtake of biomass to monitor the process itself. We analyzed



perfusion-based decellularization of small and large animal hearts to implement process analytics to whole-organ decellularization.

**Methods:** Rat and ovine hearts were decellularized by detergent based coronary perfusion. Decellularization efficacy of ovine hearts was assessed by biomass outtake analysis, histology, DNA- & protein quantification and western blotting. The decellularization process itself was evaluated by subjecting perfusates to protein content analysis and rheological assessment, thereby comparing decellularization kinetics for rodent and human-scale hearts.

**Results:** Total biomass wash-out corresponded to 18% of wet organ weight. Histological evaluation demonstrated microscopic acellularity and a reduction of non collagenous proteins by  $80 \pm 20\%$ . DNA could be reduced by  $90 \pm 8\%$ . Remnants of  $8 \pm 5\%$  desmin but no cardiac actin could be detected in decellularized ECM by western blotting. Total protein decomposition kinetics revealed in principal an exponential characteristic with outtake rates of 0.1 to 0.04 h<sup>-1</sup>, that ended in a limitation and proved to be congruent between ovine and rodent hearts. Rheological evaluation further confirmed the outtake kinetics by revealing perfusates viscosity and its content of higher-molecular structures.

**Conclusion:** For the first time we illuminate the kinetics of perfusion-based whole organ decellularization while presenting a methodology that may enable tailoring individual decellularization processes to minimize adverse effects to the matrix. Moreover these results demonstrate that donor organ size has minor impact on the biomass outtake during perfusion decellularization.

#### Repopulation of Decellularized Mouse Pancreas with Human Embryonic Stem Cells-derived Pancreatic Progenitor Cells

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Type I Diabetes affects over 1 million people in United States. While human embryonic stem cells (hESC) have emerged as an alternative cell source for  $\beta$ -cell replacement, a major gap remains in cellular immaturity partly due to the lack of supportive 3D micro-environment. We hypothesize that perfusion-decellularization of whole pancreas could yield a 3D architecture and complex natural pancreas extracellular matrix (ECM) suitable for the pancreatic differentiation of hESC.

Cadaveric pancreata (n=8) were perfusion-decellularized with detergent and characterized for acellularity, preservation of ECM and microstructures. hESC-derived pancreatic progenitor (PP) cells ( $3 \times 10^6$  cells) were generated following previous published protocol and seeded into the 3D pancreatic scaffold via vasculature. The recellularized 3D construct was cultured on a perfusion bioreactor and evaluated via immunostaining and qRT-PCR after 9 days.

Characterization of the decellularized pancreas revealed complete removal of cells, preservation of ECM proteins and 3D architecture. Engraftment of hESC-PP (positive staining for hNuclei marker) was observed, and low apoptotic cells were detected (<10% TUNEL positive). Engrafted cells were also found positive for early pancreatic marker genes (PDX1, NKX6.1, NGN3, ISL1) and maturation markers (MAFA, INSULIN) - confirming the differentiation of engrafted hESC-PP into insulin-producing cells.

Perfusion-decellularization of pancreas efficiently removes cells while retaining ECM protein and microstructure. This perfusable 3D whole organ scaffold was supportive of pancreatic differentiation of hESC. Further studies will be evaluated to establish the suitability of acellular pancreatic ECM as a scaffold to transplant donor islets and achieve euglycaemic state.

#### Multicellular Spheroids of Human Microvascular Endothelial Cells form Functional Blood Vessels When Injected into a Dermal Pocket Wound Model

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**Background:** Angiogenesis is crucial during wound healing. Here we investigate as proof of principle the use of an autologous cell-based therapy for the potential treatment of chronic wounds by injecting human dermal blood microvascular endothelial cells (hMEC) as multicellular spheroids into wound models to promote angiogenesis for healing. Multicellular spheroids promote cell survival by maintaining cell-cell contact to avoid anoikis.

**Method:** *In vitro*: Spheroids of 1,000, 2,000, 5,000, 10,000 and 20,000 hMEC were formed and cultured in fibrin gel for three days to assess capillary-like sprout formation. *In vivo*: Dermal pocket wounds were made in the backs of severe combined immunodeficiency mice and injected with either  $1 \times 10^6$  hMEC in single cell suspension or  $1 \times 10^9$  hMEC as spheroids of 10,000 cells. After seven days, the wounds were sectioned and labelled with anti-human CD31 to assess for human capillary formation.

**Results:** *In vitro*: hMEC spheroids sprouted capillary-like structures *in vitro*. After three days in culture, spheroids of 1,000 to 20,000 cells showed no significant differences in the average number of sprouts observed per plane (5–11; n=3) nor in the average lengths of the sprouts (123–193  $\mu$ m; n=3). *In vivo*: Wounds injected with hMEC spheroids showed blood vessels which stained positive to anti-human CD31 and contained mouse blood in the lumen, while human cells are yet to be detected in wounds injected with hMEC as single cell suspensions.

**Conclusion:** hMEC spheroids formed functional blood vessels in a preliminary wound model. The assessments of hMEC spheroid injection into chronic wound models are ongoing.

#### Self Organizing Vascularized Cardiac Scaffolds from Human Atrial Appendages

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We investigated human left atrial appendages (LAA) as a promising new cell source for vascularized cardiac tissue scaffolds.

LAAs were minced and cells harvested after outgrowth and MACS sorted for CD31. Cell populations (whole cell mix, CD31- and CD31+) were characterized by immunofluorescence and FACS. CD31+ endothelial cells (haaEC) were tested in compressed collagen I scaffolds primed with I) human dermal fibroblasts or II) LAA whole cell mix creating a completely autologous construct. After 24 h of incubation and removal of a central needle placeholder, the resulting core channel was seeded with haaEC and continuous perfusion was initiated with 1 ml/min. Patches were examined over 14 days regarding morphology and cell viability/proliferation. EC marker expression and synthesizing activity were investigated immunohistochemically.

Whole cell mix (WCM) and CD31- cell immunofluorescence showed mostly  $\alpha$ -SMA positive cells as well as an overall Connexin43 positivity and CD105 positive subpopulations. HaaEC were positive for VE-Cadherin, TIE2, VEGFR2, Connexin43, CD105 and showed broad Ki67 staining matching 2D culture proliferation in contrast to WCM and CD31- poorly expressing Ki67. HaaEC inside the core channel showed good attachment, survival and proliferation during the whole investigation period, regardless of scaffold cell priming. WCM in II) showed interstitial proliferation of all subpopulations under 3D conditions unlike in cell culture flasks. Strikingly, embedded haaEC showed self-organization potential and built vessel like structures as confirmed by immunostainings.

Human atrial appendages are a new cell source of cardiac primed cells providing both endothelial and interstitial cellular components within autologous prevascularized scaffolds for cardiac tissue engineering.

### Injectable Hydrogels with Double Network Formation to Promote Angiogenesis

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Human adipose-derived stromal cells (hASCs) possess tremendous potential for multiple regenerative medicine therapies including neovascularization. Unfortunately, direct injection by syringe needle into ischemic tissue typically results in only 5% cell viability, in part due to mechanical membrane damage during injection. We demonstrate that pre-encapsulating cells in a weak hydrogel ( $G' \sim 50$  Pa) provides significant protection from membrane damage during injection. However, this stiffness is much lower than that of native adipose tissue ( $G' \sim 2000$  Pa), which we hypothesized would promote secretion of paracrine, pro-angiogenic factors. Therefore, we designed a next-generation family of injectable hydrogels that are soft *ex situ* ( $G' \sim 50$  Pa) for enhanced cell transplantation and undergo double-network formation *in situ* to achieve a range of stiffnesses ( $G' \sim 100 - 2000$  Pa). hASCs injected through a 28-gauge syringe needle were significantly protected from membrane damage within all hydrogel formulations compared to delivery in medium alone. Following injection, 3D cell proliferation over two weeks was significantly enhanced within stiffer hydrogels. Similarly, pro-angiogenic gene expression and vascular endothelial growth factor (VEGF) secretion were significantly upregulated from hASCs encapsulated within stiffer hydrogels. These hASC-laden hydrogels are being further investigated for their ability to support proliferation and tubule formation of surrounding endothelial cells *in vitro* and for functional neovascularization in a murine model of peripheral arterial disease. Together, these data suggest that hydrogel mechanical properties may need to be separately optimized for each phase of cell transplantation, with weaker gels preferred during implantation and stiffer gels favored during neovascularization.

### Human Cell Conditioned Media and Extracellular Matrix Reduce Inflammation and Support Hyaline Cartilage Formation

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Hypoxia induced multipotent stem cells secrete a human extracellular matrix (hECM) that contains components associated with stem cell niches in the body and scarless healing of fetal skin including laminins, osteonectin, decorin, hyaluronic acid, collagen type IV, SPARC, CXCL12, NID1, NID2, NOTCH2, tenascin, thrombospondin, fibronectin, versican, and fibrillin-2. This material can be manufactured reproducibly under GMP conditions and has been shown to support proliferation of hESCs and MSCs and *in vitro* studies have demonstrated that it does not stimulate human dendritic cell activation. In a thrombin induced inflammatory environment in an *ex vivo* rabbit spinal disc model the hECM significantly down regulated elevated IL-1, IL-6, TNF, MMP3, and ADAMTS4 gene expression while upregulating aggrecan expression in both the annulus fibrosus and nucleus pulposus tissue ( $p < 0.001$ ). *In vivo* studies were performed in rabbits to study its potential as a scaffold for MSC growth and differentiation into cartilage and bone. Osteochondral defects were left untreated or injected with the hECM slurry. At week 8, the controls showed scarring and poor tissue growth while the defects treated with hECM showed excellent mature bone and hyaline cartilage which demonstrated a tide mark and integration into adjacent cartilage. No inflammatory reactions were seen in the hECM treated defects. Goat studies are underway for treatment of 7 mm osteochondral defects which support an IDE filing. This data demonstrates that the bio-engineered hECM has significant potential as a novel orthobiologic to reduce inflammation and support cartilage regeneration and healing in both the articulating joint and intervertebral spinal disc.

### High-throughput Polymer Screening Identifies Materials Suitable for Cardiac Progenitor Cells Growth

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After acute myocardial infarction, the resident cardiac progenitor cells are insufficient in regenerating the lost myocardial cells. *In vitro*, the fraction of cardiac cells expressing CD117 (C-Kit) has been proposed to have potential in cardiac regeneration. In this study we applied a high-throughput polymer microarray screening method to select substrates supporting the growth of human C-Kit<sup>+</sup> cells. A library of 384 polyacrylates (PAKs) was contact printed (qArray-mini, Genetix, UK) onto -coated slides. The C-Kit<sup>+</sup> cellular fraction of cardiac cells isolated by enzymatic digestion of human auricle fragments, were obtained by flow cytometry sorting (anti-CD117 antibody, BD Biosciences) and expanded in transwell for 4 passages [1]. Cells isolated from 3 independent donors were seeded (2.5E+05 cells/array) and cultured for 48 hours on triplicate replica arrays. Fluorescence staining for Phalloidin,  $\alpha$ -SMA, PhosphoHistone-H3 (PHH3) and DAPI were performed. Computer based (ImageJ) cell nuclei quantification, indicated four PAKs promoting cell adhesion (cell/spot ranging from 28.0 $\pm$ 5.2 to 95.1 $\pm$ 11.6, mean $\pm$ SE), data also supported by actin cytoskeleton polymerization. Moreover, the presence of cells positive to PHH3 staining after only 48 hours of culture on these substrates was indicative of cell proliferation. In conclusion, our data identify, for the first time, PAKs that sustain human cardiac progenitors adhesion and growth. This screening establishes criteria for a novel class of materials potentially exploitable for combined approaches in cardiac healing and/or regeneration.

### Endometrium Tissue Culture for *In Vitro* Embryo Implantation

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Recent progress of *in vitro* fertilization technology has much improved the infertility caused by ovary or ovulation. However, the reason for infertility caused by uterus or after embryo transplantation is still unclear. In this study, *in vitro* embryo development model to investigate embryogenesis mechanism and to improve the infertility treatment has been developed by co-culture of embryo and endometrium in mouse model.

Mouse embryos fertilized *in vitro* were cultured from ova to blastocysts. Then the blastocysts were cultured by F0 medium on collagen gel until embryo implantation and trophoblasts extension was confirmed. Mouse endometrium tissue was isolated and cultured in DMEM containing 10% FBS, progesterone, and estradiol. As a control, endometrium tissue was cultured in DMEM containing 10% FBS only. Change of endometrium by the hormones was observed histologically by HE staining. Furthermore, the cultured embryo and endometrium tissue were co-cultured in microtube in DMEM with FBS and hormones for *in vitro* implantation.

HE staining of the endometrium culture with hormones indicated its change from proliferative phase to secretory phase. Adhesion of embryo onto endometrium was observed by co-culture of embryo and endometrium tissue. However, the experiments could not confirm embryo implantation *in vitro*. Further progress may be needed for clear observation of the embryo implantation on cultured endometrium tissue.

### Advanced Wound Care Strategy to Enhance Angiogenesis Combining a Prolly Hydroxylase Inhibitor with Lysophospholipid

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**Background:** The prolyl hydroxylase inhibitor ciclopirox olamine (CPX), and the lysophospholipid sphingosine-1-phosphate (S1P) both have been reported independently to be pro-angiogenic. We previously demonstrated *in vitro* that combining both compounds generates angiogenic synergy potentiating endothelial cell (EC) sprouting<sup>1,2</sup>. We unravel here complementary mechanisms *in vitro* and confirm *in vivo* angiogenic efficacy in a rat model.

**Findings:** Leveraging on CPX and S1P's complementary pathways in stabilizing HIF-1 $\alpha$ , we show that their combination further increased HIF-1 $\alpha$  expression from 2-fold (S1P), 10-fold (CPX) to 15-fold (CPX + S1P). Downstream MMP-2 protein expression was also increased from 1.3-fold (S1P or CPX) to 2.5-fold (CPX + S1P). CPX also potentiates EC sensitivity to S1P by post-transcriptionally increasing S1P receptor 1 and 3 protein expression by 2.5-fold. Both receptors are crucial in mediating accelerated EC migration<sup>3</sup>. Subcutaneously implanted PVA sponges were loaded transdermally with CPX, S1P and CPX+S1P on alternate days. Lectin perfusion showed that the factors potentiated the length of functional vessel infiltration into the sponges reaching from from 2-fold (CPX or S1P, respectively) to ~5-fold (CPX + S1P) in comparison to vehicle controls.

**Conclusion:** CPX+S1P is a powerful angiogenic combination that holds promise for wound care, and might be efficacious particularly in diabetic ischaemic ulcers, where HIF-1 $\alpha$  stabilization is impaired<sup>4</sup> and S1P plasma concentration decreases with progression of diabetes<sup>5</sup>. *In-vivo* work to assess the potency of this combination in diabetic wound healing is underway.

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## Spatial Control of Wnt signalling for Orthopaedic Tissue Engineering

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Wnt signaling plays a crucial role in a variety of biological processes, including embryogenesis and stem cell maintenance in adult mammals. Disregulation of Wnt causes a wide spectrum of diseases. Enhancing tissue repair by activating endogenous adult stem cells holds promise for a variety of disorders, however, the progress of exploiting Wnt signaling for regenerative medicine has been hindered by difficulties in producing sufficient quantities of recombinant Wnts for systemic administration. Moreover, the potential risks of off-target activation of this pathway cannot be ignored. Therefore, the development of techniques for the localized targeting and modulation of Wnt signaling may provide a suitable therapeutic option. *In vivo* and *in vitro* studies have revealed that Wnt/ $\beta$ -catenin signaling is responsive to mechanical loading. Mechanical forces have been demonstrated to be critical factors in embryogenesis and tissue remodeling. Our group has demonstrated previously how nanomagnetic actuation can be used to remotely apply highly localized forces, in the piconewton (pN) range, directly to membrane mechano-receptors of mesenchymal stem cells mediated by functionalized particles and control local cellular responses. Here, we demonstrate how nanomagnetic actuation of peptide-functionalized MNPs can modulate the canonical Wnt signaling cascade *in vitro* with up regulation of downstream pathways and how this strategy can be employed to set up spatial cues in tissue engineering hydrogel approaches and *ex vivo* organ culture systems such as the chick femur. In addition, we report

how localization of Wnt binding can establish spatial gradients of differentiation of mesenchymal stem cells in hydrogel systems.

## An Endochondral-ossification Based Approach to Vascularisation and Repair of Bone Defects using Hypertrophically-primed Bone-marrow Derived Stem Cells in Combination with Collagen-based Scaffolds

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Traditionally, bone tissue engineering involves *in vitro* culture of scaffolds under osteogenic conditions, mimicking the process of intramembranous ossification (IMO). However, due to avascular necrosis within these constructs, failure often occurs. Recapitulating endochondral ossification (ECO), whereby chondrocytes undergo hypertrophy, subsequent release of pro-angiogenic factors, vessel invasion and mineralisation of the cartilaginous precursor, may offer possible solutions to this drawback. In this regard, the aim of this study was to develop an *in vitro* model of ECO using two collagen-based scaffolds which have shown potential for tissue repair, namely collagen-glycosaminoglycan (CG) and collagen-hydroxyapatite (CHA) scaffolds. We hypothesised that stimulating mesenchymal stem cell (MSC) hypertrophy would improve scaffold vasculature *in vivo* and ultimately enhance bone formation. CG and CHA scaffolds were produced by lyophilisation and subsequently cultured with rat MSCs in chondrogenic medium for 21 days with the eventual inclusion of hypertrophic medium for 14 days *in vitro*. These were compared to constructs cultured in osteogenic medium for 35 days, thus mimicking the process of IMO. The tissue-engineered constructs were then implanted into 7 mm calvarial and 5 mm femoral defects on male Fischer rats for 8 weeks. The results revealed evidence of hypertrophic-like cartilage synthesised within ECO-based constructs which sequentially supported significantly greater bone formation compared to the IMO-based constructs and empty defect controls. This study highlights a novel approach in the repair of bone defects by utilising developmental processes and demonstrates how a major challenge in cartilage tissue engineering, MSC hypertrophy, can be utilised to the benefit of bone tissue engineering.

## Co-transfection of Decorin and Interleukin-10 Genes Additively Silences TGF- $\beta$ 1 Related Pro-fibrotic ECM Gene Expression in Human Tenocytes

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Tissue extracellular matrix remodeling during tendon repair is driven by increased activity of transforming growth factor beta 1 (TGF- $\beta$ 1), which also regulates progressive fibrosis. In this study, it is hypothesised that co-delivery of decorin and interleukin-10 plasmids in a collagen hydrogel system can downregulate TGF- $\beta$ 1, abolishing fibrillogenesis. The specific objective of this study is to obtain a controlled delivery of anti-fibrotic genes to tenocytes in a hydrogel platform to downregulate TGF- $\beta$ 1 and expression of fibrosis related genes without affecting cell phenotype. Hydrogels were prepared with 1.5% collagen solution. TransIT<sup>®</sup>-LT1 transfection reagent was complexed with pDNA (decorin or interleukin-10). The polyplexes were mixed with the hydrogel solution at 4:1 v/v ratio. pDNA release from hydrogels was quantified using Picogreen<sup>™</sup> assay. Transfection of the primary human tenocytes and expression analysis was performed using RT-PCR and immunocytochemistry. Phenotypic characterization was performed using flow cytometry. Statistical analysis was performed using one-way ANOVA with p<0.05. 80% plasmid DNA release was seen over seven days. Transfection of tenocytes with decorin and interleukin-10 resulted in 12-fold

and 8-fold increase in gene expression respectively. Downregulation of TGF- $\beta$ 1 and collagen types I and III, fibronectin and elastin was confirmed at day four and seven using RT-PCR. Flow cytometry analysis confirmed phenotypic maintenance of transfected tenocytes.

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#### Co-culture Systems for Hierarchical Vascular Networks

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Creating perfusable vascular networks is critical for enabling the survival of thick engineered tissues destined for implantation. In addition, good models of angiogenesis and vasculogenesis would aid understanding of numerous pathogenic processes, and could be useful for drug testing, including anti- and pro-angiogenic agents, as well as studying the interactions of different cell types with the micro-vessels. In the present work, we investigated the effect of hydrogel composition and different cell combinations on the self-assembly of endothelial cells (ECs) into capillary-sized micro-vessels, and how more complex topologies mimicking those created by nature might be reproduced. Collagen gels with different molecular weights and geometries were employed. Using enzyme-linked immunosorbent assay (ELISA), confocal microscopy and time-lapse imaging we investigated how ECs interact with other cells (e.g., osteoblasts and pericytes) in a co-culture under normoxia and hypoxia, and revealed the optimal conditions for the establishment of hierarchical microvasculature. Collectively, our findings summarize a combination of factors affecting micro-vessel formation *in vitro*, emphasizing that the coordination of biomaterial design, proper co-culture systems and a pro-angiogenic environment are vital for improved vascular network formation.

#### Biomimetic Hierarchical Eggshell Membrane/Chitosan-Polycaprolactone Micro/Nano Fibrous Scaffold for Skin Tissue Engineering Applications

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Design and fabrication of a scaffold that can fulfill the requirements of tissue engineering is determined by interaction between cell and its microenvironment. The objective of the present work was to develop a mechanically stable, biocompatible, porous, non-immunogenic and cost-effective bilayered scaffold for skin tissue engineering (STE) applications. We have deployed microfibrillar eggshell membrane (ESM), which has a rich source of collagen, growth factors and GAGs, along with nanofibers of chitosan/polycaprolactone (PCL). A randomly arranged nanofibrillar mat of chitosan-PCL (CPL) was electrospun over the microfibrillar ESM under optimum conditions and crosslinked with each other using glutaraldehyde. The topography of the 0.27 mm thick bilayered scaffold was studied by FE-SEM and AFM, while BET technique estimated the porosity. Successful crosslinking between the fibers of CPL (150 nm diameter) and ESM (1  $\mu$ m diameter) in the scaffold was evident by FT-IR and XPS analyses, thus confirmed the bilayer structure. The matrix exhibited slow enzymatic degradation, 70% wettability, tensile strength of 11 MPa, good anti-microbial activity and thermal stability. In addition, excellent adhesion and proliferation of human dermal fibroblast (hDF) cells were observed through MTT and SEM studies. Electron micrographs showed a sheath like morphology of the hDF

cells seeded onto the scaffold after 7 days of incubation. Further, the efficiency of cell entrapment and proliferation was evaluated using Rhodamine-DAPI, H&E staining and live/dead assays. The hierarchical nano/micro fibrous bilayer scaffold mimics epidermis/dermis of human skin and holds the promise for skin tissue engineering and other biomedical applications.

#### PEGylated Fibrin Hydrogels for Delivery of Adipose Stem Cells to Deep-Partial Thickness Burn Wounds

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Current tissue engineered skin equivalents rely on thin dermal substitutes to allow for nutrient diffusion to increase cell viability and attachment. One strategy to overcome this limitation is to develop stem cell based treatments that promote angiogenesis within tissue engineered scaffolds *in situ*. We have previously shown that applying PEGylated fibrin (PEG-fibrin) hydrogels containing human adipose-derived stem cells (ASCs) to excisional wounds in nude rats enhances angiogenesis and accelerates collagen deposition.

Here, we investigate the effectiveness of ASCs in PEG-fibrin hydrogels in treatment of burn wounds in a porcine model. Deep-partial thickness burns created on the dorsum of anesthetized Yorkshire pigs were debrided on post-injury day 4 and treated with PEG-fibrin hydrogels containing allogeneic ASCs with and without meshed split thickness skin grafts (STSG). Photographs and biopsies were taken on post-burn days 10, 14, 21, 28 and 42, with euthanasia on post-burn day 42. Excised tissue was processed for routine histopathology. We delivered ASCs *in situ* at 3 increasing concentrations:  $9.8 \pm 1.2 \times 10^4$  cells/wound,  $4.8 \pm 0.43 \times 10^5$  cells/wound and  $0.99 \pm 0.11 \times 10^6$  cells/wound. We did not see any adverse effects due to ASCs delivery at the local or systemic level. PEG-fibrin hydrogels delayed contraction of burn wounds both in the presence and absence of STSG. PEG-fibrin hydrogels were apparent upon histopathology on day 10 post-burn, with blood vessel infiltration. The ultimate goal in investigating the combination of ASCs and PEG-fibrin is to generate vascularized skin equivalents for wound coverage.

#### Chitosan and its Degradation Products Promotes Peripheral Nerve Regeneration by Improving Microenvironments

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Chitosan based artificial nerve grafts were widely used to repair peripheral nerve defect and have achieved good clinical results. Our previous study showed that the chitosan constructed nerve grafts not only provide a good channel for nerve regeneration; its degradation products-chitooligosaccharides (COS) can also promote nerve regeneration, but the involved mechanism is still not very clear. By applying the chitosan based artificial nerve grafts for repairing rat sciatic nerve defects. Interestingly, we found that the levels of inflammatory cytokines changes quickly at the injury site, which was accompanied by the recruitment and disappearance of a large number of macrophages. Together, this evidence suggests that chitosan degradation products-COS may be involved in the reconstruction of the microenvironments at the injury site by affecting inflammation status. Indeed, co-culture experiments showed that COS promote Schwann cells (SCs) to release chemotactic factors, by which macrophages were recruited for secreting inflammatory cytokines. By analyzing the transcriptome and gene regulatory network, the axis of miR-327/CCL2 were identified as the main signaling pathway for mediating the release of chemotactic factors in SCs. Furthermore, the *in situ* hybridization and tissue immunohistochemistry confirmed that COS mediated chemokine secretion in the SCs via miR-327/CCL2 axis. Collectively, our results revealed that COS promotes peripheral nerve regeneration by improving microenvironments at

injury site, providing a theoretical basis for the clinical application of chitosan based grafts in the future.

### Design of Polyethylene Glycol Hydrogel to Support Angiogenesis and Folliculogenesis *in vitro*

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Cancer treatments have been shown to cause premature ovarian failure in prepubescent girls, causing lifelong sterility. A proposed solution is to cryopreserve a portion of the patients' ovarian cortex and when the patient is disease free the stored ovarian tissue can be auto-transplanted. However, there is a substantial risk of reintroducing cancer cells. We propose to engineer an artificial ovary that is free of cancer cells by isolating ovarian follicles from the tissue and providing a supportive artificial matrix that will allow for graft remodeling and vascularization.

Poly(ethylene glycol) (PEG) hydrogels were prepared at concentrations ranging from 3%-5%, cross-linked with protease-sensitive peptides and modified with 0.5 mM of the integrin binding peptide RGD. The crosslinking peptides were designed to be either matrix metalloproteinase (MMP) sensitive (GCLG-PAGCLG-PACG) or plasmin sensitive (GCYK-NSGICYK-NSCG). Ovarian follicles isolated from 14 day-old mice were co-encapsulated with human umbilical vein endothelial cells (HUVECs) and normal human lung fibroblasts (NHLFs) in 10  $\mu$ L gels ( $2 \times 10^6$  cells/mL) and cultured for 12 days. 3% PEG hydrogels were too soft to support follicle architecture but 5% hydrogels were ideal to support follicle growth and cell migration. Hydrogels cross-linked with MMP-sensitive cross-linker restricted follicle growth while the plasmin sensitive cross-linker allowed for proper follicle growth and cell migration. Furthermore, secondary follicles co-encapsulated with HUVECs and NHLFs increased endothelial tubule formation immediately surrounding the follicle during culture. We identified the matrix that can support follicle and vascular tubule development which will be further characterized as a candidate matrix for *in vivo* implantation.

### ESC Derived Mesenchymal Progenitors Exhibit Superior Growth But Inferior Immunosuppression to MSC

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Mesenchymal stem cells (MSCs) are promising candidates for cellular therapy due to their abilities to regenerate bone and cartilage and their immunosuppressive characteristics. The use of fetal bovine serum (FBS) for expansion of MSC may lead to detrimental side effects for the recipient e.g. cross species viral infections and severe immune responses. Our group has previously demonstrated that lysates from expired platelet concentrates (PIPL) can be used for the expansion and differentiation of human bone marrow derived MSC. Human embryonic derived mesenchymal progenitor (hES-MP) cells have been envisioned as a future source of MSCs, it's therefore important to develop serum-free culture supplements for them as well. Here we demonstrate that platelet lysates produced from expired platelet concentrates can be used successfully for the expansion and differentiation of hES-MP cells. hES-MP cells were expanded in DMEM-F12 cell culture media supplemented with either 10% FBS or 10% PIPL. hES-MP cells were analyzed for expansion, expression of surface markers and osteogenic, adipogenic and chondrogenic differentiation as well as their ability to suppress immune-response in an MLR assay. When expanded with PIPL hES-MP cells maintained their mesenchymal characteristics had an equivalent growth and differentiation features compared to cells expanded in FBS. However hES-MP cells demonstrated superior growth to MSC while lacking the immunosuppressive abilities. Our findings suggest that platelet

lysates produced from expired platelet concentrates can be used as an alternative to fetal bovine serum for the expansion of hES-MP cells.

### *In Vivo* Effect of the Addition of Low Doses of Bmp-2 on Bone Formation with a Construct Combining Csm and Coral Scaffold

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**Introduction:** Tissue constructs associating mesenchymal stem cells (MSCs) and resorbable coral scaffold permit to achieve bone regeneration, however it is inconsistent. Inappropriate tuning between scaffold resorption and bone formation has been identified as a critical feature. The aim of this study was to increase the osteogenic potential of MSCs-coral constructs with low doses BMP-2 to optimize in term the coupling scaffold resorption/bone formation.

**Experimental:** 30 mice were subcutaneously implanted (n=4) with constructs combining human MSCs, coral particles and rhBMP-2 at 3 different doses in fibrin gel for 8 weeks. With the controls (hMSCs-coral, coral, rhBMP-2-coral), the study included 8 groups (n=12). After sacrifice, bone and fatty marrow formation and coral resorption were documented by qualitative and quantitative histology.

**Results:** Results showed augmented implant size, bone formation and fatty marrow formation with the increasing doses of rhBMP-2. At higher doses, bone seemed less dense and abnormal as in voids-like bone lesion. At low dose, bone formation due to rhBMP-2 was lesser than with hMSCs and the addition of low dose rhBMP-2 to hMSCs-coral construct led to a synergistic effect on bone formation without fatty marrow formation. Moreover scaffold resorption was augmented in the rhBMP-2-coral implants but the association with MSCs moderated this process, thus there was also a positive effect of the association rhBMP-2-MSCs on coral resorption.

**Conclusions:** Results provided evidence that the addition of rhBMP-2 at dose slightly osteoinductive to constructs combining hMSCs and coral scaffold permitted to increase its osteogenic potential without negative effects usually resulting to rhBMP-2 application.

### Role of Sugar Chain in the Self-organization of the Pancreatic Islet Structure *In Vitro*

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Rapid aggregation of mouse  $\alpha$  cell line ( $\alpha$ TC1.6) and the mouse  $\beta$  cell line (MIN6) in a 3% methylcellulose medium induces reconstitution of a islet-like structure by cellular self-organization [1]. This dynamic rearrangement is thought to be driven by the interaction between alpha and beta cells. Sugar chain is one of the candidates which play a pivotal role in such interaction, because they are covering proteins and phospholipids on the animal cell surfaces. The objective of this study is to examine the possibility that a sugar chain participates in the mutual recognition of the cell in reconstitution of the islet-like structure *in vitro*. We found that a plant lectin, ECA (Erythrina Cristagalli Agglutinin), bound to the cell surface of MIN6. Self-organization was obstructed in aggregates using ECA-conjugated MIN6 (MIN6-ECA). Self-organization in the aggregate was rescued when MIN6-ECA was treated with excess amount of lactose, indicating that the inhibition of cell arrangement by ECA was mediated by the beta-galactoside structure. It was suggested that formation of islet-like architecture in the aggregates comprising alpha and beta cells is controlled through beta-galactoside in a sugar chain. These study is indispensable to reconstitute islets with higher functions which may help to solve donor shortage in the treatment of type I diabetes.

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### Exploring a Novel Y1 Receptor-Based Therapeutic Strategy for Bone Regeneration

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There is an unmet need for local therapies regulating both arms of bone remodeling (bone formation by osteoblasts and resorption by osteoclasts) to promote a fully functional regenerated tissue. Envisioning the development of nanoparticle-systems for local drug delivery targeting receptor-pathways for the control of formation/resorption, arises Y1-receptor (Y1R) signaling-pathway. Y1R is a Neuropeptide-Y receptor expressed by bone marrow cells. The genetic Y1R-deletion or systemic Y1R-antagonism induced a remarkable 2-fold accretion of bone mass, by exerting both anabolic and catabolic actions.

Therefore, we aim to first explore the role played by Y1R in the process of bone fracture repair using a tibia fracture model and knockout mice; and then assess how the pharmacologic Y1R-antagonism (by Y1R-antagonist BIBP3226) will affect both arms of bone remodeling, namely osteoblast and osteoclast activities *in vitro*.

Our studies demonstrated that although the process of fracture repair in germline Y1R-/- mice was slightly delayed, exhibiting a smaller callus tissue volume, the healing process was not compromised, when compared to wild-type littermates.

Additionally, the treatment of monocultures or co-cultures of osteoblasts and osteoclasts with increasing doses of BIBP3226 (0, 60, 1000 nM) revealed that BIBP3226 dose-dependently stimulated osteoblast differentiation/mineralization and osteoclasts formation/activity (number of nucleus >7). Notably, these effects were more pronounced in osteoblasts/osteoclasts co-cultures.

In conclusion, this study demonstrated an important role for Y1Rs in the dynamic interplay between bone cells, relevant for the development of bone regenerative strategies. The knowledge acquired will be translated into the design of new Y1R-based therapeutic approaches.

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### Engineering of Axially Vascularized Bone Grafts for the Treatment of Avascular Bone Necrosis

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Avascular bone necrosis leads to impaired locomotor function and severe pain and is a challenging clinical scenario. The standard of treatment includes the use of pedicled or free autologous bone grafts. Those are surgically demanding and associated with morbidity and limited availability. This study aims to combine an osteogenic/vasculogenic engineered graft to a vascular bundle to develop a pedicled bone graft substitute. Hydroxyapatite cylinders were seeded and cultured for 5 days with stromal vascular fraction (SVF) cells from human adipose in a perfusion bioreactor system. Generated constructs were inserted into devitalized bone hollow cylinders mimicking necrotic bone, wrapped in a membrane to prevent tissue ingrowth from the sides. In nude rats, a ligated arteriovenous bundle from the deep inferior epigastric vessels was created and introduced into the constructs through a central drill hole. Grafts were kept *in vivo* for up to 8 weeks. Histology and microtomography were performed.

Cell-free constructs served as controls. After 1 week *in vivo*, constructs with and without SVF were fully vascularized. Cell-based

constructs showed significantly higher (1.54-fold) blood vessel density. At 8 weeks, bone tissue was formed only in seeded constructs. Implanted cells contributed to both bone and blood vessel formation, as documented by staining for human-specific ALU sequences. The surrounding necrotic bone was revitalized in both groups. In conclusion, pedicled grafts containing SVF can form bone tissue and blood vessels within the core of a simulated necrotic bone. Longer *in vivo* implantations are required to investigate bone ingrowth into the initially necrotic area.

### Bacterial Cellulose Membranes as Applied Natural Dressing in the Treatment of Full-thickness Burns in Rats

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Burns are injuries which represent a high risk of morbidity and mortality. The wound healing of such injuries is complex, and with the intention of innovating the current treatments, new biomaterials have been studied with the purpose of stimulating cell migration and supporting the tissue repair process. Therefore, the aim of this study was to investigate the use of bacterial cellulose as a biomaterial both in its pure state and enriched with lidocaine in full-thickness burns in rats. In the experiment, thirty Wistar rats were used and distributed in three groups: control group; bacterial cellulose membrane group; bacterial cellulose membrane enriched with lidocaine. The animals were induced burn through soldering iron heated at 150°C which was then pressed to the back of the rats for 10 seconds, under anesthesia. After injury induction, the treatment was immediately done and the skin samples were collected on the tenth day of the experimental period. For the statistical analysis, the level of significance 5% ( $p \leq 0,05$ ) was used. In the groups treated with the biomaterials, a histological pattern compatible with a more advanced repair stage showing skin appendages, mild inflammatory infiltrate, better collagen fiber organization and mild immunomarking COX-2 was observed, compared with the control group. Thus, it can be concluded that the bacterial cellulose-based biomaterial both in its pure state and enriched with lidocaine provided satisfactory environment for wound bed, optimizing the full-thickness burn wound healing in rats.

### Novel Detergent for Whole Organ Tissue Engineering to Improve Implantability of Acellular Scaffold

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Whole organ tissue engineering for various organs, including the heart, lung, liver, and kidney, has demonstrated promising results for end-stage organ failure. However, the sodium dodecyl sulfate (SDS)-based protocol for standard decellularization has drawbacks such as clot formation in vascularized transplantation and poor cell engraftment in recellularization procedures. Preservation of the surface milieu of extracellular matrices (ECMs) might be crucial for organ generation based on decellularization/recellularization engineering. We examined a novel detergent, sodium lauryl ester sulfate (SLES), which has weaker chemical properties than SDS, to determine whether it could overcome the drawbacks associated with SDS using rat heart. Hearts were perfused in an antegrade fashion with either SLES or SDS. Morphological analysis using scanning electron microscopy and an assay of glycosaminoglycan content on ECMs

showed that SLES-treated tissues had better-preserved ECMs than SDS-treated tissues. To determine higher order structure surface properties as typified by the carbohydrate structure of the acellular scaffold, we used quantification of glycosaminoglycans, and glycan profiling. SLES-treated hearts showed better preservation of glycosaminoglycans than SDS-treated hearts, and glycans on hydrophilic proteins in SLES-treated hearts were similar to cadaveric hearts, in contrast with those in SDS-treated hearts. Mesenteric transplantation revealed SLES did not induce significant inflammation, as opposed to SDS. Platelet adhesion to decellularized tissues was significantly reduced with SLES. The acellular scaffold by SLES offered the niche for iPS-derived cardiac progenitors to differentiate to mature cardiomyocytes. Overall, SLES could replace older detergents such as SDS in the decellularization process for generation of transplantable recellularized organs.

### Cell-Derived Matrix for Cardiomyocyte Differentiation and Maturation

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Despite its great potentials the use of cell-derived extracellular matrix (CDM) in cardiac tissue engineering is still very rare. Here we have explored the application of CDM and its modifications for cardiomyocyte differentiation and maturation. Fibroblast-derived matrix (FDM) was produced by culturing fibroblasts (NIH3T3) *in vitro* for 5 days and decellularizing them using detergent and enzymes. Modifications to FDM substrate include (1) stiffness tuning via crosslinking with genipin and (2) combination with aligned electrospun fiber. The efficacies of the two platforms were tested separately by using H9c2 cardiomyoblast for cardiomyocyte differentiation and neonatal primary cardiomyocyte for cardiomyocyte maturation. Results revealed that FDM was more effective in advancing H9c2 cardiomyogenic differentiation than controls (gelatin and fibronectin). Interestingly, differentiation capability of H9c2 was highly upregulated once increased matrix elasticity was presented. In addition, aligned electrospun poly(L-lactide-co-caprolactone) (PLCL) fiber was prepared and combined with FDM to produce PLCL/FDM, in which FDM was deposited *in situ* on top of aligned PLCL fiber. PLCL/FDM showed a much better biophysical capability towards neonatal cardiomyocyte maturation than PLCL itself, indicating an instructive role of FDM. Taken together, CDM and tunable matrix stiffness can serve as a promising research tool to study cardiomyogenic cell behavior, differentiation, and maturation.

### Decellularized Bovine Nucleus Pulposus as a Biomimetic Scaffold for Intervertebral Disc Regeneration

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Low back pain (LBP) is a significant burden with a lifetime prevalence of 84% and estimated expenditures of \$85.9 billion<sup>1,2</sup>. Although LBP is multifactorial; it has been shown that LBP can originate from degenerating intervertebral discs (IVDs); a process which initiates in the nucleus pulposus (NP). Current treatments for intervertebral disc degeneration (IDD) are palliative and merely delay surgical management. Thus, it would be advantageous to develop a biomimetic scaffold that can help to mitigate, halt or reverse the progression of IDD by aiding in NP tissue regeneration. The objective of this research is to create a biomimetic scaffold via complete decellularization of bovine caudal NP. Initial results illustrate our ability to remove greater than 93% of bovine DNA (with no residual base-pairs present). Concomitantly, greater than 200 µg of glycosaminoglycan (GAG) per milligram of sample dry weight was maintained. Furthermore measurement of hydroxyproline (HYP) content within NP scaffolds demonstrated a GAG:HYP ratio

of 15:1, which is comparable to values reported for healthy human NP<sup>3</sup>. Histological and macroscopic evaluations of the scaffold illustrate a GAG-rich matrix which closely resembles the human NP. We continue to evaluate scaffolds for the presence of extracellular matrix components via immunohistochemistry, compressive mechanical properties and human stem cell cytotoxicity. This is the first known report demonstrating the complete decellularization of bovine NP tissue for use as a biomimetic scaffold for human NP tissue engineering.

### Transdifferentiation of Human Fibroblasts into Chondrocytes

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The present study is a proof-of-principle study that proposes an approach to transdifferentiate human neonatal dermal fibroblasts into human chondrocytes. Transdifferentiation is a process by which one mature differentiated cell can become another mature cell without going through a pluripotent state (Slack, 2007; Okada, 1991). Neonatal dermal fibroblasts are chosen because they are abundant, free of ethical concern, lack of immunogenicity (they have undeveloped HLA tissue markers and do not express HLA-DR markers (Kern, 2001; Rider *et al.*, 2008)) and they have no risk of tumor transformation. Cell-specific microenvironment instead of chemically defined culture medium will be used for transdifferentiation.

Significance of this study is that it presents a proof-of-concept for producing certain cell types that no known factors have been currently identified or reported for the production of that cell type.

This study aims to decellularize rabbit chondrocytes deposited matrix and to repopulate the acellular matrix with human neonatal dermal fibroblasts.

Histological analysis showed that rabbit chondrocytes encapsulated in collagen microspheres were able to produce a GAG-rich and type II collagen-rich extracellular matrix. After removing all rabbit chondrocytes with decellularization detergents, human fibroblasts were seeded onto the acellular microspheres. Histological analysis showed that the seeded human fibroblasts were able to transdifferentiate into human chondrocytes. Mass spectrometry was also done. It is revealed that collagen XII was deposited in rabbit chondrocytes microspheres and it was still retained after treatment of decellularization reagents. So, collagen XII might be a key factor in inducing the transdifferentiation of human fibroblasts into chondrocytes.

### Co-Delivery of siRNA and Small Molecules via Cationic Sterosomes to Enhance Osteogenic Differentiation of Mesenchymal Stem Cells

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Bone morphogenetic proteins (BMP) are well recognized as the most potent growth factor for inducing osteogenic differentiation of stem cells and have been extensively used in clinical bone repair. Noggin is a specific antagonist of BMPs that can prevent the interaction of BMPs with their receptors. RNA interfering molecules have been used to downregulate noggin expression, thereby, to stimulate BMP signaling. Furthermore, small molecules, such as phenamil, can also induce osteogenic differentiation through the upregulation of BMP/Smad signaling. In this regard, the introduction of siRNA combined with small molecules into stem cells may present synergistic enhancement on osteogenesis that can effectively complement or ultimately substitute exogenous BMP activity. Cationic liposomes have been among the more efficient synthetic gene delivery reagents. In the past decade, Sterosomes made of single-chain amphiphiles and high sterol content have been developed. For example, Sterosomes composed of stearylamine (SA) and cholesterol (Chol) display distinct properties, including positive surface charges, enhanced stability, and low cost. Herein, we report our SA/Chol Sterosomes not only

encapsulated hydrophobic phenamil with high drug to lipid ratio, but also formed complexes with siRNA molecules, making them very attractive delivery nanocarriers. In addition, we applied these nanocarriers to mouse adipose derived mesenchymal stem cells in *in vitro* two- and three-dimensional settings as well as in a mouse calvarial defect model. The co-delivery of siRNA and small molecules presents a powerful method to enhance stem cell differentiation, and may provide new strategies for the promotion of bone regeneration.

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#### Identification of Active Sequence Peptides for Enhanced Angiogenesis by Mesenchymal Stem Cells

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Human bone marrow-derived mesenchymal stem cells (hMSCs) are a promising cell source for tissue engineering and regenerative medicine. One primary hurdle of tissue engineered constructs is the limitation of nutrient and oxygen diffusion into tissue due to insufficient vasculature, thereby preventing the scalability of many new technologies. hMSCs are known to be pro-angiogenic in nature, likely due to their perivascular origin *in vivo*. hMSCs have been shown to promote the formation of new vasculature and are required for long-term stabilization *in vivo*. Several studies have demonstrated that co-culture with endothelial cells (ECs) stimulates pro-angiogenic activities of hMSCs that can be exploited for therapeutic benefit; however, the exact mechanisms that drive these events remain underexplored.

In the current study, hMSCs were co-cultured with human umbilical vein ECs (HUVECs) at different ratios for three days. Our results indicate that hMSC/EC culture stimulates an increase in angiogenic capacity for the combined cell types, and that this phenotype requires direct cell-cell contact. Gene-level expression data have suggested a number of key cell adhesion molecules that are responsible for these events, and inhibition of the binding interactions is underway to verify the role of each. Furthermore, synthesis of the active peptide sequences for the molecules-of-interest and incorporation into 3D hydrogel scaffolds will allow EC-free cultures of hMSCs that exhibit a pro-angiogenic phenotype. This ongoing work exploits the fundamental behavior of hMSCs for enhancing therapeutic activity and will be applied to next-generation tissue engineered constructs.

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#### Bioactive Scaffold-Stem Cell Strategy for Treatment of Acute Full Thickness Skin Wounds

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Mesenchymal stem cells (MSCs) have been shown to enhance skin wound healing through paracrine secretion of soluble factors that regulate inflammation [1], promote angiogenesis and tissue repair [2]. In this work, a poly (DL-lactide-co-glycolide)-type I collagen electrospun scaffold was created to facilitate the transfer of MSCs. The construct was functionalized by blending five biochemicals beneficial for wound healing: Vitamin C (Vit C), Hydrocortisone (H), Insulin (I), 3,3,5-triiodo-L-thyronine sodium (T3) and Epidermal Growth Factor (EGF), collectively known as

CHITE. The scaffold was mechanically strong, biodegradable and released approximately its incorporated factors in 12 hours. All included factors were individually tested to confirm biological functions post-fabrication. The combined CHITE factors, when released from the electrospun scaffold, were able to increase the proliferation of skin cells such as human dermal fibroblasts (HDFs) and normal human epithelial keratinocytes (NHEKs) over a period of 7 days. The released CHITE also maintained basal properties of NHEKs in culture, decreasing the expression of terminal differentiation marker cytokeratin 10. These data demonstrated the scaffold's ability *in vitro* to support the growth of skin cells. When tested on a splinted full thickness skin wound in immunocompromised rats, the MSC-scaffold composite created accelerated wound closure and improved wound granulation tissue formation and structure over SHAM controls. This work demonstrates the feasibility of the tissue engineering paradigm to combine cells, scaffold and growth factors in treatment.

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#### Selection, Isolation and Expansion of Mesenchymal Stem Cells for Scaffold Construction

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**Introduction:** Emerging applications of 3D scaffolds pre-seeded with autologous stem cells for tissue reconstruction emphasize the need for the selection of cell types, and optimum laboratory techniques for scaffold colonization. We discuss our results of animal and human mesenchymal stromal cell application for construction of the 3D scaffolds for tissue reconstruction purposes.

**Materials and Methods:** Adipose stem cells (ASC) from human, mouse, rat, rabbit, or sheep, and human umbilical cord stem cells (UCSC) were isolated and expanded *in vitro*. Cells were characterized by their phenotype (including surface markers analysis), proliferative potential (CFU-F, doubling rate) and *in vitro* differentiation ability into bone, cartilage or adipose tissue. Freshly isolated cells ("stromal vascular fraction" SVF) e.g. ASC plus endothelial progenitor cells (EPC), or expanded ASC, or expanded UCSC from Wharton jelly were used for scaffold colonization. In animal studies, stem cell-colonized scaffolds were implanted into rats, and compared with the "empty" scaffolds.

**Results and Conclusions:** 1. Freshly-isolated ADSCs (SVF) or *in vitro*-expanded ADSCs, when seeded into 3D scaffolds prior to their implantations, improved the process of angiogenesis initiated by local host cells inside the structure of scaffold.

2. *In vitro* osteogenic differentiation of scaffold-colonizing ASCs did not initiate bone formation of scaffolds implanted without contact with host bone.

3. Prevailing numbers of cells grow in scaffold in "monolayer" manner (adhering to PCL surface), the technique of scaffold colonization needs further improvement to obtain 3D cell growth type.

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#### Improving Chronic Wound Healing by Promoting Angiogenesis with Cobalt Doped Glasses

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**Introduction:** Chronic wounds fail to heal in a timely manner, severely reducing quality of life. Hypoxia regulates processes essential in tissue repair and is a key inducer of angiogenesis; therefore it is expected to promote chronic wound healing. Hypoxia can be mimicked through the Hypoxia Inducible Factor-1 (HIF-1) pathway using cobalt, which stabilises HIF-1 $\alpha$  in normal oxygen environments. Bioactive glasses are particularly interesting as therapeutic ions which are beneficial for tissue repair can be incorporated, and as the glass degrades, these ions are released into the aqueous environment. Here, novel cobalt-containing melt-derived glasses were designed to prevent the formation of calcium phosphate, making them suitable for soft tissue regeneration, and fibres were produced from these.

**Methods:** A novel technique, namely laser spinning, was used to form glass fibres without modifying the composition or amorphous structure.

**Results:** Laser spinning produced fibres with micro and nano-scale diameters, which were cotton wool-like in appearance. When incubated in cell culture media, cobalt ions were released at concentrations known to stabilise HIF-1 $\alpha$ , without the undesired burst ion release usually associated with glass particles. In the presence of glass fibres, the HIF-1 $\alpha$  concentration in keratinocytes was increased above that of cobalt chloride controls, without impacting the metabolic activity.

**Conclusion:** Glass fibres formed by laser spinning have excellent handling properties. The fibres stabilise HIF-1 $\alpha$  and are expected to promote angiogenesis in the wound bed, thus improving chronic wound healing.

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#### Controlled Release Composite Hydrogel System for Increased Adipogenesis

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Soft tissue reconstruction for the repair congenital deformities or defects from tumor resections/trauma often require adequate replacement of adipose tissue. Autologous fat grafting using lipoaspirate is minimally invasive in reconstructive surgery but results are unpredictable due to resorption with sometimes 10% volume retention. These limitations serve as motivation for developing therapies to regenerate adipose tissue within the tissue engineering field. Natural biopolymers, like collagen and hyaluronic acid, have been molded into scaffolds but struggle to resist resorption. Non-traditional biomaterials consisting of decellularized skeletal muscle and placental tissue have been used for adipose regeneration studies and show promising results. Unfortunately, these scaffolds do not match the composition of the native adipose extracellular matrix (ECM). While many tissues share similar ECM elements, it has become more evident that each tissue contain unique complex compositions and concentration of chemical constituents, which regulate cell attachment, differentiation, proliferation, migration, and survival. Therefore, adipose ECM serves as ideal scaffold material for adipose tissue regeneration. Human adult whole fat was altered to develop a decellularized adipose matrix and a pepsin digestion induced gelation. Dexamethasone-encapsulated microspheres were added to the hydrogel under the hypothesis of increasing adipogenesis. Fluorescent-tagged dexamethasone was used to monitor release *in vitro* with adipose derived stem cells at days 7, 14, and 21 days. Lipid accumulation, cellular proliferation, and cellular morphology were also analyzed in correlation with recorded dexamethasone release times. Results demonstrate increased adipogenesis in treatment groups containing composite hydrogel displaying its potential to improve adipose regeneration/retention in soft tissue reconstruction.

#### Tissue-Engineered Mitral Valve with Biomimetic Design

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The development of artificial mitral valve substitutes is challenging due to the complex anatomy of the mitral apparatus. The commercial prostheses implanted in the mitral position were originally designed for the pulmonary/aortic position and, therefore, do not reproduce the annular-ventricular continuity, which is crucial to preserve valvular durability and ventricular pumping efficiency [1,2]. Here we present a tissue-engineered heart valve specifically designed for the mitral position, recapitulating the main anatomical components of the native valve, i.e. the annulus, asymmetric leaflets and chordae tendineae. It is based on a hybrid scaffold composed of fibrin gel as cell carrier and a warp-knitted textile mesh. Human umbilical cord veins were used as cell source. The valves were fabricated by injection moulding and cultivated for 25 days under dynamic conditions. Valvular functionality was assessed in a custom-made flow-loop circulation system following ISO guidelines. Tissue analysis included histology, immunohistochemistry, transmission electron microscopy, biochemical assays, burst strength measurements and tensile tests. The valves revealed an excellent hydrodynamic performance with an effective orifice area of  $2.49 \pm 0.15 \text{ cm}^2$  (for a valve diameter of 2.4 cm), regurgitation of  $4.3 \pm 0.4\%$  and mean gradient pressure of  $2.0 \pm 1.2 \text{ mmHg}$ . Tissue analysis showed abundant collagen, elastin and glycosaminoglycans. Tissue samples from the leaflets burst at pressures ranging from 800 to 1000 mmHg and Young's moduli were 0.12–0.60 MPa. All in all, these are encouraging results towards the fabrication of a fully functional, viable prosthesis for the mitral position.

#### Bioresorbable Dermal Scaffold for Wound Repair

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Chronic wounds are defined as wounds that fail to heal within three months. In the United States, chronic wounds affect over 6.5 million patients. The standard of care is saline-soaked gauze, changed every 24–72 hours. Gauze removal disrupts newly epithelialized areas, lengthening healing and hospitalization time, and increasing the risk of post-operative infection.

The aim of this pilot study was to design an allograft scaffold for dermal regeneration. The scaffold is bioresorbable, eliminating the need for frequent changing and providing natural collagen to aid in remodeling. It is suitable for both chronic and acute wounds, and has the potential for use in reconstructive applications.

The gel-like scaffold contains collagen from sterilized human dermis. Its high porosity and degree of swelling allow control of fluid secretion while maintaining fluid transport across the wound bed. Our data demonstrate that fibroblasts and keratinocytes attach within 2 hours and are viable up to 7 days.

In an *in vivo* study, scaffolds were implanted into dermal defects in a porcine negative-pressure wound model. By 21 days the wounds were completely re-epithelialized. The grafts were replaced by well-organized and mature granulation tissue and neodermis. An early revascularization and repopulation with fibroblasts resulted in high levels of collagen fiber deposition and organization. Preliminary *in vitro* and *in vivo* data suggest that host cells infiltrated and remodeled the scaffold to lay down new extracellular matrix.

Future studies will evaluate interactions between adipose stem cells and the scaffold *in vivo*, specifically angiogenic protein release and signaling.

### Concept Learning and Epistemological Beliefs in Tissue Engineering Postgraduate Students

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**Background:** Despite their importance for curriculum designing, conceptions of learning and epistemological beliefs of tissue engineering postgraduate students have not been fully investigated to the date. The present work is focused on the evaluation of both essential elements in order to shed light on their incidence on competence acquisition.

**Methods:** Postgraduate students of a Master in Tissue Engineering. Conceptions of learning and epistemological beliefs were assessed by using modified COLI and EBQ questionnaires, in which students were asked to score their degree of agreement with each item by using a Likert scale (from 1 to 7).

**Results:** Results for the COLI analysis showed that the highest scores were assigned to items "Learning as a process not bound by time or place" (mean 6.08) and "Learning as gaining information" (5.76), where the lowest scores corresponded to "Learning as acquisition of competences" (5.23) and "Learning as a duty" (5.27). As for the EBQ, the highest scores were found for the items "Development of Knowledge" (6.36) and "Justification of Knowledge" (5.98), and the lowest values corresponded to "Certainty of Knowledge" (2.26) and "Source of Knowledge" (4.00).

**Conclusions:** These results suggest that the profile of the Master in Tissue Engineering students is preferentially focused on gaining information to develop and justify their own knowledge independently of time and place. This should be considered for future design of tissue engineering curricula.

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### Engineering Vascularized Cardiac Tissue *In Vitro*

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Engineering thick tissues can give advance a new myocardial therapy and a new *in vitro* models for pharmaceutical research. Our laboratory has fabricated sheet-shaped cells by lowering temperature from culture surfaces grafted temperature responsive polymer and attempted to fabricate thick tissue by layering cell sheets. However, the *in vitro* scaling up of thick tissues is limited due to the lack of vessels supplying oxygen and nutrition, removing waste molecules. We report a new strategy for constructing perfusable vascularized tissues using a bioreactor having collagen-based microchannels.

A tri-cultured cell sheets, which contain normal human dermal fibroblasts (NHDF), GFP-expressing human umbilical vein endothelial cells (GFP-HUVEC) and human cardiac cells from iPS cells were prepared. The tri-cultured cell sheet was placed on vascularized the collagen-gel with microchannels perfused with the culture medium. After 14 days cultivation, microscopically observation demonstrated that HUVEC formed tubular structure in the tri-cultured cell sheets and collagen gel. To check the flow in the tissue, blood was perfused to microchannel. The blood flow indicated the connection between cell sheet vascular and collagen gel vascular.

Perfusable vascularized cardiac tissue was constructed *in vitro* by cell sheet technology and the perfusion bioreactor system. These results confirmed a route to fabricate *in vitro* engineered tissue, perfusable vascularized cell sheets. This technology should lead to restore damaged cardiac tissue and successful production of accurate cardiac tissue models for pharmaceutical investigation.

### Amniotic Fluid Kidney Progenitors as a Tool to Study Podocyte-Endothelial Cell Interaction *In Vitro*

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**Introduction:** The glomerular filtration barrier is formed by podocytes, glomerular basement membrane (GBM) and endothelial cells. The possibility to establish *in vitro* system that mimics the glomerular barrier is still lacking. We demonstrated that human amniotic fluid progenitor cells (hAKPC-P) can differentiate into mature podocytes. In this study, we evaluated their ability to interact with endothelial cells in a 3D *in vitro* system to possibly resemble a GBM-like assembly.

**Methods:** hAKPC-P were co-cultured with human glomerular endothelial cells in 3D systems and analyzed by PCR, Western Blotting, immunofluorescence, TEM and SEM. In addition, response to active molecules and nephrotoxic agents was tested.

**Results:** Differentiated hAKPC-P express a full range of proteins required for the formation of a functional slit diaphragm barrier, like nephrin and podocin. When three-dimensionally co-cultured with endothelial cells, hAKPC-P form spheroids, express kidney and podocyte specific markers and form peculiar morphological structures confirmed by TEM and histology. These spheroids presented deposition of collagen IV and laminin with the same spatial deposition as *in vivo* GBM. When stimulated with angiotensin II, spheroids showed marked contraction and loss of slit diaphragm proteins and attachment to the secreted membranous proteins.

**Conclusions:** hAKPC-P exhibit the ability to interact with endothelial cells to form a GBM-like structure. These preliminary results suggest that hAKPC-P might prove useful to understand the molecular pathways that lead to the formation of the GBM, the most important structure in kidney ultrafiltration, as well as serve as a platform for testing specific podocyte and endothelial related drugs.

### Spermidine impacts Proliferation and Migration in Human Fibroblasts

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**Background:** Hypertrophic scarring following severe burn injury occurs due to a complex imbalance between interleukins and other cytokines that modulate migration and proliferation of fibroblasts and epidermal cells. Polyamines play crucial roles in cell survival and rapidly proliferating tissue. A recent study described spermidine, one of the common polyamines, as a mediator of human hair growth and a modulator of epidermal stem cell function. However, the physiology behind the impact of spermidine on fibroblasts is still poorly understood. The aim of our study was to determine the impact of spermidine on proliferation and migration of human dermal fibroblasts.

**Methods:** Primary fibroblast cell lines were established from biopsies of hypertrophic scar and non-burned skin biopsies isolated from burn patients. A primary human dermal fibroblast cell line was obtained from ATCC (American Type Culture Collection). The effects of spermidine on cell proliferation and migration were measured using a MTT viability assay and the xCELLigence system (Roche, Basel, Switzerland). Quantitative polymerase chain reaction was performed for ornithine decarboxylase 1 (ODC1) mRNA expression.

**Results:** Concentrations exceeding 100 nM spermidine killed cells within 72 hour. Migration for 8 h following 50 µM spermidine application was comparable to the sham group. There was a significant decrease of ODC1 mRNA expression following 10 µM spermidine treatment (p<0.05).

**Conclusion:** The decrease in ODC1 mRNA expression indicates that in the presence of spermidine the dermal fibroblasts may be down-regulate endogenous spermidine synthesis. Future experiments

will determine the role of spermidine in these cells, including confirmation of the levels of ODC1 enzyme activity.

### Biomimetic Superhydrophobic Surfaces Patterned with Wetttable Spots as Implantable Chips for *In Vivo* High-content 3d Biomaterials Response Assessment

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Flat patterned superhydrophobic films are herein reported as implantable platforms containing arrays of miniaturized porous scaffolds for the high-throughput *in vivo* study of biomaterials. High-throughput platforms are powerful platforms to detect hit conditions showing promising properties. However, high-content analysis of biomaterials has been mostly limited to *in vitro* testing where crucial information is lost, as the *in vivo* environment is highly complex. Conventional *in vivo* testing of biomaterials performance requires the use of high numbers of animals, leading to ethical questions and costly experimentation. In this work, an array of 36 distinct biomaterials was patterned onto biomimetic superhydrophobic platforms. Each biomaterial precursor was dispensed in individual spots with high control of shape and size. Biomaterials were processed as freeze-dried three-dimensional scaffolds and the chips were afterwards implanted subcutaneously in Wistar rats. The presence of inflammatory cells was assessed on-chip by performing immunocytochemistry in the miniaturized biomaterials to identify lymphocytes and activated macrophages. Histological sections of the tissues surrounding the implants were also analyzed. Localized and independent inflammatory responses were detected and the integration of this data with control data proved that these chips are robust platforms for the rapid screening of early-stage *in vivo* biomaterials' response.

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### Anti-CD133 Antibody Conjugation to Decellularized Human Heart Valves for Endothelial Progenitor Cell Capture

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The long-term efficacy of heart valve bio-prostheses is limited by progressive degeneration characterized by immune mediated inflammation and calcification. To avoid this degeneration, decellularized heart valves with functionalized surfaces capable of rapid *in vivo* endothelialization have been developed. The aim of this study is to verify the capacity of anti-CD133 antibody conjugated valve tissue to capture endothelial progenitor cells (EPCs). In this study, decellularized human pulmonary valve tissue was conjugated with anti-CD133 antibodies at varying concentrations and exposed to CD133+ NT2 cells in a microfluidic chamber. The amount of anti-CD133 antibody present on the tissue surface and the number of NT2 cells captured in the presence of shear stress was measured using immunofluorescent imaging. We demonstrated that both the amount of anti-CD133 antibody conjugated to the tissue surface and the number of NT2 cells captured in the presence of shear stress increased as the concentration of anti-CD133 antibody present in the conjugation procedure increased. The data presented in this study support the hypothesis that the rate of CD133+ cell adhesion in the presence of shear stress to decellularized heart valve tissue functionalized by anti-CD133 antibody conjugation increases as the quantity of anti-CD133 antibody conjugated to the tissue surface increases.

hypothesis that the rate of CD133+ cell adhesion in the presence of shear stress to decellularized heart valve tissue functionalized by anti-CD133 antibody conjugation increases as the quantity of anti-CD133 antibody conjugated to the tissue surface increases.

### Effect of Fiber Diameter on the Assembly of Functional 3D Cardiac Patches

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The cardiac extracellular matrix (ECM) has a unique 3D structure responsible for tissue morphogenesis and strong and continuous heart contractions. It is divided into 3 distinct fiber groups with specific roles and dimensions; nanoscale endomysial fibers, perimysial fibers with a diameter of 1 μm, and epimysial fibers, with thicker, several micrometers diameters. We report here on our work, where distinct 3-dimensional fibrous scaffolds, each of them recapitulating the dimension scales of a single fiber population in the heart matrix, were fabricated. We have assessed the mechanical properties of these scaffolds and the contribution of each fiber population to cardiomyocyte morphogenesis, cardiac tissue assembly and for the engineered cardiac patch function. Our results show that the nanoscale fiber scaffolds were more elastic than both of the microscale fiber scaffolds, however, cardiomyocytes cultured on microscale fiber scaffolds exhibited enhanced spreading and elongation, both on the single cell and on the engineered tissue level, when compared to nanoscale fiber scaffolds. In addition, lower fibroblasts proliferation rates were observed on these microscale topographies. Based on the collected data we have fabricated composite scaffolds containing micro and nanoscale fibers, promoting superior cell and tissue morphogenesis without compromising tissue contraction. We have shown that cardiac tissues engineered within these composite scaffolds exhibited superior functional properties, including lower excitation threshold and stronger contraction forces than tissues engineered within the single-population fiber scaffolds.

### Type I Collagen Remodeling by MMP-13 Promotes Osteogenic Differentiation of Human Adipose-derived Stem Cells

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Matrix metalloproteases (MMPs) are essential for the intra- and extra-cellular biology of stem cells, such as proliferation, survival, and differentiation through extracellular matrix remodeling. Type I collagen (Col I) is a major component of bone tissue, which is known to promote osteogenic differentiation of human adipose-derived stem cells (hASCs). However, the mechanism of the effects of Col I on osteogenesis is still not clear. It has been proven that cell-matrix interaction plays an important role in osteogenic differentiation of hASCs. In this study, we evaluated that the expression of MMPs and integrins during osteogenic differentiation in presence of Col I. As a result, Col I triggered the high level of expression of MMP-13 and ITGA3. The mRNA level of ITGA3 was reduced by MMP-13 silencing using siRNA, while the mRNA level of MMP-13 was not affected by a knockdown of ITGA3. Col I induced RUNX2 translocation into nucleus. It is known that RUNX2 is transcription factor which bind to MMP-13 promoter. We suggest that MMP-13 initiated and enhanced osteogenic differentiation of hASCs through the activation of focal adhesion molecules including ITGA3 in the presence of Col I. Furthermore, nuclear translocated RUNX2 by hASCs-Col I interaction will regulate MMP-13 expression. Consistent with *in vitro* experiment, osteogenic capacity of MMP-13 overexpressed hASCs was increased in the presence of a Col I after 14 days of mouse subcutaneous transplantation. These results collectively suggest that hASCs-Col I interaction can enhance osteogenic differentiation both *in vitro* and *in vivo* through Col I/MMP-13 positive feedback loop.

### Human Muscle-derived Induced Pluripotent Stem Cells Loaded Onto Coral Scaffolds are Osteoinductive in an Ectopic Mouse Model

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**Introduction:** Tissue-engineering bone constructs are an effective strategy for large bone defect repairing. Among them, human-induced Pluripotent Stem cells (h-iPS) constructs are appealing, as they are an inexhaustible source of osteocompetent cells.

The objective of our study was to establish h-iPS osteogenic ability.

**Materials and Methods:** *In vivo* osteogenic ability of h-iPS was assessed by implanting (i) coral particles loaded with h-iPS, (ii) coral particles, (iii) h-iPS alone (n=3) subcutaneously in mice for 2 months. Newly-formed bone was quantified by histomorphometry. Mineral composition was examined using scanning electron microscopy.

To identify cell origin in the constructs, mRNA were analyzed after 3, 15 and 30 days of implantation, using murine and human markers.

Expression of BMP-2 and BMP-4 after 3 days of culture of h-iPSc and of human mesenchymal stem cells (h-MSC) (n=3) was determined by RT-PCR. In addition, conditioned media from the supernatant of h-iPSc (CM-hiPS) was used to grow h-MSC for 21 days. Calcium deposits were assessed using Alizarin Red stain.

**Results and Discussion:** At 8 weeks, newly-formed bone was present only in the h-iPS constructs and the mineral content of the calcified structures was native bone.

RT-PCR revealed the absence of human gene expression and up-regulated murine osteogenic gene expression at day 15, indicating an indirect role of h-iPS.

Compared to h-MSC, h-iPS over-expressed BMP-2 and BMP-4, and CM-hiPS induced calcium deposition, suggesting that h-iPS promotes bone formation through this secretion.

**Conclusion:** This study demonstrates the osteoinductive potential of h-iPSc in coral construct in an ectopic model.

### The Human Pancreas as a Source of Pro-tolerogenic Extracellular Matrix Scaffold for a New Generation Bio-artificial Endocrine Pancreas

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Our study aims at producing acellular extracellular matrix scaffolds from human pancreas (hpaECMs), as a first step to the production of a new generation, human-derived bio-artificial endocrine pancreas (BAEP). In this BAEP, the hardware was represented by hpaECMs, while the software consisted in the cellular compartment generated from patient's own cells. ECM-based scaffolds obtained through the decellularization of native organs have become the favored platform in the field of organ bioengineering. However, the paradigm is now switching from the porcine to the human model. To achieve our goal, human pancreata were decellularized with Triton-based solution and characterized. Primary endpoints were: complete cell and DNA clearance, preservation of ECM components, growth factors (GFs) and stiffness, ability to induce angiogenesis, conservation of the framework of the innate vasculature, and immunogenicity. Secondary endpoint was hpaECMs' ability to sustain growth and function of human islet and human primary pancreatic endo-

thelial cells (hPPEC). Results show that hpaECMs can be successfully produced from human pancreata, maintaining their innate molecular and spatial framework and stiffness, as well as vital GFs. Importantly, hpaECMs inhibit human naïve CD4+ T cell expansion in response to polyclonal stimuli by inducing their apoptosis and promoting their conversion into regulatory T cells expansion in response to polyclonal stimuli by inducing their apoptosis and promoting their conversion into regulatory T cells. HpaECMs are cytocompatible and supportive of representative pancreatic cell types. We conclude that hpaECMs has the potential to become an ideal platform for investigations aiming at the manufacturing of a regenerative medicine-inspired BAEP.

### Gene-activated Alginate Hydrogels for Articular Cartilage and Endochondral Bone Tissue Engineering

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Alginate hydrogels are commonly used for tissue engineering applications due to their biocompatibility, biodegradability and possibility of delivery via minimally invasive approaches. This study focuses on the development of gene-activated alginate hydrogels to support non-viral gene transfer and subsequent mesenchymal stem cell (MSC)-mediated protein production that can enhance chondrogenic and osteogenic differentiation for cartilage and bone regeneration. To produce these gene-activated constructs, MSCs and nanohydroxyapatite (nHA) complexed with plasmid DNA encoding for TGF- $\beta$ 3 (pTGF- $\beta$ 3), BMP2 (pBMP2), or a combination of both (pTGF- $\beta$ 3/pBMP2), were encapsulated into alginate hydrogels. ELISA confirmed effective gene delivery and the sustained overexpression of TGF- $\beta$ 3 and BMP2. A significant increase in sGAG and collagen production in the pTGF- $\beta$ 3/pBMP2 co-delivery group was observed in comparison to the delivery of pTGF- $\beta$ 3 or pBMP2 in isolation. In addition, stronger staining for collagen type II deposition was observed in the pTGF- $\beta$ 3/pBMP2 co-delivery group. In contrast, greater levels of calcium deposition were observed in the pTGF- $\beta$ 3 and pBMP2 only groups compared to co-delivery, with strong staining for collagen type X deposition in the pBMP2 only group, suggesting this construct was supporting hypertrophy and an endochondral phenotype. Together these results suggest that the co-delivery of TGF- $\beta$ 3 and BMP2 genes has a synergistic effect on chondrogenesis of MSCs, suppressing calcification and hypertrophy, providing support for the use of these gene-activated hydrogels for articular cartilage regeneration. Future studies will explore the potential of these gene-activated hydrogels to spatially support either a stable cartilaginous phenotype or an endochondral phenotype for osteochondral defect repair.

### Transdifferentiation of Human Endothelial Progenitors into Functional Smooth Muscle Cells Following Induction with the Transcriptional Co-Activator MYOCD for Tissue Engineering Applications

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Derivation of induced smooth muscle cells (iSMCs) through direct epigenetic reprogramming of an easily acquired and expanded primary cell source opens a wide range of possibilities for their use in tissue engineering, drug testing, and disease modeling applications. We hypothesized that transient overexpression of transcriptional cofactor MYOCD induces transdifferentiation of human endothelial progenitor cells (EPCs) into iSMCs. MYOCD

overexpression in EPCs induced a robust phenotypic change similar to endothelial-to-mesenchymal transition (EMT). Within 3 days post initiation of MYOCD expression we detected significant cytoskeletal rearrangement (F-Actin, Vimentin) resembling mesenchymal cells. This transition was associated with a significant downregulation of endothelial cell surface markers (CD31, CD105) as determined by flow cytometry. iSMC derivation was associated with a significant upregulation of smooth muscle markers ACTA2, MYH11, TAGLN, and downregulation of CD31 and CDH5 as determined by gene expression analysis. Using immunofluorescence we detected expression of MYH11 and ACTA2 and absence of endothelial markers VWF and CD31. We performed microarray gene expression analysis on iSMCs, EPCs, and UASMCs. Principle component analysis demonstrated a significant similarity between iSMCs and UASMCs. Molecular pathways associated with smooth muscle contraction and cytoskeletal reorganization were upregulated in iSMCs. Using a lentivirally-delivered genetically encoded calcium indicator (RGE-CO-1) we detected calcium transients in iSMCs stimulated with phenylephrine; an effect absent in EPCs. Using traction force microscopy we also detected a significantly stronger (10x) substrate deformation in iSMCs. Finally, we utilized a coculture system (iSMCs, EPCs) suspended within a fibrin and placed on patterned PDMS substrate to induce assembly of human microvasculature.

### Injectable Thermogels with Enhanced Mechanical Properties for Cell therapy and Tissue Engineering

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**Introduction:** Injectable hydrogels present high potential for cell therapy and tissue engineering because they ensure appropriate localization, retention and survival of seeded cells and provide them a mechanically resistant support. They must present low initial viscosity for injection through catheters and needles, rapid gelation, high mechanical strength to withstand *in vivo* stress, and cell compatibility. Thermosensitive hydrogels such chitosan (CH)- $\beta$ -glycerophosphate (BGP) present interesting features but are limited by low mechanical properties. Moreover rapid gelation can only be obtained with high BGP concentrations that make the gel cytotoxic to embedded cells. We report here novel injectable thermogels with enhanced biocompatibility and mechanical properties.

**Methods:** CH was combined with new gelling agents, namely sodium hydrogen carbonate (SHC) mixed with phosphate buffer (PB) or BGP. Their rheological properties, injectability and mechanical strength were evaluated by rheometry and compression. Fibroblasts and mesenchymal stem cells survival and growth were evaluated by alamar-blue. *In vivo* implantation in rats was performed both intraperitoneally and subcutaneously.

**Results:** SHC with PB or BGP presented synergic effect leading to hydrogels with improved mechanical properties (> 20X increase of Young modulus) despite lower salt concentration. The hydrogels had physiological pH, were isoosmotic and presented a sol-gel transition temperature near 37°C which enables easy addition of cells at room temperature. They sustained cell viability and proliferation over 7 days, while chitosan/BGP-hydrogels led to significant cell death. Finally, thermogels formed a cohesive scaffold after *in vivo* injection.

**Conclusion:** This work presents a new platform with a high potential for cell therapy and tissue engineering.

### BMP-2-Loaded Heparin Microparticles Facilitate Functional Bone Formation in Large Bone Defects

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Sustained presentation of bioactive growth factors can stimulate highly localized tissue responses and promote tissue repair. Tar-

geted delivery of bone morphogenetic protein-2 (BMP-2) may improve bone repair and reduce inflammation and heterotopic ossification resulting from poor BMP-2 localization. We have previously fabricated pure heparin microparticles (HMPs) that bind and retain large amounts of bioactive BMP-2 (up to 300  $\mu$ g/mg MPs) [1]. This study was undertaken to evaluate the ability of HMPs to provide sustained BMP-2 presentation *in vivo* and improve healing in a critically sized bone defect. Constructs consisted of BMP-2 or BMP-2-loaded HMPs mixed into 2% RGD-alginate (150  $\mu$ L) and injected into a polycaprolactone mesh tube. Analysis of fluorescent HMPs (1 mg) implanted subcutaneously in rats indicated persistence of ~55% of HMPs at 6 weeks. To evaluate bone healing, constructs were implanted into critically sized rat femoral defects (n=4-5/group). 2.5  $\mu$ g of BMP-2 were loaded onto HMPs (1 mg) mixed with alginate (HMP-bound) or entrapped within alginate alone (alginate-bound). Bone volume within the defects increased over 12 weeks in both groups. Mineralized bridging occurred in all alginate-bound BMP-2 samples and 60% of HMP-bound BMP-2 samples. Biomechanical testing revealed comparable functional restoration of maximum torque and stiffness between alginate-bound and HMP-bound BMP-2 groups. Histological analysis of defects at 4 and 12 weeks revealed HMPs and alginate in close proximity to regenerated bone. This study demonstrates that BMP-2-loaded HMPs can induce functional restoration of segmental bone defects; however, optimization is required to achieve consistent bridging distribution of regenerated bone.

[1] Hettiaratchi, *et al.* (2014) *Biomaterials* 35(25):7228-7238.

### Synthesis of an Artificial Cornea Allowing *In Vitro* Colonization of the Host Cells

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Dense transparent collagen type I scaffolds were synthesized exploiting the intrinsic liquid crystals properties of collagen molecules (Giraud-Guille *et al.* 2008) to synthesize artificial cornea.

3 mg/mL collagen solutions in 500 mM acetic acid were dialyzed against a solution of precise concentrations in acetic and hydrochloric acid. When concentrated, this physico-chemical condition provided a liquid-crystal organization resembling plywood, which is the organization of the collagen fibrils in the cornea. This was verified by polarized light microscopy and second harmonic generation experiments on solutions injected and concentrated in glass micro chambers. Collagen solutions were also concentrated by centrifugation-filtration up to 90 mg/mL. These concentrated solutions were then pressed into cornea-like shape and submitted to ammonia vapor in order to induce the fibrillogenesis of collagen. The result is a transparent dense fibrillated collagen matrix. The samples were analyzed for their mechanical and optical properties, their ultra-structures and for epithelium development. Transmission electron microscopy revealed that fibrils kept the organization of the concentrated solution. Using a custom made device, mechanical tests showed that the Young modulus reached 900 kPa. Human donor limbal explants were sewed on top of the scaffolds and cultured for 14 days. Optical microscopy and immunocytochemical analysis showed the development of an epithelium with characteristics of corneal epithelial cells. Transparency was measured prior and after cell culture for wavelengths in the visible range. The average transparency in both cases was around 85%, which is similar to cornea.

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### Fabrication of Natural Extracellular Matrix Mimic using Functionalized Self-Assembled Peptide Scaffolds for Three-Dimensional Cell Culture

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The fabrication of low cost artificial scaffolds that can effectively mimic the host environment of cells are rich in potential for a range of tissue engineering and regenerative medicine applications. Recently, self-assembled peptide (SAP) hydrogels have shown promise, due to their inherent biocompatibility, propensity to form highly ordered structures, rich chemical functionality and ease of synthesis. However, to closely mimic the range of chemical and mechanical signals provided by tissue specific extracellular matrix (ECM) is still a challenge. Our research aims to engineer scaffolds that can closely imitate diverse native ECM using minimalist Fmoc-peptides that are easily functionalized in order to meet various needs.

Here, SAPs containing the fibronectin-derived peptide epitopes RGD and PHSRN were prepared using solid phase peptide synthesis modified and used to produce self-assembled scaffolds, the properties of which were controlled via ionic strength and pH change to form tuneable hydrogels. Results showed that the self-assembly conditions could be tuned to manipulate their morphological and mechanical properties, and the biocompatibility and functionality of the resultant hydrogels for *in vitro* three-dimensional (3D) cell culture of primary human mammary fibroblast cells was tested. By coassembling PHSRN with RGD, the dual peptide system showed an increased synergistic effect where improved cell attachment and proliferation were observed.

The fabrication processes of these materials contribute to control the biocompatibility, functionality and suitability for biotechnology applications including cell transplantation and tissue engineering.

### Regeneration of Hair Follicle-like Structures within 3D Biomimetic Nanofiber-formulated Microenvironment

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Full restoration of the damaged structures and functions of wounded skin remains a big clinical challenge in repairing full-thickness wounds such as chronic ulcers. In recognition of the essential pathophysiological functions of hair follicles, often missed from the healed skin, it would be a viable approach to fabricate tissue-engineered skin grafts with hair-regeneration capacity. However, the difficulty of culturing and developing follicular structures from isolated cells *in vitro* determines the need to develop an effective and innovative strategy. In this regard, this study was to investigate the use of a novel and highly controllable nanofiber-enabled cell layering approach to formulate a friendly 3D microenvironment for the aggregates of dermal papillae (DP) cells and guide their development into proto-follicular structures. DP cells isolated from rat vibrissa were expanded *in vitro* and then cultured into aggregates via hanging droplets. During the nanofiber-enabled cell assembly, DP aggregates were placed in different locations of the 3D constructs seeded with fibroblasts and keratinocytes. Upon extended culture, follicle-like structures were formed in skin grafts, but were closely related to the spatial organization of keratinocytes. Pilot studies were also performed to investigate the hair-regeneration capacity of such skin grafts upon grafting onto the full-thickness skin wounds of nude mice. Our results have shown that layer-by-layer assembled 3D constructs can potentially formulate an inductive microenvironment for DP aggregates to form a proto-hair follicle in *in-vitro* culture. Grafting of the skin grafts containing follicle-like structures onto nude mice can lead to the formation of hairs.

### Effects of Reactive Oxygen Species and Antioxidants on Macrophage Polarization

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Synthetic heart valves used to replace dysfunctional pediatric pulmonary valves have been shown to undergo oxidative damage, causing the implants to have a relatively short life within the body [1]. Oxidative stress during the inflammatory response post-implantation, mediated primarily by macrophages, can lead to the degradation of biomaterials in the body. Antioxidants have been incorporated into the valves to scavenge reactive oxygen species (ROS), which have been shown to inhibit oxidative degradation to the biomaterial [1]. However, the effects of depleting extracellular ROS in the local environment have not been evaluated for potential off-target effects on macrophage behavior. For example, in addition to pro-inflammatory behavior, macrophages also play an important role in tissue repair and healing. Following the pro-inflammatory phase (M1), macrophages later change their phenotype to become pro-healing (M2). Pro-healing macrophages contribute to repairing (M2a) and remodeling (M2c) of damaged tissues. Here we show that M1- and M2c-macrophages produce significantly higher quantities of intracellular ROS compared to M0- and M2a-macrophages, suggesting a potential role in mediating biomaterial degradation. In preliminary studies of the direct effects of ROS on macrophage behavior, M1-macrophages treated with 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 hours exhibited an upregulation of M2a and M2c markers, although this was not statistically significant. Interestingly, H<sub>2</sub>O<sub>2</sub> did not affect the behavior of M0-macrophages. These data suggest that ROS may play a role in mediating M1-to-M2 macrophage polarization, and that antioxidants may therefore potentially inhibit this activity of macrophages.

### Reference

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### Human Microvascular Endothelial Cells Implanted as Multicellular Spheroids Incorporate into the Vasculature of a Mouse Tissue Engineering Chamber

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**Background:** Two challenges in tissue engineering include the efficient formation of vascular networks for nutrient exchange, and the prevention of transplanted cells from undergoing anoikis. Our aim is to promote the survival of microvascular endothelial cells when transplanted into tissue engineering constructs and the rapid formation of capillary networks by transplanting these cells in multicellular spheroid structures.

**Method:** *In vitro*: Spheroids of 5,000, 10,000 and 20,000 cells were formed from human blood microvascular endothelial cells (hMEC) and human lymphatic microvascular endothelial cells (hLEC) and cultured in fibrin gel for three days to assess capillary-like sprout formation. *In vivo*: hMEC and hLEC spheroids of 5,000 cells were implanted into a mouse tissue engineering chamber that included the host epigastric pedicle. After two weeks, the chambers were sectioned and labelled with anti-human CD31 and anti-human vimentin to assess human capillary formation.

**Results:** *In vitro*: hMEC and hLEC spheroids sprouted capillary-like structures *in vitro* and displayed no significant differences in the average number of sprouts between 5,000, 10,000 and 20,000 cell spheroids observed per plane (hMEC: 9–11; hLEC: 11–18; n=3) nor in the average lengths of the sprouts (hMEC: 130–183  $\mu$ m; hLEC: 169–213  $\mu$ m; n=3). *In vivo*: hLEC spheroids that were implanted close to the host pedicle formed anti-human

vimentin and anti-human CD31 positive capillaries containing mouse blood in the lumen.

**Conclusion:** Human microvascular endothelial cells in multicellular spheroids contributed to the vasculature in the mouse tissue engineering model and may potentially be used in other tissue engineering and wound applications.

#### **Fibromodulin A Promising Therapeutic Molecule for Reducing Cutaneous Scar Formation**

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Scar formation affects up to 100 million patients per year, translating into annual costs approaching \$3 billion [1]. Unfortunately, current treatments for scar formation are minimally effective or have undesirable adverse effects [2–4]. Unlike adult wounds, fetal wounds heal scarlessly and are accompanied by rapid cell migration, minimal extracellular matrix deposition, and rapid myofibroblast clearance [5]. In this study, we utilized multiple loss- and gain-of-function rodent models to demonstrate that fibromodulin (FMOD) is essential for scarless, fetal-type skin repair. Significantly, for the first time, we reversed the scarring to scarless healing in late gestation rat fetus by FMOD administration. Important for future clinical applications, we validated that FMOD significantly reduced scar size not only in adult rodent models, but also in two porcine models that simulate normal and hypertrophic scar repair in humans (32±4% reduction in Yorkshire pigs and 41±9% reduction in red Duroc pigs at 8 weeks post-injury). Functionally, FMOD increased wound tensile strength and collagen architecture organization while reducing scarring. In addition, we observed significant improvements in the gross visual appearance in both pig models. Selective modulation of transforming growth factor b1-responsive signaling accelerated wound closure and induction of interleukin-1b expression resulted in subsequent myofibroblast clearance after wound closure, FMOD achieved a more fetal-like cellular response in optimizing cutaneous repair. Therefore, FMOD's collective mode of action and consistent, preclinical large animal efficacy data constitute strong preclinical findings supporting FMOD-based approaches as a successful alternative therapy that can simultaneously promote cutaneous repair while reducing scar formation.

#### **The Best Source of Isolated Stromal Cells for the Artificial Ovary: Medulla or Cortex, Cryopreserved or Fresh?**

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**Objectives:** Determine the best source of ovarian cells for the artificial ovary (AO): medulla or cortex, cryopreserved or fresh?

**Methodology:** Ovarian tissue was retrieved from 7 patients, one half processed as fresh and the other half frozen and thawed before processing. In each group, cells from the cortex and medulla were isolated separately, and their viability tested. Fifty thousand cells were encapsulated in fibrin(F12.5/T1) and grafted to peritoneal pockets in nude mice (14 in all). Grafts recovered after 7 days were analyzed by immunohistochemistry for cell survival (vimentin), proliferation (Ki67) and graft vascularization (double CD34). Cell apoptosis was analyzed by TUNEL assay.

**Results:** Cryopreservation decreased cell yield (-2,804 cells/mg, p=0.015) and viability (-9.72%, p=0.052) before grafting and had a

considerable (5-fold, p=0.2) but non-significant negative impact on OC survival in grafts. The medulla yielded many more cells (+3,841 cells/mg, p<0.001) with higher viability (+18.23%, p<0.001) than did the cortex. Moreover, grafts with cells from the medulla exhibited a statistically significant 6.44- and 2.47-fold increase in human and total vascular surface area respectively.

**Significance:** Our study confirms that cryopreservation is deleterious for ovarian cells. We found that isolated ovarian cells have the potential to proliferate and degrade fibrin scaffold *in vivo*, and reorganize into a rich vascular network, essential for follicle survival and development. Our findings also suggest that fresh medulla is the best source of cells, as they showed an increase in number and viability after isolation, and improved graft vascularization. These results are very important for construction of the AO.

#### **Development of a Decellularized Pericardium Percutaneous Heart Valve**

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**Objective:** Currently available percutaneous heart valves (PHVs) composed of glutaraldehyde fixed xenogeneic materials lack ability to grow or repair. A PHV based on decellularized pericardium recellularized with patient's own cells could be a viable alternative to overcome these drawbacks.

**Methodology:** Porcine and bovine pericardia were decellularized using a protocol combining Triton X-100, sodium cholate and endonuclease. Decellularization was verified through histology and biochemistry. PHV prototypes were fabricated by sewing the decellularized pericardium onto commercially available stents, and were tested in a pulse duplicator. Finite element(FE) computational modelling in Abaqus was used to assess the crimping, expansion and fatigue characteristics of conventional and novel stent designs, aimed at optimizing PHV performance.

**Results:** H&E staining revealed complete decellularization of the pericardium and preservation of gross native histoarchitecture. The biochemical analysis indicated that collagen content was preserved after decellularization. The pericardial leaflets of the PHV prototypes showed good coaptation under physiological pulsatile flow. FE analysis of preliminary stent designs has demonstrated uniform stress distribution in the stent after crimping and expansion, and a fatigue life of 10<sup>8</sup> cycles.

**Conclusions:** The developed prototypes provide a proof-of-concept for the feasibility of decellularized pericardium PHVs. PHV function will be optimized through incorporation of novel stents to provide superior supporting frames and reduction in the leaflet stresses. These next-generation PHVs will represent an ideal solution specially for paediatric patients, by eliminating the need to perform high-risk re-do procedures.

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#### **Osteogenic Differentiation of Human Mesenchymal Stem Cells within an Ionic-complementary Peptide Hydrogel Promotes Mineralisation *In Vitro***

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The need to regenerate missing tissues is a difficult challenge in regenerative medicine. The design of biomaterials capable of directing cellular fate to induce the regeneration of tissues such as bone

is attracting significant attention as a promising route to address the loss of tissue integrity<sup>2</sup>.

Recently peptide-based hydrogels<sup>3</sup>, and human mesenchymal stem cells (hMSCs) have been combined to regenerate a range of tissues including bone. In this study, the capability of the peptide hydrogel FEFEFKFK to support the three-dimensional (3-D) culture of hMSCs and induce their osteogenic differentiation under osteogenic stimulation is investigated. Both cellular viability and proliferation assays showed a sustained cell viability and proliferation when embedded within gel over 2 weeks of culture. Moreover, immunocytochemistry revealed that hMSCs synthesised and deposited type-1 collagen within the gel, where the highest collagen production was observed at day 12 of culture under osteogenic stimulation. When the synthesis of collagen, alkaline phosphatase and osteocalcin was quantified, it corroborated the commitment of hMSCs into an osteoblastic program. Finally, qualitative and quantitative assays demonstrated that the differentiated cells were capable of mineralising over 12 days of culture under osteogenic stimulation as was evidenced by the deposition of bone-like nodules within gel. These findings suggest that the FEFEFKFK hydrogel not only succeeded to support the 3-D culture of hMSCs, but it also prompted the cell differentiation into human osteoblast-like cells. The subsequent mineralisation by these cells under osteogenic stimuli, clearly demonstrates that this peptide hydrogel is a promising scaffold for bone regeneration applications.

#### Engineering a Heparan Sulfate-based Medical Device for Use in Spinal Fusion

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Due to their instability and poor delivery kinetics, high doses of growth factors are frequently required for the successful treatment of many type of skeletal injuries. As such, there is high demand for therapeutic strategies that stabilize these factors, prolong their bioactivity and lower the efficacious dose. Likewise, carrier systems used to deliver growth factors have been suboptimal and often lack the mechanical strength necessary for load-bearing applications. To address these shortcomings we developed a spinal fusion device consisting of bone morphogenetic protein 2 (BMP-2) complexed with a heparan sulfate (HS) variant previously shown to enhance bone healing (Murali *et al.*, 2013) in combination with a silane-modified PCL-TCP/collagen scaffold (sPTC) that has enhanced load-bearing capacity. The efficacy of this novel combination product was first examined *in vitro*, followed by *in vivo* proof of concept studies using rodent ectopic bone assays, and then a porcine anterior interbody fusion model. Assays (proliferation, mRNA transcripts, ALP activity) with progenitor cells *in vitro* showed higher ALP induction and enhanced osteogenic gene expressions (RUNX2, Col1a2, ALP, OCN) when the fusion device contained BMP-2/HS complexes, compared to devices containing BMP-2 alone. This translated to a 2-fold increase in the amount of ectopic bone when sPTC/BMP-2/HS combination devices were implanted into a rat hamstring muscle model as determined by 2D radiography, 3D  $\mu$ CT and histological analyses. Further, preliminary studies of spinal fusion showed that the sPTC/BMP-2/HS combination device resulted in the formation of a fusion mass, highlighting their potential for clinical application.

#### Substrate Stiffness Manipulated by a Simple Approach Dictates Differentiation of Human Mesenchymal Stem Cells

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Stiffness of biomaterial substrates plays a critical role in regulating cell behavior. While electrospun fibers have been extensively used for tissue engineering, the variable of stiffness of an electrospun fibrous scaffold on regulation of mesenchymal stem cell (MSC) activities has not been fully investigated. In this study, aligned PLLA ultrafine fibers with the diameter of  $1.455 \pm 0.199 \mu\text{m}$  were prepared by stable jet electrospinning and then subjected to annealing treatment at 65°C or 75 °C for 3 h to create fibrous substrates with different stiffness. Human MSCs (hMSCs) were cultured on control and annealed fibrous substrates before analyzed for the expression lineage-specific transcription factors and cytoskeleton organization. Our results showed that annealing treatment did not change the diameter of electrospun fibers but increased the polymer crystallinity and mechanical property. Larger stress fibers were formed in hMSCs cultured on annealed substrates than those in the cells on control substrates. Proliferation of hMSCs was increased with substrate stiffness. The mRNA expression of *CBFA1* was upregulated while that of *SCX* and *SOX9* was downregulated in response to an increase in substrate stiffness, suggesting that increased stiffness favorably drives hMSCs into the osteogenic lineage and the mechanical environment plays a critical role in hMSCs differentiation. Taken together, we hereby demonstrate that simply using the annealing approach can manipulate stiffness of an aligned nanofibrous substrate without altering the material chemistry, and the substrates made by this approach can be used to study the effect of environmental mechanics on MSC differentiation.

#### Colony Morphology Distribution of Mouse Embryonic Stem Cells Cultivated on Poly(Lactic-Co-Glycolic Acid) Scaffolds

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The aim of this study has been to evaluate the colony morphology distribution of mouse embryonic stem cells (mESCs) (C57Bl/6) cultivated on poly(lactic-co-glycolic acid) (PLGA) electrospun scaffolds. To produce the scaffolds, the PLGA was dissolved in dichloromethane:ethanol (8:2) to create a 20% (w/v) solution. The average diameter of the fibers of the scaffolds was calculated. Half of the scaffolds were hydrolyzed with NaOH (hydrophilic) and the other half were non-hydrolyzed (hydrophobic). A total of  $1.5 \times 10^4$  cells/cm<sup>2</sup> was seeded and the analysis was performed after 2, 7 and



14 days. For colony morphology analysis, the cells were stained with DAPI and Phalloidin (Confocal microscope), the cell viability was assessed by MTT test and the pluripotency markers were analyzed by immune staining. Statistical analyses were performed by the Kruskal Wallis test ( $P < 0.05$ ). The average diameter of the fibers of the hydrophilic and hydrophobic scaffolds was  $3.593 \mu\text{m}$  and  $3.664 \mu\text{m}$ , respectively. The colony morphology analysis showed that the number of colonies and the total area occupied by the mESC in both scaffold groups presented no statistical difference, as well as in the cell viability test. The pluripotency markers were positive for all the groups. This taken into account, it is possible to conclude that both hydrophobic and hydrophilic scaffolds are equally suitable for maintaining mESC culture. Therefore, scaffolds can be considered an important tool for cell therapy and can contribute significantly to the studies of mESC maintenance in scaffolds.

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#### Plasmid DNA-loaded Polycaprolactone/Pluronic F127 Membrane for Long-term BMP-2 Delivery and Effective Osteogenesis

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Recently, the bone regeneration using tissue engineering technique and local gene delivery system have been considered as one of the promising therapeutic methods. In this study, we prepared plasmid DNA (with BMP-2 encoding)-loaded polycaprolactone (PCL)/Pluronic F127 membrane to enhance the osteogenic differentiation of adipose stem cells (ASCs). For effective plasmid DNA release from the membrane and the following transfection into the cells of plasmid DNA, the PCL membrane was hydrophilized by Pluronic F127 and the plasmid DNA was complexed with polyethyleneimine-polyethylene glycol (PEI-PEG). The pDNA/PEI-PEG complex was continuously released from the PCL/Pluronic F127 membrane for a long period of time (more than 3 months). From the *in vitro* transfection assays, it was observed that the pDNA/PEI-PEG released from the membrane was efficiently transfected into the ASCs, inducing the osteogenesis effectively.

#### Application of a Novel 3d Biofunctional Scaffold for Hips-msc Fibrogenesis

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Fibroblasts are the most common cells in connective tissue which provides structural and metabolic support for other tissues and organs. Scarcity and damaged fibroblasts is one cause of impaired healing. We developed a novel 3D biomimetic scaffold for stem cells augmentation and differentiation to fibroblasts. The 3D scaffold PFP-C in this study was prepared using electrospun PCL fibers which embedded in PEG-fibrinogen (PF) hydrogel and infiltrated with connective tissue growth factor (CTGF). *In vitro*, PFP-C scaffold showed good biocompatibility confirmed by the human induced pluripotent stem cells derived mesenchymal stem cells (hiPS-MSCs) attachment, infiltration, and proliferation throughout the construct. Real-time PCR was used to identify the gene expression patterns of cells in the scaffold. PCR showed that the mRNA expression of MSCs markers (CD26, CD29, CD106), adipogenic marker aP2, chondrogenic marker Col II and early osteogenic marker ALP were down regulated on day 14, whereas

fibroblasts markers (FSP1, Col I, FN1) were up regulated. Collagen staining, flow cytometry and confocal microscopy in further demonstrated hiPS-MSCs differentiation towards fibroblasts by protein level. The performance of the scaffold is further evaluated *in vivo*. PFP-C scaffolds seeded with hiPS-MSCs were implanted in the dorsal subcutaneous site of SCID mice. 6 weeks later the mice were sacrificed, the grafted implants were analyzed with histologic and real-time PCR examinations. *In vitro* and *in vivo* results showed this 3D scaffold provided a suitable environment and well fibroblasts differentiation from hiPS-MSCs. This 3D scaffold could be potentially used in cell-based therapies for connective tissue related disorders.

#### Development of a Cell-seeded, Bioartificial Lung Assist Device

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**Introduction:** COPD is the 4th leading cause of death worldwide. Extracorporeal lung assist devices that promote oxygenation and/or CO2 removal can improve lung protection and increase quality of life. As a drawback these systems limit patient mobility. In addition, long-term use of these devices is frequently accompanied by thromboembolic complications and device fouling due to inappropriate material properties of the blood contacting surfaces. Our goal is to develop the first wearable miniaturized lung assist device with improved hemocompatible surfaces.

**Methods:** We designed prototypes of new, miniaturized hardware components with an optimized geometry to minimize hemolysis and thrombogenicity. To improve the hemocompatibility of the gas exchange membranes polymethylpentene (PMP), we seeded human dermal endothelial cells onto PMP hollow fibers, either coated with heparin/albumin, or following covalent functionalization with heparin/REDV. Seeding efficiency and cell coverage were quantified microscopically.

**Results:** We successfully miniaturized all hardware components and developed a new design of the gas exchanger, yielding improved blood distribution concomitant with adequate gas exchange *in vitro* and *in vivo*. Following seeding of stacked PMP fiber mats, the endothelial cells formed a confluent monolayer on the fibers, which was maintained for 5 days on the heparin/REDV coated fibers under both static and dynamic *in vitro* testing conditions in a novel perfusion bioreactor system.

**Conclusion:** We successfully designed and implemented an advanced concept of a miniaturized wearable lung assist device, which is currently being tested in a porcine model. Future studies will test these devices in a first-in-man trial.

#### PLGA-Hyaluronan/Fibrin Matrix Induces Bone Formation Through Endochondral Ossification in a Transgenic Mouse Calvarial Defect Model

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**Introduction:** Most bones are developed and repaired through endochondral ossification. However traditional scaffold designs supports bone formation through intramembranous ossification (IO) which limits vascular formation leading to graft core necrosis and thus poor bone formation. We have developed a hybrid matrix of PLGA-hyaluronan/fibrin that supports cartilage-mediated bone formation.

**Materials and Methods:** hBMSCs encapsulated in either control (PLGA-scaffold alone) or hybrid matrix (PLGA scaffold+70% hyaluronan and 30% fibrin) and cultured in chondrogenic media for one week. Constructs were implanted in a mouse (NSG/Col3.6Tpz) calvarial-defect model (n=6). Hypertrophic-cartilage template and

bone formation were determined histologically 4 and 8 weeks post implantation.

**Results:** Hybrid matrix induced a significantly richer sGAG at both week 4 and 8 compared to control shown by toluidine blue staining. X-ray images showed bone formation in hybrid matrix at week 4 and increased by week 8. Histological staining revealed that the newly formed bone is a result of the host osteoprogenitor recruitment. The control group did not show any mineral and thus new bone formation throughout the study.

**Discussion and Conclusion:** PLGA/HA-FB matrix showed great potential to induce cartilage-mediated bone and vascular formation. Our study shows the feasibility of developing an effective tissue engineering therapies for segmental bone defect repair and regeneration through endochondral ossification.

### Basic Research Toward a Cylinder-type Regenerative Airway (bio-air-tube)

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**Aim of the study:** An anatomically mimicked, cylinder-type regenerated trachea (BIO-AIR-TUBE) was fabricated from engineered cartilage and an in-body tissue-engineered collagenous connective tube (BIOTUBE). Previous research demonstrated that BIOTUBES are useful for engineered cardiovascular tissue. The aim of the present study was to determine the short-term functionality of BIO-AIR-TUBE.

**Material and Methods:** Chondrocytes were isolated from the auricular cartilage of New Zealand white rabbits, and seeded onto collagen sponges shaped with cartilaginous rings arranged on a 6-mm diameter silicon tube. The constructs were embedded in dorsal subcutaneous pouches in rabbits for 1 month, after which time, they generated anatomically mimicked cylinder-type tracheae (BIO-AIR-TUBES). Tracheal resection of three cartilaginous rings per rabbit was performed in the cervical trachea. The BIO-AIR-TUBES were implanted into defective airway by end-to-end anastomosis. Poly-L-lactic acid (PLLA) biodegradable stents were inserted into the implantation site.

**Results:** BIO-AIR-TUBE with PLLA stent implantation (n=3) maintained airway structure for up to 10 days after implantation. BIO-AIR-TUBE without PLLA stent implantation (n=3) induced airway stenosis less than 7 days after implantation. Histologically, the internal architecture exhibited hypertrophy.

**Conclusion:** BIO-AIR-TUBE may be useful for developing cylinder-type engineered tracheae. Biodegradable stents were helpful to maintain such an engineered airway structure.

### Peptide Amphiphiles as an Anti-aging and Anti-wrinkle Agent

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One of the most predominant symptoms of skin aging is wrinkle formation, which results from both intrinsic aging and environmental damage, such as chronic exposure to UV radiation. Current anti-aging and anti-wrinkle materials often induce a toxic response, which results in inflammation, to increase tissue growth under the skin. Although effective, a much safer and more effective way to alleviate the effect of aging and thereby wrinkle formation needs to be developed. Several biomimetic peptide sequences (KTTKS, GQPR, GHK etc.) have been identified as structural mimics of collagen type I to be effective in stimulating the synthesis of key

constituents of the extracellular matrix by fibroblasts, however, they typically suffer from poor skin penetration. Incorporation of cell penetrating peptides (CPPs) has been an emerging strategy to transport a variety of cargo across cellular membrane in a dose dependent manner. In this study, peptide amphiphiles that consist of both CPPs and biomimetic sequences were designed, characterized, and tested for their ability to promote skin growth in a healthy manner. The ability of these peptide amphiphiles to penetrate the skin, to scavenge free radicals, and to promote fibroblast functions (specifically, increasing adhesion, proliferation, collagen synthesis, and decreasing elastase and collagenase synthesis) was determined. In this manner, this study identified a new nanomaterial to regenerate skin in a healthy manner for numerous applications from wound healing to cosmetic surgery.

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### Bacterial Cellulose Membrane Containing Ibuprofen for Wound Repair

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Bacterial cellulose membranes (BC) has been widely studied for medical applications due their properties such as biocompatibility, non-toxicity and non-allergenic. BC is a natural polymer with a complex three-dimensional organization in nanometric scale. Nevertheless BC allows adding other molecules, e.g., drugs. In this context the ibuprofen molecule is an optimal drug for favoring the wound repair, because this drug act as anti-inflammatory. Therefore, the aim of this study was to evaluate the *in vitro* release profile of ibuprofen molecule from BC. The BC membranes were obtained from cultures of wild strains of *Gluconacetobacter xylinus*. Highly hydrated BC membranes were cut to a standard size and soaked in a ibuprofen solution (0.5 mg/cm<sup>2</sup>) for 24 hours. After this period, dried BC membranes were obtained by drying at 28°C. To evaluate the therapeutic potential of BC-ibuprofen, after the incorporation of ibuprofen was performed the study of the *in vitro* release in PBS using Franz diffusion cells apparatus and monitored by Ultraviolet Spectroscopy. The maximum release of ibuprofen was 752 mg around 4 and 8 hours, corresponding to 85% of the ibuprofen theoretical value incorporated into the BC, where this amount is greater than that found in medicinal products for topical use. This release feature may be interesting for the treatment of acute conditions due to a drug release faster. According to the results, the BC-ibuprofen incorporated can be a potential material for skin injuries.

### Effect of High-molecular Weight Hyaluronic Acid on Osteoarthritic Chondrocytes in an *In Vitro* Co-cultivation System with M1-macrophages

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**Purpose:** Osteoarthritis is described by an imbalance between anabolic and catabolic processes in the affected joint. This dysregulation of metabolism not only affects the cartilage tissue with the embedded chondrocytes, but also the cells of the synovial membrane border the joint, like synovial macrophages. The purpose of this study was to evaluate the effects of a high-molecular weight hyaluronic acid in a co-cultivation system of osteoarthritic chondrocytes and M1-Macrophages.

**Methods:** THP-1 cells were seeded into cell culture inserts and were differentiated to Macrophages using 100 nM PMA before activate them by addition of IFN- $\gamma$  and Lipopolysaccharide. Osteoarthritic chondrocytes were seeded into a 6-well culture plate and cultivated for 3 days before adding 10% hyaluronic acid and the cell

culture inserts. The expression of chondrocyte-specific genes as well as the synthesis of sGAG of different culture conditions was analyzed. Also the release of pro-inflammatory and anti-inflammatory cytokines in the supernatant was measured.

**Results:** Analysis of gene expression showed that hyaluronic acid did not suppress anabolic-encoding genes like collagen type II or aggrecan, while catabolic-encoding genes stayed on a low expression level. This tendency of the positive effect of hyaluronic acid was also shown by the measurement of cytokines, as pro-inflammatory cytokines decreased in comparison to the control group and anti-inflammatory cytokines increased. Also the analysis of sGAG synthesis showed better results.

**Conclusions:** These results suggest that the used hyaluronic acid has a chondroprotective effect in the present inflammation model as it decreases pro-inflammatory cytokines and increases anabolic factors.

#### Use of Induced Endothelial Progenitor Cell from Adipose-derived Stem Cell to Promote Angiogenesis of Ischemic Skin Flap

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**Purpose:** To facilitate the survival of ischemic skin flap by endothelial progenitor cell induced from adipose-derived stem cells (ASC) and to investigate both *in vitro* and *in vivo* effect of angiogenesis.

**Methods:** P3 ASC are treated with endothelial growth factors for 3 days to induce early endothelial progenitor cell (early EPC). Laminar shear stress is then given in continued flow system to induce late EPC. Ischemic skin flap (10×3 cm) is created on back of Sprague-Dawley rats with special designed skin flap chamber to obstruct collateral circulation. Therapeutic cells (1×10<sup>5</sup> cells) are injected equally into skin flap 3 days before surgery. Histologic analysis of ischemic flap is investigated 7 days after surgery. *In vitro* angiogenesis is analyzed using PCR, Dil-LDL uptake and tube formation assay. *In vivo* angiogenesis is analyzed using tissue PCR and immunohistochemical staining.

**Results:** Induced early and late EPC cells have signal increase in Flt and VEGF gene expression. Assays of Dil-LDL uptake and 3-D tube formation also confirm EPC characteristics. Seven days after surgery, significant increase of flap survival including high capillary density is observed in early EPC group, approximating therapeutic benefit of human umbilical vein endothelial cells. Tissue PCR of survived flap confirms increased expression of angiogenesis signals (Flt, PECAM, VEGF).

**Conclusions:** Using ischemic flap model, we demonstrate the therapeutic effect of induced EPC from ASC to improve flap survival, in terms of angiogenesis. This potentiates the clinical application of autologous cell therapy using adipose-derived stem cell for clinical ischemic disease.

#### Different Culture Conditions Modulate Immunological Properties of Adipose Stem Cells

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The potential of human adipose stem cells (ASCs) for regenerative medicine has received recognition due to the ease of isolation and their multilineage differentiation capacity. Additionally, low immunogenicity and immunomodulation capacity make them a relevant cell source considering immunosuppressive therapies and allogeneic stem cell treatments.

In the current study, immunogenicity and immunosuppression of ASCs were determined through mixed lymphocyte reactions. The immunogenic response was analyzed after cell isolation and expansion

in fetal bovine serum (FBS), human serum (HS) supplemented medium, and in xeno-free and serum-free (XF/SF) conditions. Additionally, the immunophenotype as well as the cytokine secretion (CXCL8, CXCL9, CXCL10, CCL2, CCL5, IL-2, IL-4, IL-6, IL-10, IL-17A, TNF- $\alpha$ , and IFN- $\gamma$ ) of cells was analyzed.

ASCs were weakly immunogenic when expanded in any of the three serum conditions.

The significantly strongest suppression was observed with cells expanded in FBS conditions, whereas higher ASC numbers were required to display suppression in HS or XF/SF conditions. Statistically significant differences in cytokine secretion were observed between different culture conditions, and between direct versus indirect co-cultures. The characteristic immunophenotype of ASCs was maintained in all conditions. However, in XF/SF conditions significantly lower expression of CD54 (ICAM-1) and higher expression of CD45 (LCA) was observed at low passage number.

Although culture conditions have an effect on the immunogenicity, immunosuppression and cytokine secretion of ASCs, our findings demonstrated that ASCs have low immunogenicity and promising immunosuppressive potential whether cultured in FBS, HS or in XF/SF conditions.

#### Allogeneic Human Dermal Fibroblasts for Remodeling Scar Contractures: A Phase I/II Clinical Trial

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**Background:** Wound contracture creates significant morbidity following burn injuries. Viable human dermal fibroblasts may promote remodeling of scar tissue. ICX-RHY-013 (Intercytx Ltd., United Kingdom) is an investigational medicinal product comprised of viable allogeneic human dermal fibroblasts. The primary objective of this FDA Phase I/II study is to determine safety and tolerability, and the secondary objective is to evaluate improvement in burn scar contractures.

**Methods:** In phase I, four normal, healthy subjects with stable abdominal surgical scars underwent treatment into designated areas within the scar with no injection, vehicle only, or injections of ICX-RHY-013 in escalating doses up to 5 million cells/cm<sup>2</sup> with a repeat dose at 4 weeks. The scar was excised and stained with hematoxylin/eosin or FISH for the Y chromosome. In phase II, nine patients with stable burn scars were treated with ICX-RHY-013 injections at one of three doses: 2.5 million cells/cm<sup>2</sup>, 5 million cells/cm<sup>2</sup>, or 2.5 million cells/cm<sup>2</sup> with a repeat dose at 4 weeks. Patients were followed for 12 weeks. Scar scales, quality of life questionnaires, range of motion, disability indexes, complications, physical examinations, and photographs were collected.

**Results:** No serious adverse events were reported. Pain, swelling and redness at the site of injection resolved within 8 hours of treatment. Hematoxylin/eosin stain demonstrated normal scar cell architecture and FISH analysis revealed no Y chromosome containing cells. The Vancouver scar scale improved from 9.00±1.1 to 8.25±1.2 from baseline to week 12 ( $p=0.024$ ).

**Conclusions:** Allogeneic human dermal fibroblasts are a safe, feasible and promising treatment for scar contractures.

#### Spatiotemporal Gene Expression Patterns as a Function of BMP-2 Dose in Early Segmental Bone Defect Regeneration

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Successfully healing large bone defects is challenging, often leading to the use of supraphysiological doses of bone

morphogenetic protein-2 (BMP-2), which is associated with adverse effects including heterotopic mineralization and edema. Previously, using a proportionately high dose of BMP-2 in a rat bone defect model, we observed heterotopic mineralization in the adjacent soft tissue. Therefore, the current objective was to examine the effects of BMP-2 dose on gene expression in the bone defect and surrounding muscle. We hypothesized high dose BMP-2 would elicit elevated osteogenic and inflammatory gene expression.

Critically sized rat femoral segmental defects were treated with 2.5 µg (low dose) or 30 µg (high dose) BMP-2 in RGD-alginate hydrogel injected into a poly(ε-caprolactone) nanofiber mesh. Bone and muscle samples from operated limbs and contralateral, unoperated limbs were harvested at 3, 7, 14, and 21 days. cDNA was analyzed by real-time microfluidic qPCR.

Higher expression of chemokine genes was observed in bone defects and operated muscle (days 14–21) with low dose BMP-2. In bone defects, elevated inflammatory gene expression occurred at day 3 with low dose BMP-2 but was delayed to day 7 with high dose BMP-2. Further, high dose BMP-2 resulted in increased osteogenic gene expression in bone defects at day 3, and heightened expression of inflammatory genes in both operated muscle (day 14) and intact muscle (day 7), indicating local and systemic effects, respectively. In conclusion, these spatiotemporal differences in gene expression may, in part, explain the heterotopic mineralization and tissue swelling seen clinically with high doses of BMP-2.

#### Ectopic Implantation of Juvenile Osteochondral Tissues Recapitulates Endochondral Ossification

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**Objectives:** Subcutaneous implantation in mouse is an ectopic model frequently used to understand the *in vivo* fate of tissue engineered (TE) cartilage and bone. We hypothesized that this model can recapitulate endochondral ossification associated with skeletal development. We tested this hypothesis by studying the changes in juvenile osteochondral tissues implanted ectopically for a period of 10 weeks.

**Methods:** Osteochondral tissues were cored from the femoral condyles of 2 month old calves, sectioned 1 mm thick perpendicular to the articular surface, and implanted subcutaneously in SCID mice. Tissues were evaluated at time zero, 4 weeks and 10 weeks following implantation by µCT and histologic analysis.

**Results:** Following implantation, the thick layer of immature cartilage gradually remodeled into endochondral bone. Cartilage was maintained near the superficial permanent zone, and underwent ossification in the deep transient zone. Whereas most cartilage loss and bone formation occurred within 4 weeks, extensive remodeling and trabeculi formation occurred between 4 weeks and 10 weeks. Histologic evaluation confirmed vast presence of osteoclasts that was attenuated by 10 weeks. The lack of a subchondral component severely limited the progress of endochondral ossification, which suggests an essential role of bone marrow cells. Quantitative µCT analysis revealed remarkable similarities in microarchitectural profiles of native and ectopic bones.

**Significance:** Ectopic subcutaneous model recapitulates endochondral ossification during skeletal development and could thus serve as a simple and effective model for evaluating the phenotypic changes in TE osteochondral composites.

#### The Use of Autogenous Fat Grafting to Address Craniomaxillofacial Volume Loss and Contour Deficits Secondary to War Trauma

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**Purpose:** Craniomaxillofacial (CMF) trauma represents almost a third of all battlefield injuries. CMF war-related injuries can result in significant facial volume loss, scarring, soft tissue atrophy, and contour deficits. Our group will report on the use of autologous fat grafting and role of adipose derived stem cell (ADSCs) therapies to address CMF combat-related defects.

**Methods:** A retrospective review was completed to identify war-related craniofacial injury patients treated with autologous fat grafting. Data analyzed included mechanism of injury, Injury Severity Score (ISS), types of defects treated, outcomes, and complications.

**Results:** Eleven Wounded Warriors underwent autologous lipotransfers for craniofacial deformities. Regions treated included the temporal, malar, oral commissure, orbit, lower lip, eyelid, nasal dorsum, tear trough, and forehead. All patients reported subjective improvement in their craniofacial defect and aesthetic appearance. Resorption of engrafted fat was observed within all patients, with the average long-term retention of fat grafts being estimated between 40–60 percent of initial grafted volume. Subjective improvements were had in facial volume, scar softening and appearance, as well as contour restoration.

**Conclusions:** Autologous fat grafting can improve facial volume deficits, scar appearance, and contour deformities associated with combat-related CMF injuries. This pilot study provides the basis for further investigation of autogenous fat grafting and ADSC therapies to craniofacial as well as other combat injury-related sequelae where soft issue volume enhancement may be of interest. Future studies of significance will include application of autogenous fat grafting +/- ADSCs to address burn and upper or lower extremity contour, scarring, or volume-related deficits.

#### Covalent Linking Growth Factors to Biomimetic Scaffold has a Positive Effect on Bone Formation

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The FDA have recently reiterated that growth factor treatments should not be used as a treatment of choice in growing patients i.e. children, due to serious injuries which can arise from supraphysiological doses of the growth factors being released from the scaffold in a burst release fashion. To overcome this problem, researchers have developed a biomimetic scaffold from a combination of chitosan (CS) and hydroxyapatite (HAP) which is similar to the composition of natural bone. To this scaffold, osteogenic (BMP-4) and angiogenic (VEGF-a) growth factors were covalently attached to the polymer phase of the scaffold using a UV initiated crosslinking reaction. FTIR analysis of the construct before and after UV irradiation showed that the amide I and OH groups of CS had reduced in intensity indicating that these groups were involved in the covalent linking reaction. The optimal scaffold strength was found to be at ratios of HAP:CS 2:1(w/w) and these scaffolds retained their shape/integrity for up to 10 weeks. Elution assay cytocompatibility testing indicated approximately 100% cell viability in comparison to tissue culture plastic. Growth factor retention in the scaffold was excellent. Scaffolds which had an initial BMP-4 and VEGF-a loading releasing a physiological doses of BMP-4 (0.38- 1.15 ng) and a VEGF-a concentration of 38 – 11.5 pg after 10 days as measured using Elisa. *Ex vivo* computed tomography following 8 weeks implantation in a rodent femoral defect model indicated a significant increase in bone formation in the defect for growth factor laden constructs compared to empty scaffolds.

### Development of a Biomimetic 3D Printed Hydroxyapatite-Collagen-Polycaprolactone Scaffold for Bone Defect Treatment

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Biomedical scaffolds used for bone tissue regeneration should have various properties including appropriate bioactivity, mechanical strength, and morphologically optimized pore structures. The objective of the current study is to prepare a three-level hierarchical hydroxyapatite-collagen-polycaprolactone (HA-Col-PCL) scaffold for bone tissue regeneration using biomimetic synthesis. Porous PCL scaffold with a lay down-pattern of 0°/60°/120° was fabricated by 3D printing technique, then functionalized three dimensionally with different concentration of collagen gel (1,2,3 wt%) by vacuum infusion and subsequently lyophilized, finally a third level of HA layer was achieved by biomimetic mineralization. The biomimetically functionalized three-level hierarchical HA-Col-PCL scaffold was characterized with scanning electron microscope, x-ray diffraction and Fourier transform infrared spectroscopy, and the mechanical properties of the scaffolds were also evaluated. The results demonstrated that the biomimetic scaffolds have favorable mechanical properties with a compressive modulus of  $40.8 \pm 3.4$  MPa, porous collagen network homogeneously distribute among the PCL strips and coating with a layer of bone like HA (Ca/P =  $1.65 \pm 0.24$ ). The biocompatibility and osteoinductivity of the biomimetic scaffolds were evaluated *in vitro* and *in vivo*. The biomimetic 3D functionalization significantly increases cell seeding efficiency and promotes cells proliferation and increases alkaline phosphate activity compare to native PCL scaffold *in vitro*. The biomimetic ornamented scaffolds were also implanted into a critical-size segmental bone defect of rabbit radius. X-ray, micro-computed tomographic imaging, histological analysis demonstrated that the biomimetic scaffold could repair the segmental bone defect in 12 weeks. The results suggest that the biomimetic ornamented HA-Col-PCL scaffold exhibits good mechanical properties and osteogenic capacity as a promising substitute for bone defect treatment.

### Fabrication of Fluorescent Nanofibers for Monitoring Wound Healing *In Vivo*

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The basement membrane of a human skin is a specialized extracellular matrix providing compartmentalization along with cell attachment and signalling factors. The dermal layer comprises mostly of nano/microfibrous collagens while the epidermal layer is a compact sheet of cell layer. For successful development of skin graft, it is important to reciprocate the nano/microscalar architecture of tissue matrix as well as its biological cell recognition character. Nanofiber morphology facilitates good cellular response owing to their high surface area, interconnected pore, simplicity, environmentally friendly nature, cost-effectiveness, scalability and tailor able mechanical properties. Amongst various biopolymers used, chitosan is promising due to its biocompatibility, tailorable biodegradability, non-antigenicity, antimicrobial activity and wound healing potential. Polycaprolactone (PCL) and gelatin was electrospun with carbon nano dots to make fluorescent nanofibres.

The microstructures of samples were evaluated by scanning electron microscopy (SEM). FTIR spectroscopy, mechanical properties, swelling behaviour, biodegradation kinetics of matrices was studied for their suitability as tissue engineering scaffold. Cell ad-

hesion and cytocompatibility assay for the sample was carried out using primary fibroblast cells isolated from foreskin. MTT assay, SEM, rhodamine/DAPI staining, DNA quantification was done.

SEM images and fluorocent images revealed fluorescent nanofibrous scaffold which promoted cell growth and proliferation. The nanofibers thus formed had a nano-microfibrillar architecture as desired. Furthermore, the physico-chemical characterization adds to the suitability of the scaffold for skin tissue regeneration application.

### Matrix Remodeling Regulates Neural Progenitor Cell Phenotype in Engineered Elastin-Like Protein Hydrogels

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Matrix properties such as stiffness and cell-adhesive ligand presentation have been implicated as important regulators of stem cell differentiation. Recent work has suggested that the ability of encapsulated mesenchymal stem cells to degrade their surrounding matrix is at least partially responsible for the cells' ability to sense and respond to their three-dimensional environment. In this study, we investigate the effects of hydrogel crosslink density on the ability of hippocampal-derived neural progenitor cells (NPCs) to remodel the matrix and maintain a characteristic NPC phenotype within three-dimensional cultures. NPCs were encapsulated in hydrogels prepared from engineered elastin-like proteins (ELPs), as these materials permit decoupled control of matrix mechanics and biochemistry. Direct tuning of the covalent crosslinking stoichiometry resulted in ELP matrices with elastic moduli spanning a relevant range for neural cells,  $E \sim 0.5$ – $1.5$  kPa. When NPCs were encapsulated in these ELP matrices, NPC proliferation, metabolic activity, and expression of NPC, neuronal, and glial lineage markers increased with decreasing crosslink density. Degradation of fluorescently-labeled hydrogels also varied with crosslink density, as lower crosslink density gels were more extensively degraded by encapsulated NPCs. Inhibiting metalloprotease-mediated matrix degradation decreased proliferation and expression of NPC, neural, and glial markers. Accordingly, permitting matrix degradation and remodeling prior to induction of differentiation resulted in improved cell viability and controlled differentiation into either astrocytes or electrically-active neurons that respond to treatment with GABA and glutamate. These results suggest that matrix degradation and remodeling is required for both phenotypic maintenance and differentiation of NPCs encapsulated in 3D hydrogel constructs.

### Modulation of Cell Microenvironments with Oxygen Tension and Macromolecular Crowding: A Multifactorial Approach Towards *In Vitro* Organogenesis

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Advancements in tissue engineering have enabled the development of scaffold-free substitutes<sup>1</sup>. Despite efficacious *in vitro* and *in vivo* results, very few products have been commercialised, primarily due to prolonged time required to develop an implantable device. It has been demonstrated that macromolecular crowding (MMC)<sup>2</sup> and low oxygen tension<sup>3</sup> enhances deposition of extracellular matrix (ECM). Here, we assessed the potential of combining MMC technology with low oxygen tension. Human skin fibroblasts (WS-1) were cultured under MMC conditions [75 µg/ml carrageenan] in a range of fetal bovine serum, human serum concentrations (0.0–10%) and oxygen tension (0.5%, 2%, 20%). SDS-PAGE demonstrated that MMC significantly increase type I collagen deposition ( $p < 0.0001$ ). Thermo-responsive pNIPAAm failed to produce intact cell-sheets, whilst coating with 65% NIPAM/35% N-tert-butylacrylamide facilitated detachment of intact ECM-rich cell sheets. Complementary Immunocytochemistry (ICC) for mass

spectrometry validation confirmed the enhanced deposition of collagens (I, III, IV, V, VI) and other ECM molecules (laminin, fibronectin, hyaluronic-acid, decorin, lysyl-oxidase), without changing other tested proteins. Moreover, ICC demonstrated that MMC with 2% oxygen supply significantly increase collagen-I, III, IV, V and fibronectin deposition ( $p < 0.001$ ) at all tested serum concentrations. This study shows that modulation of the *in vitro* microenvironment with polydispersed MMC and low oxygen tension enhances ECM deposition, even under low serum supply.

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#### Immobilized Peptides with TGF- $\beta$ 1 Affinity Promote MSCs Differentiation

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TGF-beta plays an important role in the process of chondrogenesis and cartilage regeneration; however, uncontrolled release of TGF-beta may cause adverse effects on cell morphology. Here, we hypothesize that chitosan scaffold with crosslinked peptides has TGF- $\beta$ 1 affinity and due to the spatial as well as temporal interactions between MSCs and TGF- $\beta$ 1, chondrogenesis of MSCs may be induced, leading to the accumulation of cartilage-like ECM *in vitro*.

Firstly, we modified chitosan by reacting it with butanedioic anhydride, and then used EDC/NHS crosslinking method to bind peptides to the N-succinyl chitosan scaffolds, successful modification of peptides and the surface morphology of the scaffolds were characterized by NMR and SEM, whereas the control release rate of TGF- $\beta$ 1 was detected by ELISA assay. After 21 days in culture, the chondrogenic potential of MSC's was assessed by studying the cartilage related genes (COL1A1, COL2A1, COL10A1 and ACAN) and the TGF-beta signaling pathway related proteins (Smad2/3, pSmad2/3).

In this study, NMR results showed that the peptides were successfully bound to the chitosan scaffolds whereas the SEM micrographs displayed that the modified scaffolds consisted of homogeneous pore structures. The control release studies revealed that the scaffolds were able to avoid the burst release of TGF- $\beta$ 1, which may potentially promote chondrogenesis and in turn inhibit hypertrophy.

In conclusion, this scaffold may be a potential material for *in situ* cartilage defect repair and regeneration.

#### The Interaction between Human Blood Endothelial Cells and Polycaprolactone Scaffolds

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Endothelial cells grown out from human blood (BOECs) are a promising cell source for the cellularization of tissue engineered heart valves (TEHVs). We have previously shown that polycaprolactone (PCL) is a suitable material for scaffold construction for TEHVs due to its biodegradability, apparent biocompatibility and the ease of fabrication in the form of anisotropic scaffolds. However, its pro-inflammatory effect on human blood endothelial cells remains unknown. In the current study, we assessed the effect of culturing BOECs with PCL on viability, cytokine (CXCL8) and vascular hormone (endothelin-1) release.

**Methodology:** BOECs were cultured from blood of healthy donors. PCL films prepared by solvent evaporation method were either treated with ethanol, or plasma oxidized at 30 w and 0.1 mbar for 30 min before coating with extracellular matrix proteins and cell

seeding with 10,000 cells/well. Viability was measured using alamar blue assay and CXCL8 and endothelin-1 by ELISA after 72 hrs.

**Results:** Compared to controls (cells on glass coverslips), there was approximately a 40% reduction in the number of viable cells that remained on each of the PCL preparations. Incubation of BOECs for 72 hrs on PCL films gave no significant increase in the levels of CXCL8 or ET-1 released into the media, even when the reduction in cell viability was taken into account. The release of other cytokines is currently being investigated.

**Conclusions:** These results are promising and indicate the potential of BOECs as seeding cells to populate PCL. Nevertheless, it may be necessary to biofunctionalize PCL scaffolds to enhance the attraction of BOECs.

#### Stem Cell Factor Expressing Feeder Cells Enriches Interstitial Cells of Cajal for Engineering Functional Intestinal Smooth Muscle

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Smooth muscle layers in the intestine move autonomously due to pacemaker cells called interstitial cells of Cajal (ICC). Stimulation of ckit via stem cell factor (SCF) is essential for ICC maintenance. However, primary ICC cultures tend to undergo extensive phenotypic changes, including the apparent loss of the ion channels responsible for the pacemaker activity, within a few days even in the presence of exogenous SCF. To overcome the challenge, SCF-expressing feeder cells and scaffold systems were proposed to enrich the ICC population. Our study demonstrated that STO feeder cells not only 1) significantly enriched the immunomagnetic sorted ICC, but also 2) successfully induced the spontaneous contractility of enzymatically digested intestinal smooth muscle cells in culture for over two weeks. This is one of the first systems that cultures smooth muscle cells with spontaneous contractile activity. Therefore, our study achieved an essential step toward engineering functional intestinal smooth muscles.

#### Repair Large Size of Swine Articular Osteochondral Defect in Weight-bearing Areas with Chondrogenically Induced Autologous BMSCs

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Previously we demonstrated the feasibility of using bone marrow-derived mesenchymal stem cells (BMSCs) combined with polyglycolic acid/poly(lactic acid) (PGA/PLA) scaffold to repair swine articular cartilage defects. However, these efforts met only limited successful rate because of cell leakage, scaffold triggered inflammatory reactions, and poor mechanical strength. We propose that *in vitro* chondrogenic induction may help to address these issues. In the current study, The results showed that constructs revealed little cartilage-like features at 2 weeks, then started to form cartilaginous tissues at 4 weeks, and became mature and stable at 8 weeks. After 6 months post operation, constructs induced for 4 weeks and 8 weeks formed mature hyaline cartilaginous tissue with cell density and cartilage thickness similar to those of native cartilage; while most of the constructs induced for 2 weeks became fibrous tissues. Nevertheless, successful repair rate of 4 weeks and 8 weeks constructs were only about 50%, which did not reach our expectancy. We considered that many parameters influence the long-term fate of tissue engineered cartilage in the knee, and proper defect model is also a key factor to guarantee a reasonable technique evaluation. The strategy developed in the current study provided a promising approach for clinical application of tissue engineered cartilage constructed by BMSCs.

#### Dynamic Culture in a Microfluidic Chamber Enhances Proliferation and Osteogenic Response Of Pre-osteoblastic Cells

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The limited volume of bone grafts and frequent morbidity of patients leads to the need for development of advanced therapeutic strategies for bone regeneration [1]. The aim of the present work is to study the proliferative and osteogenic behavior of pre-osteoblastic cells MC3T3-E1 in a microfluidic system that allows continuous and homogenous feeding of cells, and compare them with static culture conditions. We employed gelatin films and a fibrous collagen network as substrates for the cells, and performed a rheological characterization of collagen. Under both flow rates of 30 and 50  $\mu\text{l}/\text{min}$ , cells appear oriented along the direction of flow after 3 days, whereas cells depict a random orientation under static conditions. Using collagen fiber networks as a cell substrate, the alkaline phosphatase (ALP) activity increases 1.6-fold under a flow rate of 30  $\mu\text{l}/\text{min}$  compared to static ones. Additionally, the cell proliferation increases 3.1-fold under a flow rate of 50  $\mu\text{l}/\text{min}$  compared to static conditions. Using gelatin film as a substrate, the collagen produced in the extracellular matrix under flow rates of 30 and 50  $\mu\text{l}/\text{min}$  increases 2.2-fold and 4.4-fold, respectively, compared to static conditions. Our results indicating enhanced cell growth and osteogenic response of pre-osteoblastic cells, demonstrate the potential of microfluidic systems for the generation of autologous graft substitutes that could be readily implanted in the defect site, and accelerate the new bone formation and healing.

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### Bioactive Glass Ions Enhance the Osteogenic Differentiation of Human Adipose Stem Cells

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Human adipose stem cells (hASCs) are a promising cell source for tissue engineering applications. Of the various biomaterials used in bone tissue engineering, bioactive glass (BaG) has been shown to be especially advantageous due to its osteoinductive properties. However, it is currently not known whether the ionic dissolution products from BaGs are enough to induce the osteogenesis of hASCs. To shed light on this, hASCs were cultured in BaG ions containing extracts prepared from four different BaG compositions, a commercial glass S53P4 and three experimental glasses (2-06, 1-06 and 3-06). Bioactive glass extracts were prepared from each glass type using basic medium (BM) and osteogenic medium (OM) as an extraction basis. Based on our results the cells stay viable in all the glass extracts for the whole 14 day culturing period. At 14 day time point the mineralization in OM extracts was significantly higher than in the control as determined by Alizarin red S staining. Parallel to the increased mineralization we detected a decrease in the cell amount. Raman and Laser Induced Breakdown Spectroscopy analyses confirmed that the mineral consisted of calcium phosphates. Consistently, the OM extracts also increased osteocalcin production and collagen-I accumulation in the extracellular matrix at 13 days as determined by immunocytochemical stainings. However, regardless of the enhanced mineral formation, alkaline phosphatase activity was not enhanced by the extracts. The OM extracts could potentially provide a fast and effective way to differentiate hASCs *in vitro* prior to their utilization in clinical bone tissue engineering applications.

### Hybrid Superparamagnetic Collagen-like Peptide Microparticles applied on Bone Tissue Regeneration

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Magnetic biomaterials have been showing promising properties when applied on bone tissue engineering. Basically, weak static magnetic fields can promote osteoblasts proliferation and differentiation, growth factors expression, as well as enhanced osteointegration that boost bone tissue regeneration [1]. The development of new bioactive and biocompatible superparamagnetic devices can reduce the concerns of limited safety of iron oxide nanoparticles [2]. This research work is focused on development of novel hybrid superparamagnetic microspheres able to assist bone tissue regeneration. A biomineralization process, well-assessed to induce bio-inspired mineralization of type I collagen fibrils [2] was adapted to use with a collagen-like peptide that was mineralised with superparamagnetic, iron-substituted hydroxyapatite. Hybrid composites were synthesized and microspheres were fabricated using an emulsification process. Temperature, iron/calcium molar ratio and Fe<sup>3+</sup>/Fe<sup>2+</sup> ratios were controlled to produce hybrid microspheres with defined amount of mineral phase and adequate superparamagnetic properties. Effect of phase composition, cells interaction and magnetization was evaluated. As a result, magnetic microspheres (2–120  $\mu\text{m}$ ), mineralized with low crystallinity apatite were obtained. *In vitro* preliminary cells studies indicate the biocompatibility of the biomaterial and after 30 days immersed in buffer medium, is evident low weight loss percentage and maintenance of magnetization properties. The results suggest the potential of these hybrid superparamagnetic microspheres on bone tissue engineering.

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### Surface Markers for Adipose-derived Mesenchymal Stromal Cells: Novel Applications for Regenerative Medicine

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Cluster of differentiation (CD) markers are cell surface antigens recognized by antibodies that are used to determine the immunophenotype of a cell. Mesenchymal stem cells (MSCs) are characterized by the expression of CD105, CD74, and CD90, and absence of CD45 and CD34. Although these CD markers define a cell population with tri-lineage potential (ability to differentiate into bone, cartilage, fat), MSCs from different donors can exhibit significant differences in proliferation and trophic activity (e.g. secretion of immunosuppressive cytokines). A CD marker panel with the ability to predict the functional activity of MSC populations and not only their capacity for tri-lineage differentiation has the potential to improve their therapeutic efficacy.

To identify novel CD markers that predict the functional activity of MSC populations we applied next generation RNA sequencing to identify 551 cell surface markers expressed by clinical grade adipose derived MSCs grown in human platelet lysate. High throughput real-time qPCR was used to validate MSC specific cell surface markers across three MSC donors and different mesenchymal cells. In addition to six established markers defined by the International Stem Cell Consortium, we identified eight novel markers that distinguish MSCs from other mesenchymal cell types. Several of these markers have known immune modulatory functions. This novel panel of 14 markers was validated across 18 different AMSC donors by flow cytometry. The cell surface markers identified in this investigation have the potential to more accurately characterize the functional activity of MSC cell populations that are currently being used for clinical trials in patients.

### The Architecture of Fat Grafting: What Lies Beneath the Surface

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**Background:** Fat grafting is a powerful procedure that is limited by unpredictable volume loss. Grafted tissue survives via plasmatic imbibition until a neovascularization occurs. Fat that is deposited more than 2 mm from capillaries will undergo central necrosis. This study aims to better understand the architecture of fat deposits within the recipient bed following fat grafting.

**Methods:** Fat was harvested by liposuction and stained with methylene blue. Stained fat was grafted into 4×4×2 cm sections of pannus at graft to recipient volume ratios ranging from 1:10 to 1:1. Each tissue block was sectioned for stained graft visualization. The diameter of each deposit and the percentage with a radius greater than 2 mm was recorded.

**Results:** Average tunnel diameter at a graft to recipient ratio of 1:10 was 0.19±0.01 cm, at 1:8 was 0.24±0.01 cm, at 1:6 was 0.25±0.01 cm, at 1:4 was 0.30±0.01 cm, at 1:2 was 0.38±0.01 cm, and at 1:1 was 0.55±0.02 cm. All comparisons reached statistical significance (p=0.05) by ANOVA with Tukey post-hoc except 1:8 vs 1:6 (p=0.96). The percentage of fat parcels with a radius greater than 2 mm at 1:10 was 3.0%, at 1:8 was 5.3%, at 1:6 was 9.5%, at 1:4 was 20.9%, at 1:2 was 42%, and at 1:1 was 68.3%. All percentage comparisons were significant by Fisher's exact except 1:10 vs 1:8 (p=0.15).

**Conclusions:** As the total volume transferred increases, grafted deposits coalesce to form larger globules, likely contributing to central necrosis and subsequent volume loss in a dose-dependent fashion.

### Exploiting Peptide Self-Assembling Hydrogels as a Tunable 3D Scaffold for Cell Culture

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In the last two decades, significant efforts have been made to develop soft materials exploiting short peptides self-assembly for biomedical applications.<sup>1</sup>  $\beta$ -sheet forming peptides have been designed to form highly stable hydrogels with potential application in a range of fields.<sup>2</sup> In particular, they offer a flexible platform for the design of 3D scaffolds for cell culture.

We have developed a platform designing hydrogels with tailored properties and functionalities exploiting self-assembly of short (8–10 amino acids)  $\beta$ -sheet forming peptides, based on alternating hydrophilic and hydrophobic residues. By altering primary peptide structure, formulation and concentration the properties of these scaffolds (e.g.: modulus and functionality) can be easily controlled. We have previously shown that chondrocytes<sup>3</sup> and osteoblasts<sup>4</sup> can be cultured successfully within the peptide scaffolds.

A range of mechanical properties and charge states were investigated and the effect of these changes on fibroblast viability, morphology, mobility and phenotype was determined. Cells were shown to respond differently to each change of property, demonstrating that in the future these hydrogel properties can be tailored to accommodate different cells' needs. Our results clearly demonstrate that our peptides offer great promise for the design of tunable 3D cell scaffolds due to the ability we have to control and tailor their properties.

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### Diagnostic Scaffold to Guide Tissue Morphogenesis in Wound Healing

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Within the field of regenerative medicine and tissue engineering, there is a lack of tools for *in-situ* real-time monitoring of *in-vitro* tissue regeneration processes. To overcome this we have developed self-reporting scaffolds by incorporating pH-sensitive polyacrylamide nanosensors in optically transparent gelatin scaffolds. Fluorescent polyacrylamide nanosensors are ratiometric and pH sensitive across the physiological range from pH 3–8. They comprise a polyacrylamide matrix and contain 3 entrapped fluorophores; Oregon Green (OG), 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethylrhodamine (TAMRA). OG and FAM exhibit an additive fluorescent response across the physiological pH range, while TAMRA emits a pH independent fluorescence.

Gelatin is a natural bio-polymer that is prepared by partial hydrolysis of collagen and can form optically transparent, biodegradable and biocompatible gels. Incorporation of pH-sensitive nanosensors and control of gelatin concentration and cross-linking conditions allow an optimal self-reporting scaffold to be prepared, capable of reporting changes in pH while maintaining the structural requirements to support cell growth and proliferation. Experimental results demonstrate that the self-reporting gelatin scaffold can support cell growth and proliferation while fluorescent and live-cell microscopy methods are used to simultaneously track cell migration and to follow changes in the pH microenvironment. This new diagnostic scaffold will enable improved understanding of *in-vitro* conditions, which can, in turn, generate a better understanding of *in-vivo* conditions within the body for wound healing, tissue regeneration and ageing.

### Biosilicate and Low Level Laser Therapy Improve Bone Repair in Osteoporotic Rats

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The aim of this study was to investigate the effects of a novel bioactive material (Biosilicate) and LLLT on bone fracture consolidation in osteoporotic rats. Forty female Wistar rats were submitted to the ovariectomy, to induce osteopenia. Eight weeks after surgery, animals were randomly divided into four groups: bone defect control group; bone defect filled with Biosilicate group; bone defect filled with Biosilicate and irradiated with LLLT, at 60 J/cm<sup>2</sup> group; bone defect filled with Biosilicate and irradiated with LLLT, at 120 J/cm<sup>2</sup> group. The size of particle used for Biosilicate was 180–212 micrometers. The animals were submitted to laser irradiation (830 nm, 100 mW, at 60 J/cm<sup>2</sup> and 120 J/cm<sup>2</sup>, with energy of 1.7 J and 3.4 J, respectively) at a single point on the bone defect for seven sessions, on alternate days. The results demonstrated that the LLLT, with fluences of 60 J/cm<sup>2</sup> and 120 J/cm<sup>2</sup> stimulated the expression of COX-2 in the circumjacent cells of the biomaterial, increased of the collagen deposition and the biomechanical bone properties. Morphometric analysis revealed that the animals with bone defects filled with Biosilicate and irradiated with laser, at 120 J/cm<sup>2</sup> showed a higher amount of newly formed bone compared to the other groups. Thus, the LLLT, mainly in fluency 120 J/cm<sup>2</sup> in contact with Biosilicate improved the bone repair process in osteopenic rats. These findings are fundamental in elucidating the biological mechanisms involved in the repair of fractures with difficult consolidation, especially those associated with bone metabolic disease processes, such as osteoporosis.

### Fabrication of Biomimetic Vascular Scaffolds using Vascular Corrosion Casts for Reconstruction of Pre-vascularized Engineered Tissues

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One challenge common to tissue engineering of most solid organs is maintaining cell viability in large constructs. *In vitro* pre-vascularization of the engineered constructs enhances delivery of oxygen and nutrients, which increases cell survival and function. Biofabrication and microfluidics techniques have been applied to the creation of vascular networks, but limitations still exist with respect to the size and complexity of the resulting vessels. Simple branching vessel-like structures can be produced, but they do not replicate the organization seen in native tissues. In addition, these methods are often complicated and expensive. To this end, we aimed to create a simple and novel method for the fabrication of biomimetic microvascular scaffolds for pre-vascularization of engineered tissue constructs. We achieved this by utilizing vascular corrosion casts as a polycaprolactone (PCL)-based physical mold for the creation of the vascular scaffolds. Vascular corrosion casts are capable of capturing the structure of the entire vascular network of an organ, including artery to capillary-sized features, making the resulting collagen-based scaffolds a copy of the native anatomy. Our vascular scaffolds can support endothelialization and incorporation into hydrogel-based renal constructs. Kidneys have unique vascular features that are necessary for function, making them an ideal target for our biomimetic scaffolds, but the nature of this process means that the vascular scaffolds produced are tissue-specific, making them widely applicable in the field of tissue engineering. We believe this method can be used to create simple, cost effective, biomimetic vascular scaffolds for pre-vascularization of 3D engineered tissue constructs.

#### Implications of Ineffective Biologic Scaffold Decellularization upon the Host Response

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Biomaterial-mediated tissue repair is a complex process that depends upon the immune response. An association has been established between macrophage phenotype and clinical outcome following biomaterial implantation. While early signs of chronic inflammation including foreign body reaction and a predominant M1-proinflammatory macrophage phenotype are associated with scar tissue formation, a predominantly M2-macrophage response is associated with constructive/functional tissue remodeling. Macrophage phenotype has been shown to be partially dependent upon the presence of immunogenic cellular remnants in biologic scaffolds. Toll-like receptors (TLRs) are involved in critical aspects of host defense mechanisms. The objective of the present study was to assess the effect of specific cellular components on the host response and to evaluate the involvement of TLRs in this process. Collagen scaffolds were supplemented (i.e. spiked) with three different concentrations of either DNA, mitochondria, or cell membranes. The acute immune response and remodeling outcome were assessed *in vivo* using an abdominal wall defect model in the rat. The effect on macrophage phenotype and TLRs were assessed *in vitro* using wild-type and TLR-deficient macrophages. Supplemented scaffolds showed induction of M1-macrophage phenotype *in vitro* and *in vivo*, and TLRs appear to be involved in this process. Despite known effects of immunogenic cell remnants on the host response, standardized quantitative criteria for decellularization assessment have only recently been described. As a result, the amount of cell debris retained in commercially available products varies significantly, and may be responsible, at least in part, for the variations observed in tissue remodeling outcomes in the clinical setting.

#### Hasc and Nfc Membranes. New Promising Candidates for Wound Healing Treatment

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The field of wound care is always expanding with new advances but still none avoids the inflammatory reaction post-trauma. Given their immunomodulatory properties, human adipose stem cells (hASC) have emerged as efficient candidates to reduce inflammation. The main roadblocks to increase their success in clinical applications are the risk carried by animal reagents used in hASC culture, as well as the lack of appropriate vehicles for deliver the cells to the injured area. To fight zoonosis, we have developed an animal-free protocol for hASC isolation and maintenance. Wood-derived nanofibrillar cellulose (NFC) is a natural promising material. We propose the use of xenogeneic-free hASC to relax chronic dermal inflammation, using as vehicle for cell delivery a removable NFC dressing and helping the auto regeneration.

hASC were seeded on NFC membrane. After 7 days and 3 passages in culture, *in vitro* assays were carried out to study if the culture on NFC affects different parameters of hASC as morphology (by SEM and TEM), viability (Alamar Blue - AB), multipotent gene profile expression, cytokine release and proliferation by Ki-67. Cell toxicity was also evaluated by tunnel assay. Wound repair was measured by using SCID mice. Immune rejection against the dressing was checked in NGS humanize mice. *In vivo* assays demonstrated the wounds treated with hASC- NFC dressing healed faster and presented normal skin structure. Humanised mice assay showed the absence acute rejection symptoms both in the healed area or systemically.

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#### Hybrid Organoid Consisting of Extracellular Matrix Gel Particles and Cells for Functional Liver Tissue Construction

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**Introduction:** Cell transplantation is one of alternative therapies of orthotopic liver transplantation because of a chronic donor shortage. However, hepatocytes are prone to losing their biological function. In our study, we tried to establish a technology, termed 'hybrid organoid', using heparin-conjugated gel particles combined with cells. This technology allows structures to form with high local cell density and low total cell number. In this study, Embryonic day 14 fetal liver cells (FLCs) and hepatocyte consist hybrid organoid was evaluated by transplantation.

**Experimental:** Cells and heparin gel particles-packed polyurethane foam (HG-C) and cells embedded polyurethane foam (C-C) were developed. Hepatocytes or FLCs was seeded in HG-C and C-C respectively. Samples were transplanted in hepatectomy (PH)-treated rat. Cell viability was evaluated by H&E stain at 7 days after transplantation. Liver function related gene expression was evaluated by real time PCR (RT-PCR). Furthermore, FLCs-filled HG-C was transplanted in PH-treated NAR, and albumin concentration in serum was evaluated at 0,3,5,7 days.

**Results and discussion:** From the results of H&E stain, cell viability in HG-C was higher than that in C-C in both hepatocyte and FLCs transplantation. FLCs contained samples showed a higher viability than hepatocyte contained samples caused by the proliferation ability of FLCs. Gene expression of albumin and CYP was confirmed by RT-PCR. Moreover, in the NAR transplantation, albumin concentration in serum increased in 3 days. These results indicated that hybrid organoid benefit albumin synthesis and cell viability during

transplantation. Therefore, functional liver tissue was formed by hybrid organoid transplantation.

#### Amnion Membrane Hydrogel Accelerates Skin Wound Healing

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**Objective:** Amniotic membrane patches have been implemented as dressings for skin wounds for many years. However, application of freeze-dried or cryopreserved patches is not optimal for wounds that are irregularly-shaped and/or of variable depth. Herein we describe a novel hydrogel composed of hyaluronic acid (HA) and amniotic membrane components that forms a gel upon deposition *in situ*. Our objectives were to 1) Develop a novel hydrogel that is easy to manufacture, store and apply to full thickness burns and wounds; 2) Compare the amnion hydrogel to commercially available wound healing products in a large animal model, and 3) Evaluate delivery strategies and product composition for future clinical and commercial translation.

**Methodology:** Porcine skin wound model: A total of 72, 4.0 × 4.0 cm full thickness wounds were created on the dorsal skin (n=9). Various compositions of amnion-membrane-derived products were evaluated for their potential to accelerate wound healing, and compared to several other commercially available wound healing products.

**Results:** Amnion membrane-derived powder and solubilized preparations were easily applied to full thickness wounds deposited as a liquid and forming a stable gel *in situ*. In this animal model, all amnion hydrogel groups showed significantly faster wound healing compared to the other treatments, with a histological and extracellular matrix composition most similar to healthy skin.

**Significance:** These results suggest that amnion hydrogel may be an effective product to accelerate wound healing.

#### Tissue Engineered Vascular Grafts: The Potential of Urinary Bladder Matrix Tubular Scaffolds

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The development of arterial substitute materials for the improved treatment of cardiovascular diseases is vital. One such material is urinary bladder matrix (UBM); an extracellular matrix material. UBM contains a highly important factor for vessel and endothelium formation, an intact basement membrane. The main aim of this research is to investigate the potential of UBM as a vascular substitute using experimental techniques. Tubular UBM scaffolds were seeded with human aortic endothelial cells [1], and then placed in a novel bioreactor exposed to an average patient flow condition. The scaffolds were examined for cellular growth and gene expression of matrix metalloproteinase 2 (MMP2), vascular endothelial growth factor (VEGF) and tissue inhibitor matrix metalloproteinase 2 (TIMP2), genes involved in remodelling and angiogenesis. Gene expression of MMP2 remained unchanged in the flow-condition, VEGF showed slight differences between control and flow samples and TIMP2 was down-regulated in the flow-condition. These results indicate a possible remodelling response of UBM in steady flow and showed good cellular growth. The scaffold materials were also assessed using tensile-mechanical tests and pressure-diameter analysis [2]. These results showed that the biomaterial has good mechanical strength and also has superior compliance properties compared to synthetic vascular grafts. These findings demonstrate the potential for the use of UBM in tubular arterial form, and show the materials potential as a vascular graft replacement.

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#### RHEB Gene Regulating Chondrocytes Phenotypic and Molecular Behavior during *In Vitro* Culture via SOX9 and p27 followed by Enhancing Cartilage Tissue Regeneration

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**Objective:** As articular cartilage has a limited ability for self-repair, successful cartilage regeneration requires clinical-grade chondrocytes with innate characteristics. Here, we investigated a novel role of the RHEB gene in the control of senescence and differentiation through the modulation of oxidative stress in the chondrocytes, a hallmark of osteoarthritis.

**Methods:** Chondrocytes were isolated from human knee articular cartilage. Senescence and oxidative stress were observed by SA-β-Gal staining and MitoSox and MitoTracker, respectively. The molecular mechanism of the activity of RHEB was elucidated by PCR and western blotting. RHEB localization was confirmed by immunocytochemistry and cell fractionation. *In vitro* and *in vivo* cartilage tissue formation was induced in pellet culture and in mice, respectively, and was further evaluated by immunohistochemistry and PCR.

**Results:** Serial expansion of chondrocytes led to senescence, dedifferentiation, and oxidative stress. RHEB regulated senescence, dedifferentiation, and oxidative stress, increased the expression of COL2 via SOX9, and down-regulated the expression of p27 via MCL1. RHEB-overexpressing chondrocytes successfully formed cartilage tissue *in vitro* as well as *in vivo* with increased expression of GAG matrix and chondrogenic markers.

**Conclusions:** RHEB maintained the innate chondrogenic properties of chondrocytes by expressing SOX9 and inhibiting p27 expression for a long period of time, resulting in increased cartilage formation during *in vitro* culture as well as in mice. Thus, RHEB expression represents a potentially useful mechanism in terms of cartilage tissue formation from chondrocytes, by which chondrocyte phenotypic and molecular characteristics can be retained through modulation of cellular senescence, dedifferentiation, and oxidative stress.

#### Large-Scale Vascularized Adipose Tissue Engineered *In Vitro* using Decellularized Porcine Jejunal Segments in a Custom-Made Bioreactor System

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The survival and the achievable size of engineered adipose tissue are crucially defined by the extent of vascularization, representing a key and limiting factor. Decellularized jejunal segments may, therefore, represent a suitable scaffolding system with preexisting capillary structures, which can be repopulated with human microvascular endothelial cells (hMVECs), and a luminal matrix applicable for the adipogenic differentiation of human adipose-derived stem cells (hASCs). Hence, utilizing a custom-made bioreactor system, this co-culture system was characterized in terms of vascularization and adipose tissue development.

The capillary structures of porcine decellularized jejunum were seeded with hMVECs and cultured in a flow-through bioreactor system with a physiological pulse. After two weeks, the lumen of the

jejunum was seeded with hASCs. Finally adipogenic induction was initiated and continued for three weeks.

Successful repopulation of the vasculature with hMVECs was monitored. Substantial adipogenesis of hASCs was observed within the jejunal lumen in contrast to uninduced controls. Expression of key adipogenic marker genes was also found to increase over time upon induction. The development of major extracellular matrix components of adipose tissue such as laminin and collagen IV was shown in induced samples. Close proximity of vascular structures and adipogenically differentiating hASCs could be demonstrated.

The presented work represents a novel approach for the engineering of vascularized adipose tissue constructs. In on-going work, vascularization and adipogenesis will be optimized by varying culture conditions in order to foster homogeneous tissue development.

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### Titanium Surface Modulation of the Inflammatory Response During Osseous Healing

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**Objectives:** This study aimed to characterise the early *in vivo* inflammatory cytokine response to modified titanium surfaces during osseous healing.

**Methodology:** Sandblasted and acid-etched (SLA) or hydrophilic-modified SLA (modSLA) titanium discs were used to cover calvarial defects in rats. Exudate within the defect and beneath the discs was collected for cytokine (IL-1a, IL-1b, MCP-1, IL-10, IL-12p40, MIP-2, RANTES and TNFa) analysis 1, 4 and 7 days post-surgery. CD11c (M1) and CD163 (M2) immuno-fluorescent staining was used to determine the phenotype of titanium adherent macrophages. Samples of the healing defect were prepared for histological analysis.

**Results:** After one day of healing, high levels of all the inflammatory cytokines were observed. Levels fell by day 4, apart from MCP-1, which decreased only on the modSLA surface. The level of TNFa decreased on both surfaces, however the extent of the decrease was significantly ( $p < 0.05$ ) more so on the modSLA surface. At day 7 the level of MCP-1 on the modSLA surface decreased further while the levels of this cytokine on the SLA surface were maintained at previous levels. TNFa at day 7 on both surfaces was maintained as seen at day 4. Compared to modSLA, more CD11c + ve (M1) macrophages were seen on the SLA surface at days 4 and 7. These results correlate with increased new bone formation on the modSLA surface by day 7.

**Significance:** This study suggests the modSLA surface can modulate the adherent macrophage phenotype, pro-inflammatory cytokine secretion and bone formation in the early stages of osseous healing.

### Repair of Segmental Bone Defects in Osteoporosis Microenvironment using Allogenic Fetal Bone Marrow Mesenchymal Stem Cells

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Traditional bone tissue engineering (BTE) strategy has so far focused on the feasibility of various bone defect repair in recipients with normal physiological function. However, huge numbers of bone fractures occur due to the bone metabolism disorders such as osteoporosis, and very few articles regarding BTE treatment effect under pathologic environment. This research mainly investigate the repair possibility of critical-sized segmental bone defects in a rabbit osteoporosis model using allogenic fetal bone marrow mesenchymal stem cells (BMSCs), and discuss the potential influences of osteoporotic condition on the treatment efficacy. Fetal rabbit BMSCs were

harvested and expanded *in vitro*, and then seeded onto decalcified bone matrix (DBM) scaffolds. After osteogenic differentiation, BMSC/DBM constructs were inserted into critical-size radial defects created in ovariectomized (OVX) rabbits group and non-ovariectomized age-matched (non-OVX) group respectively. Blank DBM was used as a blank control repairing non-OVX rabbits. The *in vivo* repairing procedure was recorded by X-ray at 1 month and 3 month time point. After that, all animals were sacrificed and radius were examined by Micro-CT and histology analyses. Notably, we find fetal BMSC/DBM constructs have successfully bridge the defects in OVX group, though the new bone formation volume is less than that in non-OVX group. Meanwhile, TRAP staining suggests a positive activity of osteoclasts in OVX repair position. Therefore, we concluded that BTE strategy based on allogenic fetal BMSC could receive a well but compromised therapy effect under osteoporosis microenvironment, and systematic osteoporosis treatments may also be required in further BTE strategy research.

### Induction of Elastin Synthesis by Synthetic Modified Messenger RNA

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Elastin is an important extracellular matrix protein, which provides elasticity to the tissue, such as blood vessels, skin, and alveolus. The absence or progressive destruction of the elastin as a result of a hereditary condition or an acquired disease requires the de novo synthesis of elastin to restore the elasticity of the affected tissue. Therefore, we examined the induction of elastin synthesis by the application of synthetic modified messenger RNA (mRNA). In this study, Ea.hy 926 cells, human fibroblasts, and mesenchymal stem cells (MSCs) isolated from thymus of a patient with Williams-Beuren syndrome were transfected with tropoelastin (TE) coding mRNA. Using Fastin assay and dot blot analyses, TE synthesis was analyzed. The transfection of cells with 2.5  $\mu$ g mRNA led to significantly higher elastin production. The elastin amount in Ea.hy 926 cells was approximately 55  $\times$  higher, in fibroblasts 45  $\times$  higher, and in MSCs 35  $\times$  higher than in negative controls. In further analyses, *in vivo* tests are performed. Using TE coding mRNA, the synthesis of TE, which is the soluble monomeric precursor of elastin, was successfully demonstrated *in vitro*. This auspicious method using exogenous delivery of synthetic modified mRNA without genomic integration can be used for treatment of hereditary diseases, in which elastin is not synthesized, and acquired diseases to induce de novo elastin synthesis, e.g. in blood vessels and skin, to restore the function.

### Evaluation of MSC Source on Angiogenesis in 3D Co-Culture

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Osteogenesis and angiogenesis are coupled during bone formation and healing. Pre-vascularization as a strategy for clinical-scale tissue engineering relies on this co-dependence in attempt to generate large volume bone scaffolds for implantation. Although the osteo-differentiation of MSCs has been well characterized for a variety of cell sources, the potential of these MSCs to induce angiogenesis in co-culture with ECs has not. We aim to characterize angiogenic response of multiple commercially-available MSC sources with two distinct EC populations. Endothelial cells were obtained as commercially-available HUVECs or umbilical cord-blood derived endothelial colony-forming cells (ECFCs). EC aggregates were co-cultured with MSCs, purchased from Lonza or RoosterBio, either in suspension or within the aggregate. Cells were embedded in fibrin hydrogels as previously described and cultured for up to 3 weeks under media conditions optimized for network formation. Samples were fixed, and stained for CD31 (HUVEC), UEA lectin (ECFC) or  $\alpha$ SMA (MSC).

Extensive, interconnected vessel structures were formed in all four combinations. The highest vessel structure area was observed using HUVECs and Lonza MSCs co-aggregates, resulting in a total area of 1.6 mm<sup>2</sup>. RoosterBio MSCs resulted in significantly larger diameter vessels when cultured in suspension with ECFC aggregates compared to Lonza MSCs (28.0 ± 4.4 μm vs 16.2 ± 1.7 μm, p < 0.001). In conclusion, we describe the angiogenic potential of MSC sources in distinct co-culture systems.

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### M2C Gene Expression Signature Revealed by RNA-Seq Indicates a Dominant Role in Human Cutaneous Wound Healing

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Macrophages play a pivotal role in cutaneous wound healing. Compelling evidence suggests that while macrophages undergo a phenotypic shift from M1-to-M2 during normal wound healing, they are stalled in the M1 state in diabetic ulcers, resulting in chronic inflammation and impaired healing. Although two main M2 macrophage subtypes, known as M2a and M2c, appear to exhibit distinct characteristics *in vitro*, their functions *in vivo* remain largely unknown because of a lack of specific markers to differentiate between them. To address this knowledge gap, we conducted whole transcriptome next-generation sequencing (RNA-Seq) analysis of primary human macrophages polarized *in vitro* to the M1, M2a, and M2c phenotypes (n=4 donors). We identified ~900 previously unknown M2c-specific markers. We applied this new M2c gene expression signature to analyze temporal changes observed during acute wound healing in humans *in silico*, using publicly available data sets. We found that M2c markers are more positively associated with healing compared to M2a markers. Next, in a pilot study of ten patients with chronic diabetic ulcers, we tracked expression of a single marker of the M1 phenotype (IL1B) and a single marker of the M2c phenotype (CD163) in debrided wound tissue obtained over the course of 4 weeks. Wounds that ultimately failed to heal by 12 weeks showed a significant increase in IL1b/CD163 ratio, while wounds that healed showed a decreasing ratio (n=5 healing and n=5 non-healing, p<0.01). Collectively, these results indicate that the M2c phenotype plays a dominant role in human cutaneous wound healing.

### ATRA and EGF Media Supplementation Effects on Human Adipose-derived Stem Cells Differentiation towards Urothelial Cell Lineage

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Currently, a reliable system for differentiating transplantable adult stem cells for urological tissue engineering does not exist. In pursuit of this goal, human adipose-derived stem cells (hADSC, Lonza) were cultured in keratinocyte serum-free media (KSFM) supplemented with epidermal growth factor (EGF) in the presence or absence of all-trans retinoic acid (ATRA). Phase contrast and fluorescence microscopy provided morphological evidence of cell differentiation with hADSCs cultured in KSFM with EGF exhibited comparable morphology to UROtsa cells, while those cultured with DMEM and ATRA showed a spread, umbrella-like epithelial morphology. The results of RT-PCR with custom-designed primers demonstrated that, compared to the DMEM control, cells in KSFM with EGF expressed urothelial markers, CK-20 and UPIb, at nearly 14-fold and 4-fold higher respectively, and exhibited a significant decrease in stem cell markers CD90 and CD105. The western blotting and immunofluo-

rescence with anti-CK17, anti-CK20, and anti-UPIb antibodies revealed upregulation of urothelial markers at the protein-level that were consistent with mRNA expression. Together, these results have indicated that defined KSFM supplemented with growth factors is key to push the hADSCs towards a urothelial lineage. Since this method achieves this goal without bovine-derived products or use of a secondary urothelial culture for conditioned media, we can significantly facilitate the use of hADSCs as a stem cell source for regeneration of the urothelial layer.

### Contractility Mediated Wound Closure in Mesenchymal 3D Microtissues

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Many insights on the cellular interactions and signaling networks underlying wound healing are based on healing of scratches in monolayers of epithelial cells in 2D culture systems or wounds in *Drosophila* embryos. In contrast, how fibroblasts participate in wound healing is less well understood. Unlike epithelial cells lining surfaces and lumen *in vivo*, fibroblasts are ensconced in fibrous ECM and therefore don't display apical-basal polarity. Hence, the influx of cells into the site of injured ECM, the closure of the wound, and the subsequent ECM stiffening are all components of the process that are not captured by planar *in vitro* models for wound healing. To overcome this limitation, we present in this work a novel wound healing model to evaluate healing of microsurgically-induced wounds in an engineered 3D microtissue of fibroblasts embedded in a type I collagen matrix. Using our microfabricated tissue gauge technology, we generated arrays of 3D microtissues suspended between flexible cantilevers that simultaneously constrain the microtissues and report microtissue contractility in real-time. With a microdissection knife mounted to a microrobotic manipulation platform, we damaged the microtissues and monitored gap closure in the next 24 h with time lapse microscopy. Using pharmacological reagents and fluorescently labeled fibronectin and collagen, we demonstrated that gap closure by fibroblasts is mediated by cellular contractility and fibronectin scaffolding through α5β1 integrin signaling. These studies introduce a new model for studying wound healing, and highlight important mechanistic differences between gap closure by fibroblasts embedded in 3D matrix versus adherent to a planar surface.

### Regeneration of Whole Meniscus using Bone Marrow-derived Mesenchymal Stem Cells in a Dog Total Meniscectomy Model

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The meniscus injury is common in clinic. Only a few literatures have reported the entire replacement of meniscus in large animals with chondrocytes. However, due to the disadvantages of chondrocytes such as poor proliferation ability, cell senescence and differentiation, it was limited in clinical use. The bone marrow mesenchymal stem cells (BMSC) has the advantages of sufficient source, convenient acquisition, small trauma and strong proliferation, so in this study we chose BMSC as the seed cell to reconstruct tissue engineering meniscus. The experiment groups (n=10) were replanted with BMSC-PGA constructs after 6 weeks induction *in vitro* to replace entire medial meniscus on dog total meniscectomy models, while the control groups (n=10) only cut off the meniscus on left legs and replanted blank holders on right. All groups were tested after 6 months. The results showed that in experiment group cartilage-like tissue had formed and articular surface showed smooth, while in control group no tissue appeared on left legs and fibrous-like tissue repaired on right ones, meanwhile the articular surface all abraded. Histological and immunohistochemical analyses got the same results

as above. Besides, mechanical testing showed that the repair tissue had certain mechanical strength in experimental groups, but much worse in control groups. From above we know the replacement of medial meniscus with BMSC-PGA constructs after 6 weeks induction could form fiber cartilage like tissue, it also has certain joint protection function and delays the onset of osteoarthritis. It should provide an evidence of the entire replacement of meniscus with tissue engineering methods in clinical therapy.

### Dermal Fibroblast Conditioned Medium (DFCM) for Skin Wound Healing

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Severe skin injury affects patient's quality of life even death. Skin regeneration requires the presence of wound healing mediators such as cytokines, chemokines, growth factors and extracellular matrix (ECM). Supplementation of these factors have been considered as alternative treatment modalities for skin injuries. Fibroblasts are well known for their ability to secrete essential factors for skin regeneration, which can be collected during fibroblasts culture as DFCM. This study aimed to characterize and evaluate the potential of DFCM in skin wound healing. Human skin samples were collected with patient's consent. Fibroblasts isolated from skin tissue were cultured until confluence and incubated with serum free keratinocyte-specific and fibroblast-specific culture medium to collect DFCM, and designated as DFCM-KM and DFCM-FM, respectively. Analysis of the protein composition of DFCM was performed via 1D-SDS-PAGE, mass spectrometry, ELISA and western blot. Efficiency of wound healing was evaluated by means of keratinocyte attachment, proliferation and migration in scratch assay. The proteomic analysis confirmed the presence of wound healing mediators such as hepatocyte growth factor, vascular endothelial growth factor, interleukin-6 and 8, intercellular adhesion molecules-1, monocytes chemoattractant protein-1, C-X-C motif ligand-1 and 5, fibronectin, collagen type I, II, III and IV. Supplementation of DFCM-KM was found to significantly increase the keratinocyte attachment and proliferation, whereas DFCM-FM significantly increased the rate of wound healing. These results suggest that supplementation of DFCM could be used for the treatment of skin injuries.

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### 3D Patterned Cell-Instructive Substrates: Towards the Formation Tissue Mimetics

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Physiologically relevant tissue models would be desirable to study physiological and pathological processes under highly standardized conditions. It is hypothesized that the spatial arrangement of cells, extra-cellular matrix components and soluble or matrix associated biological cues is sufficient for the formation of tissue mimetics.

Modular building blocks (Streptavidin and Protein A) that allow the stable immobilization of biological cues by affinity interactions were designed to be compatible with factor XIII (FXIII) cross-linkable PEG and fibrin hydrogels, recombinantly expressed and purified. Platelet derived growth factor (PDGF) and bone morphogenetic protein (BMP) were tagged by recombinant expression or chemical modification, respectively. PEG hydrogels containing matrix-immobilized growth factors were patterned using different techniques such as printing, layer-by-layer assembly or electrochemistry.

Biotin or Fc-tagged growth factors were efficiently captured by hydrogels, containing specific affinity sites. Such growth factor immobilization could be employed to for 3D-patterned cell-instructive matrices. Hydrogels containing patterns of BMP-2 were shown to stimulate the osteogenic differentiation of MSCs or C2C12 cells in locally defined areas. Hydrogel encapsulated MSCs responded to 3D PDGF-BB gradients by spreading, proliferation and migration. This concentration dependent, localized response was designed to mimic the recruitment process of perivascular cells to newly forming vessels.

By mimetic naturally occurring matrix growth factor interactions, stable micro-patterned substrates can be generated, which control the local behavior of cells. The patterning of different growth factors in specific locations holds great promise for the generation of more sophisticated 3D tissue models relying on both investigator controlled assembly and self-organization processes.

### Recellularization of Whole Porcine Kidneys with Human Epithelial and Endothelial Cells

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Whole organ engineering is a promising alternative for organ transplantation. Murine, porcine and human kidneys have been successfully decellularized and rat kidneys have previously been recellularized with human umbilical vein endothelial cells (HUVECs), rat neonatal kidney cells, and murine stem cells [1, 2]. In this study previous technology was scaled up to porcine kidneys as a model of human-sized kidneys. Decellularization of porcine kidneys was optimized using pressure control and tonic cycles and the effects of freezing/thawing conditions on decellularized extracellular matrix were investigated [3]. Sterilization methods for whole kidneys were also optimized to improve cell adhesion and proliferation. Human renal cortical tubular epithelium (RCTEs) cells and HUVECs were used to repopulate the epithelial and endothelial niches of renal tissue, respectively. More than  $6 \times 10^8$  endothelial or epithelial cells were perfused through the whole decellularized porcine kidneys for 30 min and then media perfusion was continued for at least 7 days. Histological staining demonstrated that tubules were repopulated with epithelial cells and endothelial cells were located in the complicated vasculature of the kidney. Metabolic activity of reseeded RCTEs was determined by resazurin reduction assay. The epithelial cells were viable and active throughout the kidney. This study demonstrated the feasibility of regeneration of whole human-sized kidneys using decellularized porcine kidneys and human cells.

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### A Combinatorial Approach using Phenamil and BMP-2 to Enhance Osteogenic Differentiation of Adipose-derived Stem Cells and Bone Repair

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Reconstructing mandibular bone is still a challenging clinical problem. Tissue engineering approaches based on osteoinductive factors such as bone morphogenetic proteins (BMPs) are promising alternative options for bone reconstruction. However, clinical bone repair requires use of supraphysiological BMP dose that often causes worrisome complications. Hence it is necessary to develop a strategy

that can complement BMP activity to maximize osteogenesis while reducing BMP dose requirement. Phenamil is a small molecule that is able to modulate BMP signaling and stimulate osteogenesis. Here, we developed a novel approach combining phenamil and BMP-2 to complement osteogenic activities of BMP-2. The ability of phenamil to promote osteogenic differentiation of adipose-derived stem cells (ASCs) was observed with the addition of different concentrations of BMP-2 *in vitro*. We further evaluated the complementary strategy of phenamil + BMP-2 to enhance bone regeneration in a rat mandibular defect model by using apatite-coated poly(lactic-co-glycolic acid) (PLGA) scaffolds fabricated to slowly release phenamil and BMP-2. Our results showed that phenamil can cooperate with BMP-2 to enhance osteogenic differentiation of ASCs. The enhanced osteogenesis can be explained by synergistic increase of BMP-Smad signaling. After 8 weeks post-operation, the combinatorial group significantly promoted mandibular bone repair compared with the groups treated with phenamil or BMP-2 alone as confirmed by micro-computerized tomography and histology analysis. Importantly, this approach reduced BMP-2 dose but not affected bone healing efficiency. These results suggest that the combinatorial approach using phenamil and BMP-2 may provide more efficient and cost-effective therapy for bone repair.

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### Antibacterial MgO Nanomaterials for Improved Orthopedic Tissue Engineering Applications

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Regeneration of complex orthopedic tissues (such as ligaments, bones, and the tendon-to-bone insertion site) is problematic due to a lack of suitable biomaterials with the appropriate chemical and mechanical properties to elicit formation of tissues with similar structure, organization, and functionality to natural tissues. Additionally, a non-trivial fraction of implanted biomaterials contract bacterial infections, which can lead to implant failure, secondary surgeries, and the spread of infection to other tissues throughout the body. To address these issues, the current study investigated magnesium oxide (MgO) nanoparticles as novel materials to improve orthopedic tissue regeneration and reduce bacterial infection.

Here, MgO nanoparticles and hydroxyapatite (HA) nanoparticles were dispersed within poly-L-lactic acid (PLLA) composites and then tested for their mechanical properties, surface roughness, degradation characteristics, antibacterial properties, and their ability to support the adhesion and proliferation of fibroblasts and osteoblasts.

Results showed for the first time that nanocomposites containing both HA and MgO nanoparticles performed best with respect to osteoblast proliferation and mechanical properties. Increases in alkaline phosphatase expression and vinculin focal adhesions indicated the ability of MgO to enhance the osteogenic properties of HA composites. Further, varying MgO concentrations offered tunable composite degradation kinetics, and the supernatant from degraded composites containing MgO nanoparticles supported greater osteoblast proliferation compared to non-MgO composites. Bacterial experiments with *Staphylococcus aureus* showed that MgO nanoparticles exhibit powerful bactericidal efficacy, suggesting that MgO nanoparticles should be incorporated into scaffolds for orthopedic tissue engineering to improve cell functions and reduce the risk of bacterial infection with limited antibiotics usage.

### Glycosaminoglycan Binding Cell Penetrating Peptides for Efficient Gene Delivery

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A major scientific goal is the development of non-viral drug delivery platforms for the delivery of exogenous DNA to cell nuclei. Many of these technologies cannot overcome challenges in low transfection efficiency, cytotoxicity and/or serum-inhibition limiting

*in vivo* efficacy. If these issues could be resolved then powerful technologies such as *in vivo* cell programming or gene correction could be employed therapeutically.

Cell penetrating peptides (CPPs) are intracellular delivery vehicles that can carry biologically active molecules into cells. In previous work we have shown that when a CPP (8R) was conjugated to a heparan sulphate-glycosaminoglycan (HS-GAG) binding domain (P21) the delivery of a reporter cargo into cells increased by two orders of magnitude. This synergistic increase in transduction was termed GAG-mediated enhanced transduction (GET).

To demonstrate the utility of this protein for delivery of therapeutic molecules we designed and synthesised a DNA-binding GET protein termed P21 LK15 8R. The reporter gene (pSIN GFP) was delivered into cells. Results demonstrated GET-mediated transfection efficiencies of  $38.1 \pm 1.8\%$  in serum conditions. We also compared the transfection of GET protein with a commercial transfection reagent Lipofectamine 2000. Cells treated with Lipofectamine 2000 showed inhibited cell growth and lower cell viability than cells treated with P21 LK15 8R. Following a 3 day serial transfection, 4-fold more GFP positive cells were detected using GET gene-transfer compared to Lipofectamine 2000.

In conclusion we have developed a non-cytotoxic and serum-resistant transfection system that could potentially be applied *in-vivo* for control of cell behaviour and correction of gene defects.

### Saloplastic Membranes as Green Devices for Soft Tissue Regeneration

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Implantable devices must exhibit mechanical properties similar to native tissues to promote appropriate cellular behavior and regeneration. Herein, we report a new membrane manufacture method based on the synthesis of polyelectrolyte complexes (PECs) that exhibit saloplasticity, i.e. variable physical-chemistry using salt as a plasticizer. This is a Green Chemistry approach, as PECs generate structures that are stabilized solely by reversible electrostatic interactions, avoiding the use of harmful crosslinkers completely. Furthermore, natural polyelectrolytes - chitosan and alginate - were used. Upon mixing them, membranes were obtained by drying the PECs at 37°C, yielding compact PECs without resorting to organic solvents. The plasticizing effect of salt after synthesis was shown by measuring tensile mechanical properties, which were lower when samples were immersed in high ionic strength solutions.

Salt was also used during membrane synthesis in different quantities (0 M, 0.15 M and 0.5 M in NaCl) yielding structures with no significant differences in morphology and degradation (around 15% after 3 months in lysozyme). However, swelling was higher (about 10x) when synthesized in the presence of salt. *In vitro* cell studies using L929 fibroblasts showed that cells adhered and proliferated preferentially in membranes fabricated in the presence of salt (i.e. the membranes with lower tensile strength).

Structures with physical-chemical properties controlled with precision open a path to tissue engineering strategies depending on fine tuning mechanical properties and cellular adhesion simply by changing ionic strength during membrane manufacture.

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### Fabrication of Polycaprolactone-Nukbone® (PCL-NKB) Scaffold by 3-D Plotting System for Bone Tissue Engineering

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The fabrication of biological substitutes that maintain improve or restore damaged tissues and organs has been addressed through tissue engineering for many years [1]. Recent advances in 3D-fabrication

technologies have made possible to create tissue substitutes from natural, synthetic materials or from a combination of both in a single step [2]. The objective of this study is to create scaffolds made from polycaprolactone (PCL) and a blend of PCL-bovine bone filling Nukbone® (NKB) [3] through rapid prototyping (RP). The scaffolds are fabricated using a 3-D Bioplotter® System (EnvisionTec GmbH, Germany) and are used towards bone regeneration. This study demonstrates the feasibility of the RP technique to produce tailor-made porous scaffolds with interconnecting pores that allows cell ingrowth and vascularization. In this work, bioplotting parameters to produce scaffolds with desired morphological characteristics were investigated. Successful 3D printing of scaffolds was achieved using a combination of parameters that involve deposition speeds between 0.3 and 1 mm/s, and pressure dispensing range of 3 to 4.8 bar when using three different diameter nozzles (0.2, 0.3 and 0.4 mm). Scanning electron microscopy (SEM) was used to observe the dependence of pore size on the nozzle size, the distance between strands and resulting morphology. The resulting scaffolds were characterized with infrared spectroscopy and mechanical testing. Additionally, selected scaffolds with a total volume of 500 mm<sup>3</sup> were seeded with osteoblast to study cell proliferation within the pores towards their potential use as bone substitute in tissue engineering applications.

### The Effect of Stretching on the Osteogenic Differentiation and Mechanical Properties of Human Adipose Stem Cells

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Mechanically induced signal cascades are known to affect stem cell fate, but the significance of this stimulus on the osteogenic differentiation of human adipose stem cells (hASCs) remains unclear. In this study, we investigated the effect of long-term stretching on the attachment, osteogenic differentiation and mechanical properties of hASCs *in vitro*. Tailor-made, pneumatic cell stretching devices with polydimethylsiloxane membranes were used to expose hASCs to cyclic equiaxial stretching for 10 days. Osteogenic medium was used to support differentiation. Cell attachment and focal adhesions in stretched and static samples were visualized by using immunocytochemical vinculin staining after 0, 3 and 6 days of stretching. The effect of stretching on proliferation and osteogenic differentiation were analyzed quantitatively by using CyQUANT and alkaline phosphatase activity assays at 0, 6 and 10 days. Moreover, mechanical properties of hASCs, in terms of apparent Young's modulus and normalized contractility were obtained by atomic force microscopy based indentation and computational approaches. Our results indicated that stretching restrained proliferation and might promote the osteogenic differentiation of hASCs. Stretching also modified cell morphology by reducing cell size and enhancing orientation. The focal adhesions were located not only at the edges of cells as those in static culture condition, but also throughout the cell/substrate interface. Additionally, apparent Young's modulus and normalized contractility increased from days 0 to 10, and were higher with stretched samples. These results suggest that stretching increases the stiffness and modifies focal adhesions of the hASCs and it could be exploited to enhance the osteogenic differentiation.

### A Predictive Fluid-structure Interaction Approach to Physiological Mechanical Conditioning in Vascular Tissue Engineering

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The *in-vitro* simulation of physiological mechanical conditioning through bioreactors plays a crucial role in the development of

functional tissue-engineered blood vessels. Thus, we developed a scaffold-specific fluid-structure interaction (FSI) model under pulsatile perfusion provided by a bioreactor, in order to estimate the flow rate that ensures physiological circumferential deformations and wall shear stresses (WSS) on cells seeded on scaffolds. The 2D-axiallysymmetric FSI model, computed by COMSOL Multiphysics 4.1, represents two domains: the former represents the tubular scaffold, defined as a linear viscoelastic material characterized by its elastic modulus and shear relaxation modulus, previously obtained by uniaxial stretch tests. The latter domain represents the culture medium, defined as an isotropic, homogeneous, incompressible, and Newtonian fluid. A pulsatile and parabolic velocity profile is prescribed at the model inlet, while calculated pressure is prescribed at the scaffold outlet. The pressure is estimated at the scaffold end by solving boundary ordinary differential equations, in relation to construct deformations and to the downstream lumped-parameter hydraulic circuit of the bioreactor. The results show that for the considered flow rate, circumferential deformations ( $\leq 10\%$ ) and WSS ( $\leq 2$  Pa) fall within the physiological range, except for the scaffold edges. Therefore, by tuning the flow rate provided by the bioreactor circuit, the physiological conditioning of the scaffold is achieved, which properly drives its *in-vitro* maturation. Consequently, besides saving experimental time and costs, the FSI model is pivotal for *in-silico* evaluations of physiological mechanical stimulations of tissue-engineered scaffolds using mechanical properties experimentally obtained and a proper tuning of the bioreactor working setup.

### Micropatterned Substrates for *In Situ* Spatiotemporal Control of Human Neural Stem Cell Tissue Morphogenesis

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When differentiated *in vitro*, human pluripotent stem cells (hPSCs) can spontaneously form tissues that recapitulate early stages of developmental morphogenesis (1). This is highlighted by the ability of hPSC-derived neural stem cells (NSCs) to spontaneously self-organize into highly polarized rosette structures thought to be mimetic of the *in vivo* neural tube (2). During development, all central nervous system (CNS) tissues are derived from the neural tube, thus controlling the self-organization of NSCs into rosettes is critical to fully harnessing the morphogenetic capacity of NSCs for generating CNS tissues.

Here, we present our development of culture platforms that enable *in situ* spatiotemporal control of hPSC-derived NSC rosette formation and tissue morphology (3). We engineered culture substrates with micropatterned PEG brushes capable of undergoing copper-free click reactions. Using peptide-clickable ligand bioconjugates, we demonstrated *in situ* conversion of inert PEG brushes, which initially confined NSC tissues to a microscale circular morphology, into biospecific, cell-adhesive substrates that permitted radial tissue growth. This progression in tissue morphology change mimics early development, and allowed us to control neural rosette formation and generate arrays of tissues with architectures analogous to neural tube slice cultures *de novo*. Such *in vitro* arrays of organotypic CNS tissues will significantly facilitate and expand modeling of neurological disorders as well as neurotoxin screening and drug discovery applications.

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### Experiment on the Cartilage Defects Repair by Ph-sensitive Chitosan-hyaluronic Acid-chondroitin Sulfate Nanoparticle Loading Gdf-5 Plasmid

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**Introduction:** Cartilage defect caused by articular cartilage degeneration is a common disease. In this research, nanoparticles loading GDF-5 plasmid will be prepared by chitosan, hyaluronic acid and chondroitin sulfate, which will be used to repair cartilage defects on the molecular level.

**Methods:** The nanoparticles were prepared by electrostatic adsorption theory under 55°C. Physical properties and biological function of the nanoparticle were tested, and the nanoparticles were injected into the rabbit articular cavity to observe the influence on the occurrence of osteoarthritis.

**Results:** The average diameters of nanoparticles were  $630 \pm 200$  nm, which had excellent morphology, loading efficiency and low cytotoxicity. The red blood cell rupture study, release and degradation study were all proved that the nanoparticles have pH-sensitive. The nanoparticles loading GFP could successfully transfect the chondrocytes, and the transfection efficiency was similar with the liposome. And the nanoparticles loading GDF-5 could improve the secretion of specific chondrocyte extracellular matrix significantly. The occurrence of osteoarthritis which injected the nanoparticles loading GDF-5 had been obviously decreased.

**Conclusion:** The nanoparticles loading GDF-5 with excellent physicochemical properties, low cytotoxicity and high transfection efficiency could effectively delay the occurrence of osteoarthritis in animal model.

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### Cervical Total Disc Replacement with Tissue-Engineered Composites in an *In Vivo* Canine Model

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The current surgical options to treat the most severe cases of disc degeneration include spinal fusion and total disc replacement (TDR) with prosthetics, but these approaches pose risks of altering spine motion and causing subsequent degeneration. Whole tissue-engineered intervertebral discs (TE-IVD) using composite constructs that resemble the native annulus fibrosus (AF) and nucleus pulposus (NP) have shown promising results *in vitro* and *in vivo* with murine models. This work evaluated TE-IVDs produced via pre-established methods as a TDR alternative in a canine cervical spine model.

Cervical disc cells from skeletally mature beagles were cultured for 2–3 weeks. Composite TE-IVDs were produced by injecting NP cell-seeded alginate into customized molds and surrounding them with 2 layers of an AF cell/collagen gel solution; then they were cultured for 2 weeks. Fourteen beagles underwent discectomy and were divided into a TE-IVD implanted group (n=12) and a sham group (n=2). Dogs were imaged post-operatively at 2, 4 and 16 weeks, followed by histological assessment.

Implanted TE-IVDs produced increased T2 signal from 2 to 4 weeks, while the discectomized segments were shown as black discs in quantitative MRIs. At 4 weeks, disc height indices were 71% for the implanted segment and 49% for the discectomized, relative to their adjacent healthy control discs. TE-IVDs maintained NP hydration and height of the treated segment during 4 weeks, as they promoted proteoglycan-rich extracellular matrix and proliferation of chondrocyte-like cells. Evaluating the biological replacement of degenerated discs in a larger animal model significantly impacts the clinical translation of TE-IVDs.

### A Microfluidic Platform for the Establishment of Programmed Co-culture Models using an Automatic Cell Mixing Approach

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*In vivo*, tissues are maintained and repaired through interactions between the present (different) cell types, which communicate with each other through both the secretion of paracrine factors and direct cell-cell contacts. In order to investigate and better understand this dynamic interplay among diverse cell populations, we must develop new *in vitro* co-culture strategies that enable us to recapitulate such native tissue complexity. In this work, an innovative microfluidic cell mixer was computationally designed and experimentally validated that features the ability to mix non-diffusive particles (i.e. cells) in a programmed manner. The cell mixer allowed for sequentially mixing two cell types to generate reverse linear concentration co-culture patterns. Once validated, the mixer was integrated in a perfusion microreactor as an upstream module to deliver mixed cells to five downstream culture units, each consisting in ten fluidically connected circular microculture chambers. The platform established spatio-temporally tunable osteogenic co-culture models, for investigating the role of pre-osteoblastic cells (SAOS2) on human mesenchymal stem cell (hMSC) osteogenic differentiation. An increase on expression of alkaline phosphatase in sequential (downstream) chambers, consistent with the initial linear distribution of SAOS2, suggests not only osteoblastic cell-driven hMSC induction towards the osteogenic phenotype, but also the importance of paracrine signaling. In conclusion, the microreactor combines the ability to establish co-culture models in a high-throughput and fully automated fashion, with the additional advantage of maintaining cells in culture under perfused medium to explore paracrine factor impacts, representing a promising tool for directing multi-cellular tissue formation for tissue engineering applications.

### Breast Tissue Engineering: Decellularized Scaffolds Derived from Porcine Mammary Glands

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**Introduction:** Decellularization of tissues provides inductive extra-cellular matrices (ECM) for organ reconstruction. We investigated effectiveness of different decellularization protocols of porcine mammary glands with the purpose of prospective breast tissue engineering.

**Methods:** Porcine mammary glands were cut in homogeneous samples (10x10x2 cm) and processed according to three different decellularization protocols (A, B, C) via multiple chemical treatments (A: 0.02% trypsin, 0.05% ethylenediaminetetraacetic acid-EDTA, 3% Triton X-100, 4% deoxycholic acid; B: collagenase 3 mg/g, 0.02% trypsin, 0.05% EDTA, 10 U/mL, 10 U/mL lipase; C: collagenase 3 mg/g, 0.05% EDTA, 4% sodium deoxycholate, 1% sodium dodecyl sulfate, 0.9% NaCl in TRIS-HCl containing protease inhibitors). Obtained specimens were analyzed by macroscopic (morphologic) and microscopic methods (hematoxylin and eosin-H&E, immunofluorescent labeling with 4',6-diamidino-2-phenylindole-DAPI, quantitative measurement of DNA and DNA fragment size).

**Results:** Histological structure of porcine glands resembled human glands. Glands could be molded to required shape and adjacent glands could be harvested together. Size varied (average: 20x40 cm in length and 3 cm in height). Blood supply was based on reliable vascular pedicles. Decellularization protocols had variable effectiveness: all samples showed macroscopic evidence of decellularization preserving original morphology. DAPI, quantitative measurement of DNA (below 50 ng/mg dry tissue weight) and of DNA fragment size (below 200 base-pairs) showed effective reduction of immunogenic components in each protocol. At histological analysis (H&E) protocol A preserved a morphology more closely resembling native architecture of ECM and preserving vascular/ductal networks. Protocols B and C slightly damaged and altered histological structure.

**Conclusions:** Decellularization of porcine mammary tissue represents a novel and reliable preliminary approach for breast tissue engineering.



### Collagen - Alginate Microspheres Scaffolds Promote Neovascularization and Cardiac Regeneration

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Though promising results have been obtained in the past few years with scaffolds, glues, tissue constructs and patches implanted on the heart after ischemic events, these systems still suffer from poor vascularization, resulting in a time-limited efficacy of the cardiac grafts. To promote the invasion of host endothelial cells into engineered tissue implants, we designed an acellular collagen - alginate microspheres scaffold. Alginate microspheres with narrow-dispersed diameters in the range of 50–450 µm were produced (VarJ30 Bead Generator, Nisco) by varying alginate concentration, extrusion velocity, nitrogen pressure and nozzle size. The microspheres, unloaded or loaded with growth factors (VEGF and b-FGF, loading efficacy: 40%–60%), were dispersed in the acellular collagen scaffolds in different densities (10 mg/mL–100 mg/mL). A burst release (60%–80% of the immobilized growth factors) was observed after 24 h, followed by a low but constant release (3–5%) over 7 days. Nevertheless, the microspheres provided adequate signaling for branched network formation of HUVECs immobilized in the collagen - alginate microspheres scaffold. Time-lapse imaging showed the *in vitro* formation of endothelial networks after 12 h of culture, with network length, width and area increasing in the presence of microspheres loaded with 660 ng/mL VEGF. Preliminary implants of unloaded collagen - alginate microspheres scaffolds on healthy rat hearts confirmed the ability of host endothelial cells to grow into the alginate microspheres-containing scaffold. Future *in vivo* experiments with a rat myocardial infarct model will assess the potential of these scaffolds to protect and maintain the contractility of damaged hearts.

### Biomimetic Nanofiber-enabled Rapid Formation of Skin Substitutes for Full-thickness Wound Repair

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Repair and management of large extensive wounds like burns remain a major clinical challenge. Wound closure within a clinically acceptable time with appropriate restoration of epidermis is essential to prevent dehydration and minimize infection. The aim of this study was to investigate the possibility of fabricating skin substitutes via a bottom-up nanofiber-enabled cell assembly approach and using such substitutes for full-thickness wound repair in nude mice. Following a layer-by-layer (L-b-L) construction of polycaprolactone (PCL)/collagen (3:1 w/w, 8% w/v) nanofibers using electrospinning technique, fibroblasts and keratinocytes were rapidly assembled together into 3D constructs, in which fibroblasts and keratinocytes were located in the bottom and upper portion respectively. A square full-thickness wound was surgically created on the back of mouse and wounds were grafted with either bi-layer skin substitutes, dermal substitutes, acellular nanofiber meshes or autografts. Results showed that 14-day-cultured skin substitutes facilitated a rapid wound closure with complete epithelialization comparable to autografts. Good mechanical strength obtained and complete wound closure with the appropriate regeneration of epidermis achieved. Biomimetic nanofiber-enabled cell assembly allows for rapid formation of functional skin substitutes that can effectively heal acute full-thickness wounds in nude mice and provides a promising therapeutic platform to treat burnt wounds within clinically acceptable time windows.

### Skin Recovery in a Murine Based Chimney Wound Model Under Human Embryonic Stem Cell-Derived Endothelial Cell Transplantation

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As wound contraction in the cutaneous layer occurs rapidly in mice, mechanical means are typically used to deliberately expose the wound in order to properly investigate healing by secondary intention. Previously silicon rings and splinting models were attempted to analyze histological recovery but prevention of surrounding epidermal cell migration and subsequent closure was minimal. Here, we developed an ideal chimney wound model to evaluate epidermal regeneration in murine under hESC-EC transplantation through histological analysis encompassing the three phases of regeneration; migration, proliferation, and remodeling. Human embryonic stem cell derived endothelial cells (hESC-EC) were transplanted due to possessing a well-known therapeutic effect in angiogenesis which also enhances epidermal repair in order to depict the process of regeneration. Following a standard 1 mm biopsy punch, a chimney manufactured by modifying a 1.7 ml micro tube was simply inserted into the excisional wound to complete the modeling process. Under this model, the excisional wound remained fully exposed for 14 days and even after 4 weeks, only a thin transparent layer of epidermal tissue covered the wound site. This approach is able to more accurately depict epidermal repair in relation to histology while also being a user-friendly and cost-effective way to mimic human recovery in rodents and evaluate epithelial repair induced by a form of therapy.

### Osteoblast Phosphoproteome Analysis in Early Response to Topological Cues from Titanium Surfaces

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Titanium implants with micro-rough surface topography have been shown to exhibit enhanced early osseointegration. However, the underlying mechanisms are not fully understood and require further investigations to determine the molecular signalling pathways leading to improved implant integration. As protein phosphorylation is an important part of cellular signal transduction, we sought to ascertain the early phosphorylation events triggered in osteoblasts by micro-rough titanium surface topography. An antibody phosphor-array, containing more than 580 well characterised phosphor-antibodies, was used to examine this effect. An *in vitro* cell culture model was established, in which human primary osteoblasts were cultured on titanium discs with topographically modified micro-rough surfaces. A polished titanium surface acted as a control. It was found that osteoblasts responded differently to the micro-rough surface as early as 2 hours after seeding, as evident by changed cytoskeletal structures and long dendrite like filopodia protruding from the cells. Furthermore, the rough surface was found to increase cell attachment, and decrease cell migration and proliferation. The phosphoproteome analysis revealed 60 differentially regulated phosphorylation sites in response to the rough surface. Gene ontology analysis revealed highly enriched terms related to protein phosphorylation, cytoskeleton/cell adhesion, response to stress or stimulus, cellular trafficking, cell cycle, and metabolism. Cell migration and cell differentiation terms were less abundant. Taken together our data has revealed that the early osteoblast response to titanium topography involves cytoskeletal changes that trigger further downstream signalling pathways and subsequent cell fate determination.

### Optimization of Porcine Kidney Decellularization Methods for Long-Term Implantation

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End-stage renal disease (ESRD) is a major problem globally. Kidney transplantation is currently the only definitive solution for the treatment of ESRD, however transplantation is severely limited by the shortage of available donor kidneys. Recent progress in the development of bioengineered whole organs based on decellularization/recellularization of the native organs has enabled pre-clinical *in*

*in vivo* studies using small animal models; however, these *in vivo* studies have been limited to short-term assessments. To maintain renal function long-term, vascular patency of bioengineered kidneys is necessary to deliver nutrients and oxygen to the implanted renal construct. Previously, we developed a decellularization protocol that effectively removes cellular components from normal pig kidneys and the decellularized kidney scaffold maintained vascular patency short-term after implantation (less than 2 hr). To address the limitation of short term assessment, this study aims to optimize decellularization methods that can preserve functional vascular architecture for long-term implantation. We compared four different decellularization protocols (1% Triton X-100, 0.25% and 0.5% SDS, Triton X-100 and SDS) and assessed the effects of the decellularization on the maintenance of pig kidney's vasculatures using angiography, vascular corrosion casts, and scanning electron microscopy (SEM) analysis of the casts. Particularly, the vascular casting technique was efficiently used to analyze normal morphology and functional architecture of a vascular luminal structure. Our results demonstrate that the decellularization protocol using a combination of Triton X-100 and SDS was most effective in preserving microvasculatures of the renal scaffold and that the optimized method can contribute to vascular patency for long-term implantation.

#### Affinity-binding Cytokine Platforms to Promote the *In Vitro* Maturation of Ovarian Primordial Follicles

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*In-vitro* maturation (IVM) of early stage follicles has been a challenging task. Up to now, most efforts in this field have been focused on cultivation of secondary follicles in various hydrogels as a strategy for promoting maturation. Primordial follicles, the most infant, yet abundant follicular population, hold a great potential for women fertility preservation. Therefore, we are devising strategies to promote their maturation to fully functional follicles. One strategy is the co-cultivation of the primordial follicles with ovarian stromal cells within macroporous alginate scaffolds supplemented with growth factors/cytokines, presented as affinity-bound to the matrix. The affinity binding of the growth factors/cytokines to the alginate matrix has been achieved via their interactions with alginate-sulfate or hyaluronan-sulfate. Surface Plasmon Resonance (SPR) analysis proved the affinity binding of the sulfated polysaccharides to cytokines critical for folliculogenesis, such as the binding of sulfated HA to BMP-4 ( $K_D = 33.6$  nM) and BMP-15 ( $K_D = 12.5$   $\mu$ M). Macroporous alginate scaffolds impregnated with the sulfated polysaccharides promoted the maturation of porcine primordial follicles. This was demonstrated by a dramatic increase (Over 400%) in follicular diameters (reaching up to 130  $\mu$ m) and formation of visible cuboidal multi-layered granulosa cell support. These results lay the basis to utilize the affinity binding platform for the delivery and presentation of cytokines and growth factors important for follicle maturation, which may hold a great promise for the preservation of female fertility.

#### A New Pericardial Decellularized Scaffold for the Manufacturing of Cardiovascular Substitutes

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**Objectives:** To overcome the current limitations of glutaraldehyde (GA)-treated cardiovascular bioprostheses in term of poor biocompatibility and enhanced risk of structural damage, decellularized biological tissues are emerging as promising alternatives. A mandatory checkpoint in the utilization of new biomaterials is the assessment of their biocompatibility, cytotoxicity and appeal for human endogenous cells repopulation. The goal of this research is the assessment of new biocompatible and functional cardiovascular scaffolds composed of decellularized bovine pericardium.

**Methodology:** Bovine pericardium (BP) was decellularized with TRITON-X100 and TauroDeOxyCholic acid (TRITDOC procedure). Histological, biomolecular, biochemical and biomechanical analyses were employed to assess the TRITDOC-decellularized BP. *In vitro* biocompatibility of the TRITDOC-BP was investigated by cellular, biomolecular, immunological and histological analyses and the results were compared to those obtained with commercial pericardial bioprostheses.

**Results:** TRITDOC-BP is completely acellular, with an optimal maintenance of the extracellular matrix structure and unaffected biomechanical properties. The scaffold retains a high biocompatibility in terms of *in vitro* macrophage polarization, cytotoxicity and complement activation compared to commercial pericardial bioprostheses, together with the complete elimination of the antigen alphaGal, responsible for hyperacute rejection of xenogenic biomaterials. Furthermore, it can be completely repopulated by human Mesenchymal Stem Cells from adipose tissue with no sign of calcification.

**Conclusions:** TRITDOC-BP represents a more biocompatible alternative to the currently available GA-treated scaffolds and fulfills the basic immunological and structural requirements for the employment in the construction of cardiovascular substitutes.

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#### MALDI-IMS-MSI for the Analysis of 3D Tissue-Engineered Psoriatic Skin Models

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Tissue-engineering has enabled the development of skin models. These living skin equivalents [LSEs], derived from primary human skin cells, self-assemble to form stratified layers comparable to human skin. LSEs offer treatment for burns patients following serious injury, avoiding the need for meshed skin grafts and donor skin. Additionally LSEs are used for toxicity screening with the ability to replace animal models for cosmetic and drug development. While there are still limitations to LSEs they provide a model for direct quantification of drug treatment and efficacy towards skin. MALDI-MS imaging affords proteomic and lipidomic analysis associated within psoriatic and normal skin constructs. We have also used MALDI-MSI to image the effectiveness of drug treatments towards tissue-engineered psoriatic skin constructs. We have previously applied MALDI-MSI to the study of both *ex-vivo* human skin and 3D skin models.<sup>1-4</sup> Much progress is being made towards the validation of lipids and other small molecules with the use of MALDI-MS. MALDI-MS affords a closer look, and increased understanding, for drug metabolism and distribution.<sup>3-5</sup> Following treatment with pro-inflammatory cytokine IL-22 epidermal differentiation within the LSEs successfully modelled psoriasis *in vitro*. Additionally it was possible to observe the effect of therapy drug treatments towards both psoriatic skin and normal skin. The depth of penetration was observed. It was also possible to observe the psoriatic character of the LSEs through histology and immunohistochemistry profiling. The use of high mass accuracy and ion mobility separation capabilities enabled separation of isobaric species and precise profiling of the skin constructs.

### Maintenance of *In Vivo* Osteogenic Capacity during 3D Bioreactor Expansion of Human Mesenchymal Stromal Cells

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The expansion of bone-marrow mesenchymal stromal/stem cells (BM-MSC) on perfused 3D ceramic scaffolds leads to more extensive and more reproducible *in vivo* bone formation than monolayer culture on 2D polystyrene and subsequent seeding on ceramic scaffolds. We investigated here the influences on the osteogenic potential of i) the 3D vs 2D culture environment ii) the scaffold substrate material and iii) the formation of a 3D niche by deposition of extracellular matrix during expansion. Freshly isolated bone-marrow nucleated cells were cultured on 2D polystyrene dishes or in perfusion bioreactors on 3D polystyrene or ceramic scaffolds. Bone formation was examined in an ectopic nude mouse model by extracting BM-MSC after expansion and reseeded onto a fresh ceramic scaffold or by direct implantation of the constructs following culture. Whereas BM-MSC expanded in 3D, both on polystyrene and on ceramic, deposited a dense bone matrix *in vivo*, cells cultured in 2D had lost their osteogenic potential. The amount of bone produced was significantly higher after expansion on ceramic than on polystyrene scaffolds, but no difference was observed between extracted cells and directly implanted constructs. These findings suggest that i) the bone formation capacity can be better maintained in a 3D culture environment, ii) this effect can be further increased by the use of a ceramic substrate material, and that iii) the creation of a 3D niche is of lesser importance. The preservation of osteogenic potential during 3D expansion of BM-MSC in perfusion bioreactors opens the perspective for a streamlined production of large-scale bone grafts.

### Ex Vivo Tissue Vascularization via Sprouting Angiogenesis and Endothelialized Microchannels Formed using a Novel 3D Printer

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Development of a vascularization strategy capable of producing hierarchically arranged microvessels of varying diameters within engineered tissue constructs remains a major hurdle to the formation of functional tissues *ex vivo*. To address this, we are exploring the strategy of generating endothelialized branching channels within angiogenic hydrogels; these simple small artery/vein-like vessels can then be stimulated to undergo sprouting angiogenesis with the inclusion of mesenchymal cells in order to form capillary-like vessels.

To enable this strategy, we have focused on two main aspects of this approach. Firstly, we evaluated type I collagen and fibrin as rapidly-polymerizing biomaterials to produce angiogenic hydrogels with patterned channels. The combination of 0.5–3.0 mg/mL collagen and 2.5 mg/mL fibrin as a scaffold material was found to have several key advantages compared to either material alone. For example, collagen-fibrin provided more mechanical integrity than fibrin alone for forming microchannels and resisted mesenchymal cell-mediated contraction better than collagen alone ( $p \leq 0.001$ ). As well, fibrin and fibrin-collagen supported sprouting angiogenesis during mesenchymal cell co-culture, whereas collagen did not. Secondly, we have constructed a 3D bioprinter to produce complex geometries of hierarchical channels within collagen-fibrin hydrogels. This custom extrusion-style 3D printer has a print volume of 10 cm × 10 cm × 5 cm (l/w/h), a theoretical maximum resolution of 164 μm, and five extruder heads. Through combining our 3D printing

system with angiogenic biomaterials, we aim to generate thick constructs containing both small artery/vein-like vessels and capillaries in order to better recapitulate physiological tissues.

### Osteoblast-Osteocyte Differentiation within a Three Dimensional Matrix is Determined by Matrix Stiffness and Intercellular Separation

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In this study, we test the hypothesis that a bone tissue engineering strategy that determines the optimal three dimensional (3D) physical environment and cell separation distance might provide an effective approach for bone regeneration *in vitro*. 3D microbial transglutaminase gelatin hydrogels were prepared at low (0.58 kPa) and high matrix stiffnesses (1.47 kPa). MC3T3-E1 pre-osteoblastic cells were encapsulated within these hydrogels at varying cell densities (0.25, 1 and  $2 \times 10^6$  cells/ml) and cultured up to 56 days. Cell morphology was examined to identify cell dendrites, which are phenotypic of osteocyte differentiation, using phalloidin-FITC. Biochemical analyses were performed to determine cell number, alkaline phosphatase expression (ALP) and mineralisation at specific time points (2.5 hours, 21, 56 days).

After 2.5 hours dendrite formation was induced in ~50% of cells cultured within the lowest stiffness matrices, whereas no dendrite formation was observed at higher stiffness and cells remained spherical. After 56 days of culture, dendritic cells formed an interconnected network within matrices of a lower stiffness at a high cell density ( $2 \times 10^6$  cells/ml). However, in matrices of a high stiffness, cell dendrites did not connect with neighbouring cells. Additionally, mineralisation increased in high cell density groups, along with a downregulation in ALP activity. This study shows for the first time that the 3D physical nature of the ECM and the cell separation distance within this environment contribute greatly towards osteocyte differentiation and the formation of the osteocyte network *in vitro*, providing a method for future development of bone tissue engineering constructs with an osteocyte network in place.

Session: Poster Session 2, Sunrise

Date and Time: Wednesday, September 9, 2015,  
7:00 AM - 8:00 AM

### Changes in the Mechanical Properties of Clinical Meshes for Pelvic Organ Prolapse Repair Following Implantation

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Pelvic organ prolapse (POP) is the descent of one or more of the pelvic organs into the vagina and includes uterine, vaginal vault, and anterior or posterior vaginal wall prolapse. It affects millions of women worldwide and is predominantly associated with vaginal delivery, and other risk factors including hormonal effects, obesity and age. Treatment for POP includes the implantation of a synthetic mesh to augment the ruptured vaginal wall. Clinical POP meshes are predominantly non-degradable and manufactured from polypropylene (PP).

In this study a number of clinical PP meshes, ranging in weight and stiffness, together with a partially degradable mesh, were assessed for changes in mechanical properties pre and post-implantation, using a rat

abdominal hernia model. Following 30 days implantation significant decreases in explanted mesh stiffness and breaking load, with increased permanent extension following cyclic loading, were determined for some mesh types, with these differences more prevalent after 90 days. The mechanical properties of the mesh-tissue complex (MTC), formed from the mesh and attached tissue, was also mechanically assessed. We found no correlation between pre-implant mesh weight and MTC biomechanical properties, or estimated tissue properties. However, the lightest mesh, which had pre and post-implantation mechanical properties closest to the native rat tissue, formed an MTC of comparable stiffness and strength to the rat tissue after 30 days implantation. These changes in mesh mechanical properties must be considered when introducing new mesh designs into POP surgery to avoid future clinical complications.

#### Enzyme-responsive Hybrid Hydrogels Based on Chondroitin Sulphate and Multi Arm PEG

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3-D hydrogels with controlled architectures and tunable stiffness have been fabricated, both via affinity-based and covalent mechanism. Their rational tailoring towards different tissue engineering applications by adjusting mechanical and biological properties in a modular fashion has emerged as promising tool [1].

Chondroitin sulphate (CS) was shown to bind to BMP-4 and have a role in osteoblast differentiation and mineralization [2,3]. Here, we investigated a hybrid hydrogel composed of 8-arm polyethylene glycol (PEG) and CS as delivery vehicle for tissue engineering approaches. Our extracellular matrix mimicking hybrid hydrogels containing specific biological cues was based on: First, synthesis and characterization of proteolytically degradable gel precursors with factor XIII (FXIII) substrate sequences and second cross-linking via the transglutaminase FXIII. CS was functionalized with a matrix metalloproteinase (MMP) sensitive lysine donor peptide Ac-FKGG-GPQGIWQG-ERCG-NH<sub>2</sub> (Lys-MMP) and 8-arm PEG-vinyl sulfone was functionalized with a glutamine acceptor peptide H-NQEQVSPL-ERCG-NH<sub>2</sub> (TG). The subsequent crosslinking was achieved by mixing the hydrogel precursors TG-PEG and Lys-MMP-CS in presence of FXIII and under physiologic buffer conditions.

Our results show, that network properties of the CS-PEG hybrid hydrogels depend on the degree of substitution of CS with Lys-MMP, the concentration of gel precursors and the stoichiometric ratio between the two components of the hydrogel system. Resulting differences in matrix stiffness, crosslink density and degree of swelling influence cell viability, proliferation, differentiation and migration. The findings suggest CS-PEG hybrid hydrogels can be rationally tuned to guide specific cell behaviour and tissue morphogenesis.

1. Sala A 2011.
2. Tatsuya M 2008.
3. Shuqing Y 2015.

#### Magnetic Nanocarriers in Tissue Engineered Vascular Grafts

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Tissue engineered vascular grafts, including decellularized arteries have the potential to fulfill the requirements of a vascular bypass graft for small (<6 mm diameter) blood vessels. To enhance the feasibility of using a porcine derived decellularized material we focus on achieving a fully repopulated graft by investigating the use of magnetic thermosensitive drug delivery systems that can (1) lo-

cally release specific drugs under the external application of a local temperature rise, and (2) guide the cells under magnetic stimulation thus potentially facilitating cell migration and complete recellularization. We firstly developed a collagen-elastin scaffold by decellularizing porcine common carotid arteries. Secondly, magnetic nanocarriers were synthesized consisting of mesoporous silica nanoparticles (80–100 nm) with superparamagnetic iron oxide nuclei (<7 nm, as measured by TEM) incorporated into the silica structure. The resulting magnetic mesoporous nanoparticles were analysed by XRD, FTIR and TEM. Nitrogen adsorption porosimetry showed that the designed carriers present high surface area (709 m<sup>2</sup>/g) and pore volume (0.62 cm<sup>3</sup>/g), indicating the potential to host high amounts of drugs into the mesoporous network. In order to provide a thermosensitive surface coating, dipalmitoylphosphatidylcholine (DPPC)-based liposomes with a transition temperature above 41°C were combined with the nanoparticles. We envisage that combining our decellularized scaffolds with the magnetic nanocarriers will lead to the manufacture of recellularized vascular grafts for tissue engineering applications that have the potential to enhance cell survival and proliferation within the graft.

#### Particulate Decellularized Extracellular Matrix Derived from Porcine Tissues to Regulate Inflammation and Stimulate Tear Secretion in Ex-vitro Dry Eye Model

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**Purpose:** The aim of this study is to determine the effect of various particulate decellularized ECM on the inflammation and secretion in rabbit conjunctival epithelial cells and lacrimal gland acinar cells.

**Methods:** Two primary rabbit ocular cell cultures, conjunctival epithelial cells and lacrimal gland acinar cells, were challenged with IL-1 $\beta$  (20 ng/ml). Four types of particulate decellularized ECM, derived from porcine lung, cartilage, cornea and lymph nodes were then administered to the cells respectively. Cell viability, inflammatory cytokine levels and secretory function were subsequently assessed.

**Results:** IL-1 $\beta$  induced a pronounced inflammatory response in both cell types, causing elevated levels of IL-6, IL-8, IL-17, TNF- $\alpha$  and MMP-9. A statistically significant inhibitory effect of particulate decellularized ECM from porcine lymph nodes was observed in a concentration-dependent manner. IL-1 $\beta$  also induced some changes of secretion in both cell types. Particulate decellularized ECM from porcine lymph nodes could restore even increase secretion in a concentration-dependent manner.

**Conclusion:** Particulate decellularized ECM from porcine lymph nodes act as a potent anti-inflammatory agent and secretion stimulator in ex-vitro dry eye model. It may provide a new option for the treatment of dry eye disease.

#### Human Amniotic Fluid Derived Stem Cells Improve Renal Function in a Rodent Model of Chronic Kidney Disease (CKD)

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Chronic Kidney Disease (CKD) is a major medical problem that affects approximately 26 million adults in the US. Currently, the only treatment that can restore renal function is kidney transplantation. Cell-based therapies have been proposed as a means to augment and/or restore renal function. Human amniotic fluid stem cells (hAFSCs) are multipotent and possess characteristics of embryonic and adult stem cells, and therefore are a promising candidate for cell therapy. For our studies, we have developed a CKD model in an immunodeficient rat via a renal ischemia reperfusion (IR) injury. To induce CKD model, the renal pedicle (artery and vein) of male nude rats was obstructed bilaterally for 60 minutes then released to induce the ischemia reperfusion injury. Two weeks post-surgery, Gentamycin was injected subcutaneously for 5 days to maintain renal chronic disease. Serum creatinine (Cr.) and blood urine nitrogen (BUN)

levels were measured to determine a sufficient level of renal impairment. To restore functional recovery, hAFSCs were injected into the injured kidneys and improvements in renal function and renal regeneration efficiency were observed. CKD animals augmented with hAFSCs showed a significant improvement in renal functions compared with PBS injected animals within 4 to 12 weeks. Similar to the serum Cr., BUN and GFR were significantly improved in the cell-injected animals. These results demonstrate that hAFSCs improve renal function in our CKD injury model. Our experimental data supports the hypothesis that hAFSCs-based therapies can be a potential therapy for restoring renal function in diseased kidneys.

#### Transplantation of Adipose Stem Cells Plus Platelet-rich Plasma Restores the Urethral Sphincter in a Pudendal Nerve-transected Rat Model of Stress Urinary Incontinence

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**Objective:** To assess whether adipose stem cell (ADSC) plus platelet rich plasma (PRP) could promote urethral sphincter restoration in a stress urinary incontinence (SUI) rat model.

**Methodology:** Thirty five female inbred Wistar rats were used in our study. Animals were divided into seven groups (five animals per group): continent (C), sham (S), PNT (D), PNT+PBS injection (P), PNT+PBS+ADSC injection (PA), PNT+PRP injection (R) and PNT+PRP+ADSC injection (RA). Twenty five females rats underwent bilateral pudendal nerve section (PNT) to induce SUI. ADSCs were purified from fat tissue of a 4-week-old inbred male Wistar rat, labeled CM-Dil and injected into the urinary sphincter in twelve o'clock position with 70 microlitres of PBS or PRP. Four weeks after injection, cystometry was undertaken in all animals and leak point pressure (LPP) measured to assess urethral resistance function. All groups were sacrificed after cystometry, urethra sections were submitted for histology, immunohistochemistry assessment and molecular genetic analysis of SRY gen by PCR.

**Results:** LPP was increased significantly in R, RA and PA animals after implantation ( $P < 0.01$ ), but was not different from group C and S. Histological and immunohistochemical examination demonstrated increased numbers of surviving ADSCs increased muscle/collagen ratio as well as increased microvessel density at the injection sites in RA compared to PA animals (CM-Dil+). We also found SRY gene positivity in every sphincter injected with ADSC.

**Significance:** PRP may potentially improve the action of transplanted ADSC to restore the histology and function of the urethral sphincter in a SUI rat model.

#### Poly-L-lysine Coated 3d Printed Alginate Hydrogels for Cartilage Tissue Engineering

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Scaffolds for tissue reconstruction and regeneration must have appropriate structural and functional properties. Recently, hydrogels have become attractive materials applied to the repair different tissues and organs. Biodegradable and biocompatible hydrogels are crosslinked networks of hydrophilic polymers that have the capacity to retain large volumes of water. A particular advantage of high water content is easier transport of important nutrients inside the structure of the scaffold. One group of the hydrogels that have shown excellent potential in variety of biomedical applications are alginates.

The aim of the study are to overcome problems of swelling hydrogels in the crosslinking bath and change the smooth alginate surfaces. In our work, we proposed to use a coaxial dispensing system. This system make possible to deposit a solid filament by the extruding at the same time the polymeric and the crosslinking solution. The special design coaxial needle included the inner core and outer shell was used to fabricate scaffolds with controllable speed of crosslinking [1]. To modify the structure of alginate poly-L-lysine (PLL) was used.

#### Characterization of 3D-Printed Keratin Hydrogels

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In tissue engineering, there is a need for biomimetic and biomaterial-based hydrogels with tightly controlled physical properties. Here we have developed a novel keratin-based 3D printing resin for the development of hydrogels for tissue engineering applications. Although methods exist to fabricate bulk keratin hydrogels, these typically are based upon self-assembly or the addition of dopants. Our methodology of manufacturing biomaterial-based hydrogels is based upon UV-crosslinking which will allow for the control of crosslinking density to modulate the hydrogel's physical properties. To investigate the effect of crosslinking density on the mechanical strength of the printed scaffolds, we developed three printing resins with varying concentrations of keratin. Here we utilized a lithography based 3D printer, a Perfactory 4 DLP printer, to fabricate the hydrogels in a layer-by-layer fashion with a step size of approximately 100 microns for characterization. We investigated the mechanical properties with compression testing and dynamic mechanical analysis (DMA) as well as determined the swelling ratio for these hydrogels. Furthermore, *in vitro* studies were carried out to determine the biocompatibility of these additive manufactured hydrogels. Future directions will include the fabrication of patient-specific hydrogels for wound healing applications.

#### Comparison of Bone Regeneration in Critical Size Calvarial Defects Utilizing Natural Materials and Growth Factors *In Vivo*

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Novel paste-like scaffolds incorporating natural materials such as demineralized bone, decellularized cartilage, hydroxyapatite, and hyaluronan, were used to bridge the gap in fracture healing mechanics to speed bone regeneration time. This technology can have an important impact in treating traumatic brain injuries (TBI). Current TBI treatments include removal of a portion of the calvarial bone to allow brain swelling. This method requires a secondary surgery to implant a plate over the defect site once brain swelling has decreased. Our paste-like material can be implanted with the initial surgery, providing a single-stage surgical intervention to treat TBI. The paste material is able to regenerate bone in critical size defects as well as be pliable during the brain swelling process. 8 mm calvarial defects were made in rat models and paste-like scaffolds were implanted containing a combination of ECM materials and BMP-2 or VEGF. Results were analyzed after 8 weeks using microCT to assess and quantify bone formation. Contrasting and reconstruction analysis are used to track newly formed bone within the defect site and bone that has formed and migrated out of the desired area. Results indicate better bone formation in VEGF containing groups compared to BMP-2 groups alone. Combinations of BMP-2 and VEGF show promising bone formation, highlighting the importance of angiogenesis for bone formation in critical size defects. Addition of ECM materials into groups containing BMP-2 only did not have accelerated bone growth comparatively. The bone regeneration rate shows promise for applications requiring tailored bone growth.

#### Gradient Hydrogel Scaffolds for Vascularized Tissue Formation

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Neovascularization (new vessel formation) is highly dependent on gradients of soluble (chemotactic) and immobilized (haptotactic) extracellular matrix signals as well as gradients of physical structure and mechanical properties (durotactic cues). While various engineering approaches have been exploited for the creation of gradients within 3D scaffolds, 3D cellular response has been primarily limited to gradients of a single factor. The creation of scaffolds with tunable gradients of physiologically relevant chemotactic, haptotactic and durotactic cues on 3D vessel assembly has yet to be explored. We have previously developed a photopolymerization technique (perfusion-based frontal photopolymerization (PBFP)) which allows for the creation of simultaneous gradients in immobilized RGD cell adhesion peptide concentration, protease-sensitive peptide crosslinks and elastic modulus in poly (ethylene glycol) (PEG) hydrogel scaffolds. These combined gradients have been shown to result in bidirectional vascular sprout alignment and invasion in the direction parallel to the gradient. The goal of this study was to decouple gradients in cell adhesion ligand concentration from elastic modulus in order to determine the specific role of individual and synergistic gradients on vascular sprout invasion *in vitro*. Manipulation of the composition, flow rate and duration of specific prepolymer components during PBFP allowed for the creation of hydrogel scaffolds with uniform swelling ratio ( $16.7 \pm 0.7$ ) and elastic modulus ( $\_$ ) but with gradients in immobilized RGD concentration ( $(318 \pm 24.8 - 369 \pm 35.6) \mu\text{M}$  over a length scale of 1 cm. Current studies are aimed at quantifying the role of individual and combined gradients on 3D vascular sprout invasion *in vitro*.

#### Osteoinductive Activity of Decellularized Bone Matrix Hydrogel with Controlled Protein Release for Bone Regeneration

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Hydrogel scaffolds derived from the extracellular matrix (ECM) of mammalian tissues have been used to promote constructive remodelling *in vivo*. Such scaffolds have the advantage of being delivered in a minimally invasive manner, have the bioinductive properties of the native matrix, and may be used to fill an irregular shaped space. The objective of this study were: (1) to determine the biological composition and osteoinductive properties of ECM hydrogels prepared from demineralized and decellularized bovine bone; (2) demonstrate the ability of bECM as a carrier for local delivery of proteins. Bovine bone was demineralized and decellularized as previously described [1]; the resultant bone granules were solubilized and gelation was induced by neutralizing the pH and salt concentration. Mouse primary calvarial cells (mPCs) and C2C12 mouse myoblast cells were cultured on the surface of hydrogels, osteogenic differentiation was analysed by qPCR, immunohistochemistry, alkaline phosphatase (ALP) activity, and alkaline phosphatase staining in both basal and osteogenic media. The release of fluorescently labelled HSA from ECM gels of different concentrations was measured over 2 weeks. Culture of C2C12 and mPCs on bECM and bDBM gels resulted in significant increases in expression of osteogenic markers ALP, osteopontin (OPN) and osteocalcin (OC) compared to cells cultured on collagen type I for both basal and osteogenic media. bECM hydrogels demonstrated stable release of protein; prolonging the release kinetics of proteins from hydrogels was dependent on the bECM hydrogel concentration.

1. M. J. Sawkins *et al* (2013), *Acta Biomater* 9: 7865–7873.

#### Matrix Stiffness Modulates Response to Chemotherapy in Breast Cancer Cells

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Despite advances in cancer therapy, development of chemoresistance is a major obstacle to improved patient outcomes. Unraveling the pathways leading to decreased drug sensitivity is vital to

developing novel therapeutic approaches to treating cancer. Current drug testing platforms based on cells cultured as monolayers do not take into account cell-stromal interactions which may affect drug sensitivity. In this context, 3-dimensional cell culture systems incorporating biological and mechanical cues are physiologically more representative of the tumor microenvironment, and thus, could help to better predict drug response. Our lab is especially interested in understanding how mechanotransduction from the extra cellular matrix (ECM) drives tumor cell chemoresistance and metastasis.

Towards this end, we have developed biomimetic 3D cell culture scaffolds made of electrospun polymeric composite nanofibers of  $\epsilon$ -polycaprolactone (PCL) and gelatin. Matrix elasticity and composition were modulated by varying the ratio of PCL and gelatin in the scaffolds. The effect of chemotherapeutic drugs (Paclitaxel and Doxorubicin) on the metabolic activity, apoptosis, and proliferation of breast cancer cell lines (MDA MB 231 and MDA MB 468) cultured on these scaffolds was evaluated. Increased tissue stiffness has been previously shown to correlate with tumor progression; however, the role of tissue rigidity in driving chemoresistance is not fully understood. Our results show that matrix stiffness can modulate response to drugs by altering expression of critical pathways implicated in chemoresistance. Further, such matrices could be used to better understand cancer progression and help design more effective therapeutics by targeting specific pathways activated during acquisition of chemoresistance.

#### Genetic Modification of Mesenchymal Stem Cells for Targeted Systemic Cell Therapy in Critical Limb Ischemia

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Mesenchymal stem cells (MSCs) have a number of properties which suggest that they may be good candidates for the treatment of diseases characterized by tissue ischemia such as critical limb ischemia (CLI). They have the capability to regulate immune and inflammatory responses and are able to secrete angiogenic factors that stimulate the formation of new blood vessels.

One of the major challenges in cell based therapies for CLI is the bio-distribution and survival of transplanted cells, often less than 5% of the transplanted cells are retained in the target tissue. Another challenge is the route of intervention, most CLI trials use the intramuscular route of cell delivery which requires multiple painful injections to the ischemic calf/thigh muscle.

The aim of this project was to develop a next generation cell based therapy for CLI where the therapeutic product can be delivered intravenously and the cells are modified to improve their migration to target tissue and to yield therapeutic angiogenesis.

In this study we have generated lenti virally transduced MSCs over expressing CCR2 which show enhanced migration towards a chemokine, MCP-1 *in vitro*. We have demonstrated that this genetic modification of MSCs does not alter their stem cell properties and *in vitro* angiogenic potential. The *in vivo* therapeutic and pro migratory potential of CCR2 over expressing MSCs are being investigated in a mouse model of hind limb ischemia.

#### Creating Universal Donor Stem Cells for Tissue Engineering Applications

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We hypothesized that the immunogenicity of human ESCs and their derivatives can be significantly decreased by constitutive expression of shRNA against beta-2-microglobulin (B2M), the invariable non-transmembrane 12 kDa conserved light chain of HLA class I molecules. This approach can diminish the assembly and

presence of functional HLA-I molecules on the cell surface without impairing the cell's ability to differentiate into specific cell phenotypes; thus, making them and their differentiated derivatives less susceptible to recognition by allogeneic T-lymphocytes. Decreased expression of B2M was confirmed using two different primers mapping two different regions on the B2M gene. FACS showed the diminished presence of assembled HLA-I on the cell surface. Transduced cells did not lose their ability to develop into functionally active cardiomyocytes and were used to create self-contracting engineered heart tissue constructs. The expression levels of co-stimulatory molecules including CD80, CD81, CD83, CD86, FAS and FAS Ligand did not change in B2M depleted cells. Mixed lymphocyte reaction assay confirmed that transduced cells, either in undifferentiated or differentiated state largely escaped recognition by allogeneic T-lymphocytes. We conclude that stable shRNA B2M depletion is a promising approach to create a universal donor ESC that can be used to produce either cell grafts or engineered tissue constructs with improved survival and/or minimal need for co-administration of immunosuppressive drugs.

### Bioengineering Three-dimensional Culture Model of Human Lung Cancer Cells for Screening EGFR Targeted Inhibitors

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Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related death in the world, while no effective therapy has been established to significantly improve the overall 5-year survival rate of the patients. This might mainly be due to the lack of more accurate models recapitulating those *in vivo* biological events. To address this issue, here we developed a more pathologically-relevant, three-dimensional culture model of NSCLC through encapsulating immortalized A549 cells within alginate-calcium (AC) microsphere. We hypothesized that A549 cells growing in the microenvironment provided by AC microsphere would not only generate tumor cultures closely resembling *in vivo* tumor morphology, but also contribute to producing more defined NSCLC culture model that potentially enhances targeted drug evaluation. Our results showed that the encapsulated A549 cells proliferated and generated aggregates during the cultivation. Histological analysis revealed their good viability and cell junction formation, suggesting their more tissue-like phenotype compared to the monolayer cultures. A significantly increased expression of marker genes (EGFR *et al.*) was observed in the encapsulated cultures, which was consistent with their protein expression pattern by western-blotting. An enhanced drug resistant potential of the encapsulated cultures was also found evidenced by their increased IC<sub>50</sub> values of several chemotherapy drugs and target drugs when compared to the monolayer cultures. Thus, this encapsulated NSCLC culture system effectively represents an improved culture model of human lung cancer *in vitro* and might potentially provide a robust tool for target drug screening.

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### Regulation of Inflammation and Degradation of Decellularized Vascular Grafts by Crosslinking with Branched Peg

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Decellularized tissues are good tissue regeneration materials and expected to be degraded and substituted by host tissue *in vivo*. We have developed the small-diameter decellularized vascular graft by using xenogeneic tissues. The xenografts are generally cross-linked with glutaraldehyde (GA) to minimize the immune response and inhibit the matrix degradation. However, GA-crosslinked

decellularized tissue completely lose the degradation property. Moreover, it is widely known that the GA crosslinking induces a calcification. To overcome this problem, we have developed a new crosslinking agent for decellularized vascular graft. Branched polyethylene glycol activated with succinimidyl ester were designed. When the tissue was crosslinked with branched PEG, the tissue was gradually degraded in a collagenase solution (0.75 mg/ml) although the unmodified tissue was rapidly decomposed. On the other hand, the GA crosslinked tissue was not degraded under the same condition. The degradation profile was confirmed by stress-strain curve and small-angle X-ray scattering at Spring 8. When the grafts were crosslinked with the branched PEG and transplanted into subcutaneous tissue of rats, no infiltrated CD68-positive cells were observed after one week transplantation. This tendency was same as GA-crosslinked tissue. Calcification was also suppressed by the branched PEG crosslinking treatment. From these results, inflammation and degradation profile of the decellularized tissue can be effectively regulated by the crosslinking with branched PEG.

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**Disclosures:** The authors declare that there is no conflict of interests regarding the publication of this research.

### Angiogenic Characterization of Allograft Cellular Bone Matrix T. Moseley;

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**Objective:** To characterize the angiogenic properties of commercially available cellular bone allografts consisting of viable cancellous bone retaining mesenchymal stem cells (MSCs) and osteoprogenitor cells<sup>1</sup> combined with demineralized bone matrix (DBM). These allografts mimic the healing mechanisms intrinsic to autologous bone grafts<sup>2</sup>, including osteoconduction, osteoinduction, and osteogenesis.

**Methods:** Cryopreserved cellular bone matrix (Osteocel<sup>®</sup>, NuVasive) obtained from the tissue processor was thawed, rinsed with PBS, and placed into explant culture for 24 hours. RNA was extracted from the cells via homogenization and purification (RNeasy<sup>®</sup> Mini, Qiagen<sup>®</sup>). Gene expression patterns were compared to those found in RNA from primary human adult fibroblasts (Cell Applications, Inc.) using osteogenesis qPCR arrays (Qiagen). Additionally, a retrospective analysis of 581 consecutive released lots of Osteocel was conducted to determine the cell viability and yield (by enzymatic release and Trypan Blue dye exclusion) immediately upon thawing.

**Results:** Each cc of graft contained an average of 3 million viable cells with total viability of 86.5%. Gene expression of angiogenic factors from cellular allograft bone were upregulated over human fibroblasts: Platelet-derived growth factor was up 54.2×; endothelial growth factor was up 30×; fibroblast growth factor was up 10.2×; and vascular endothelial growth factor was up 5.8×.

**Conclusions:** Cells within allograft cellular bone matrix expressed high levels of angiogenic genes relative to fibroblasts, complementing previous evidence of an overall upregulation of osteogenic genes relative to bone marrow-derived MSCs. The data indicate that the cellular components contribute to the angiogenicity of the cellular bone matrix which may help drive bone formation.

### The Bone Marrow-Like Tissue *In Vitro*: Rapid Aggregation and Reconstruction of Dispersed Bone Marrow Cells

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The bone marrow (BM) is known as the location of hematopoiesis, and the function is based on three-dimensional (3D) microenvironments (hematopoietic niches) in the BM. Reconstruction of the 3D BM *in vitro* will help the understanding of the mechanisms of hematopoiesis and the BM disease. However, it was difficult in previous aggregation methods because more than 90% of the BM cells are non-adherent blood cells. The objective of in this study is

establishment of a method to reconstruct the dispersed BM cells. In previous work, we reported a rapid cell aggregation system with methylcellulose (MC) medium [1]. This method can make 3D aggregation state even using non-adherent materials. With the method, dispersed BM cells were rapidly gathered and organized as the BM-like tissues within 1-day culture. The BM-like tissues were possible to be removed from the MC medium with retaining the original shape. By hematoxyline-eosin staining, the BM-like tissues maintained blood cells at least 3 days. Macrophage existed in the BM-like tissues at the same frequency of the BM tissue. The presences of PDGFR $\alpha$ <sup>+</sup>CXCL12<sup>+</sup> cells playing a critical role in reticular niche are also confirmed. These data showed that the BM-like tissues were able to be fabricated by using the MC medium.

1. Kojima N, Takeuchi S, Sakai Y. Rapid aggregation of heterogeneous cells and multiple-sized microspheres in methylcellulose medium. *Biomaterials*, 33, 4508–4514, 2012.

### A Tissue Engineering Model to Study Breast Cancer Cell Dormancy in the Bone Marrow

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As chemotherapy fails to target dormant breast cancer cells (BCCs) in the bone marrow, three-dimensional, bioactive scaffolds that mimic the bone marrow/bone microenvironment are needed as both *in vitro* and *in vivo* models to study BCC dormancy. The majority of studies use *in vitro*, two-dimensional monolayer cultures, which do not recapitulate the *in vivo* microenvironment. Therefore, to determine the effect of the 3-D microenvironment on BCCs, this study used electrospun scaffolds made of poly( $\epsilon$ -caprolactone) (PCL) and composites consisting of PCL with uniform dispersion of nano-hydroxyapatite (HA), having aligned or random fibers. Aligned and random fibers mimic the highly organized and random collagen fibers, respectively, found in the bone matrix. Chemo-resistant BCCs, identified by cell viability and western blot analysis, showed an increase in Bcl-2, Oct-4 and Sox-2, suggesting protection from apoptosis and increase in stem-like markers. Both chemoresistant (treated) and non-treated BCCs were evaluated on the scaffolds, specifically for attachment, growth, cell cycle analysis, apoptosis proteins and stem-like markers. BCCs adhered to the scaffold fibers after 7 days. Little to no change in cell number occurred over time for non-treated BCCs on scaffolds and they also expressed stem-like markers whereas on tissue culture polystyrene (TCP), non-treated BCCs had a significant increase in cell number and metabolic activity over time ( $p < 0.05$ ). Treated BCCs did not change on TCP and the fibrous scaffolds. The 3-D scaffolds may be a useful model to examine the behavior of BCCs.

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### Efficient Differentiation of human iPS cells to Pancreatic $\beta$ -cells by Optimizing Oxygen Supply through Silicone Membrane Plates

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The ability of iPS cells to differentiate into desired cell type can be used for the treatment of type 1 and type 2 diabetes by developing pancreatic  $\beta$ -cells. Physiological environment and physico-chemical factors highly influence the differentiation. Therefore, from a biochemical viewpoint, we investigated the effect of direct and indirect oxygenations on differentiation of iPS cells to  $\beta$ -cells using poly

(dimethylsiloxane) (PDMS) and tissue culture-treated polystyrene (TCPS) plates. Formation of 5–6 and 1–2 cell layers were observed for direct oxygenation with PDMS and indirect oxygenation with TCPS, respectively. Per-cell gene expression levels of pancreatic progenitor marker PDX1 and  $\beta$ -cell marker INS were 2.5 fold higher in 10% direct oxygenation compared with 20% indirect oxygenation. Measured oxygen tensions at the cellular level in PDMS with 5, 10, and 20% oxygenations were 3.7, 8.2, and 16.9%, respectively. But in TCPS, the measured oxygen tension was much lower than at the ambient conditions. Moreover, cellular oxygen consumption was satisfied by PDMS at 5% oxygenation, whereas even 20% oxygenation was not enough to meet the cellular oxygen demand in TCPS. The improved oxygenation at lower oxygen concentrations through PDMS reduced the oxidative stress to the cells and enhanced the production of pancreatic  $\beta$ -cells per unit culture surface area. Thus, differentiation with direct oxygenation at lower oxygen concentrations is promising for 2D plate-based mass production of pancreatic  $\beta$ -cells. Additionally, it can be utilized for growing thick pancreatic  $\beta$ -cell tissues that can enhance the number of the cells to the same implantation area.

### Metabolic Demands in Early Tumors Drives Metastasis Facilitating Changes in Tissue Engineered Microvasculature

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As a primary tumor mass proliferates, it facilitates vascular changes to fulfill an ever increasing metabolic demand. The demand for blood flow of tumors is particularly high due to enhanced proliferation and glycolytic cellular respiration. Overactivity of the Wnt signaling pathway is a primary driver of colon carcinogenesis and leads to glycolytic metabolism of cancer cells. We hypothesized that an early glycolytic avascular tumor mass (<10,000 cells, equivalent to a tumor of diameter of 200  $\mu$ m) can initiate angiogenesis before reaching a size that limits oxygen diffusion. To test this hypothesis, we designed a model of the tumor microenvironment in a microfluidic device that allows growth of a tumor spheroid and a vascular network in close and controlled proximity. The microfluidic platform also allows precise delivery of concentration gradients of exogenous morphogens. We created tumor spheroids from SW620 colon cancer cells, which have elevated canonical Wnt/ $\beta$ -catenin signaling as well as genetically altered SW620 cells in which Wnt/ $\beta$ -catenin regulation of target genes was reduced. When the microvasculature in the device was exposed to VEGF and lactate, the main proangiogenic factors produced by glycolytic SW620 cells, angiogenesis was directed towards positive gradients of these factors. When tumors were grown in close proximity to the vascular tissue, wild type tumors facilitated more changes in vascular integrity and angiogenesis than tumors with genetically reduced Wnt signaling. Our results indicate that tumors in early developmental stages could initiate vascular changes mediated by glycolytic metabolism.

### Tissue Engineered Artificial Stromas for Abdominal Wall Repair: an *In Vivo* Study in Laboratory Animals

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**Background:** Several surgical meshes have been used for the surgical management of abdominal wall defects such as hernia or eventration. However, this therapeutical approach has some clinical disadvantages, including poor biocompatibility, inflammatory response and fibrosis, which may lead to intestinal adhesences and other complications. The objective of this work is to develop a more biocompatible bioengineered mesh for *in vivo* use.



**Methods:** Two surgical meshes were investigated: a partially degradable polyglycolic-polypropylene mesh (PGA-PP) and a non-degradable polypropylene (PP) mesh. In addition to the native meshes, a model of tissue-engineered artificial stroma was developed by including the PGA-PP and PP meshes in fibrin-agarose hydrogels (FAH) containing rat fibroblasts. The four types of meshes were grafted in the abdominal wall of Wistar rats and histological studies were performed after 2 and 4 weeks.

**Results:** Association of FAH with both surgical meshes resulted in less inflammation ( $p < 0.001$ ) and less fibrosis ( $p < 0.001$ ) as compared to surgical meshes alone, especially at 2 weeks. No differences were found between PGA-PP-FAH and PP-FAH. Collagen synthesis around the mesh fibers was lower in PGA-PP-FAH and PP-FAH than in the PGA-PP and PP animals, with statistically significant differences after 4 weeks ( $p = 0.021$  for PGA-PP and  $p < 0.001$  for PP).

**Conclusions:** These results support the use of FAH artificial stromas associated to the surgical meshes in order to reduce inflammation and fibrosis at the host site.

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### CRISPRi Immunomodulation for Tissue Engineering/Stem Cell Therapies Targeting Intervertebral Disc Degeneration

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Degenerative disc disease (DDD) is considered a major contributor to lower back pain (LBP), the single leading cause of disability worldwide. Progression of DDD has been associated with the presence of inflammatory cytokines in the intervertebral disc (IVD), and the restoration of the diseased disc has been of great interest in tissue engineering/stem-cell therapy. The inflammatory cytokines present provide a deleterious environment to tissue engineering strategies that target the regeneration of the IVD. We are interested in engineering the cell response of inflammatory cytokines by regulating pro-inflammatory cytokine receptor profiles via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems. We are using CRISPR interference (CRISPRi), a system shown to be highly specific and effective for gene regulation in mammalian cells. The objective of this research is to engineer the inflammatory response in engineered IVD tissue/cells through CRISPRi of type I tumor necrosis factor receptor (TNFR1) and Interleukin I receptor type I (IL1R1).

Through quantitative reverse transcriptase PCR we have demonstrated that CRISPRi can be used to knockdown TNFR1 and IL1R1 expression by 99% in HEK293T cells, and TNFR1 (80%) in human IVD cells. In order to determine our ability to reduce the inflammatory response of a cell after CRISPRi receptor knockdown, we dosed CRISPRi engineered HEK293T cells with TNF- $\alpha$  and observed significant reductions ( $\geq 70\%$ ) in NF- $\kappa$ B activity at all doses of TNF- $\alpha$  post CRISPRi. These results demonstrate the efficacy and great promise of engineering the inflammatory response in engineered tissues and cells via CRISPRi regulation of inflammatory receptors.

### Repair of Large Osteochondral Defects in Weight-bearing Area with *in vitro* Regenerated Cartilaginous Implants Based on BMSCs and PGA Scaffolds in a Porcine Model

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Previously we reported successful repair of porcine articular osteochondral defects by bone marrow stromal cells (BMSCs) combined with polyglycolic acid (PGA) scaffolds, but the overall efficacy

was compromised by cell leakage, poor mechanical property, and immune reactions toward the PGA remnants – problems usually associated with cell-scaffold constructs. We propose *in vitro* regeneration would address these issues. However, it is still unknown what would be the optimal duration for *in vitro* chondrogenesis, and whether the *in vitro* regenerated implants can repair both chondral and subchondral regions of large osteochondral defects in weight-bearing area. Therefore, in the current study, porcine autologous BMSC-PGA constructs were chondrogenically induced for 2 w, 4 w, and 8 w respectively before implantation into articular osteochondral defects (12 mm in diameter) in weight-bearing area. The results demonstrated that: before implantation, samples in 2 w group displayed little cartilaginous features with abundant PGA remnants, while the 4 w samples formed more mature cartilaginous tissue with less PGA remnants, and the 8 w samples formed the most mature cartilaginous tissue with proper mechanical property and the least PGA remnants. At 6 m post-implantation, samples in 4 w and 8 w groups repaired the osteochondral defects with cartilaginous tissue and bone, while most of the 2 w samples displayed fibrous repair with signs of inflammation. These results indicated that *in vitro* chondrogenesis for 4–8 weeks can significantly improve the repair outcome of large osteochondral defects in weight-bearing area for BMSC engineered implants. In fact, we have recently translated this strategy in clinical settings and achieved satisfactory outcomes for patients with osteochondral defects.

### Instructive Biomaterial Platform to Engineer Hematopoietic Stem Cell Activity

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The bone marrow microenvironment provides a complex set of extrinsic signals that regulate hematopoietic stem cell (HSC) behavior, hence the production of the body's full complement of blood and immune cells. An artificial marrow would have significant value for expanding HSCs or to study the onset and treatment of hematologic diseases. However, the influence of matrix biophysical properties and cell-cell signaling remains poorly understood. We describe integration of a 3D methacrylamide-functionalized gelatin (GelMA) hydrogel with a previously described microfluidic templating approach to present multiple instructive cues to investigate the impact of spatially-graded biomolecular signals on HSC quiescence vs. activation. Selective inclusion of matrix-immobilized SCF promotes improved retention of primitive HSCs within the matrix, while local patterning of stem cell factor (SCF) across the biomaterial was able to promote regionally-specific HSC retention. Further, while co-culture of HSCs with Lin+ niche cells stimulated selective lineage specification, the biomaterial matrix was able to locally modulate this effect, suggesting that biomaterial design could generate local regions within the artificial bone marrow which promote paracrine signaling versus autocrine feedback. Mesenchymal stem cells (MSCs) represent a second critical niche component, hypothesized to promote HSC quiescence and self-renewal but also capable of remodeling the biomaterial matrix, which may significantly affect long-term HSC cultures. Ongoing efforts are examining individual and co-cultures of HSCs and MSCs in the GelMA hydrogel-based artificial bone marrow in order to examine MSC-mediated matrix remodeling and resultant long-term expansion of primitive HSCs as a function of MSC co-culture and local presentation of matrix-immobilized biomolecules.

### The Analysis of pH Effect on Xanthan Hydrogels

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Hydrogels, macrogels and nanogels represent a class of three-dimensional hydrophilic polymeric networks, formed by physical and/or chemical crosslinking. Due to their unique properties hydrogels have a wide application range in various fields, particularly in

biocompatibility, non-toxicity and inertness. They are used as scaffolds for tissue engineering as well as drug carriers for controlled release drug delivery. They are also used for separation of biomolecules or cells. Xanthan is anionic in natural form due to the presence of pyruvic and glucuronic acid groups in its side chain. In this study, it was aimed to obtain Xanthan hydrogels by means of high temperature heating esterification reaction, where citric acid is used as cross linker. This study analyzed the pH-dependent swelling and mechanical specifications of Xanthan hydrogels. Thermo gravimetric analysis was done using Shimadzu DTG-50 thermal analyzer. FTIR spectra of uncross-linked and cross-linked Xanthan hydrogel particles was observed in the range of 400 up to 4000  $\text{cm}^{-1}$ . The effect of varying pH on surface morphology of hydrogels was analyzed using Scanning Electron Microscopy. As a results, hydrogels significantly swelled under high pH values and shrank under low pH. The varying pH also effected the size of pores on the surface of hydrogels. An increase in pore size of hydrogels was reported as the pH increased and the vice versa. The pH-related structural changes emphasized a promising future for hydrogels in the areas of regenerative medicine, tissue engineering and controlled drug delivery.

### Primed 3D Injectable Micro-Niches as Cell Delivery Vehicle and Enabling Low-dosage Cell Therapy for Critical Limb Ischemia

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The promise of cell therapy for repair and restoration of damaged tissues or organs relies on administration of large dose of cells whose healing benefits are still limited and sometimes irreproducible due to uncontrollable cell loss and death at lesion sites. Using a large amount of therapeutic cells increases the costs for cell processing and the risks of side effects. Optimal cell delivery strategies are therefore in urgent need to enhance specificity, efficacy, and reproducibility of cell therapy leading to minimized cell dosage and side effects. Here, we addressed this unmet need by developing injectable 3D micro-scale cellular niches (microniches) based on biodegradable gelatin microcryogels (GMs). The microniches are constituted by *in vitro* priming hMSCs seeded within GMs resulting in tissue-like ensembles with enriched extracellular matrices and enhanced cell-cell interactions. The primed 3D microniches facilitated cell protection from mechanical insults during injection and *in vivo* cell retention, survival, and ultimate therapeutic functions in treatment of critical limb ischemia (CLI) in mouse models compared with free cell-based therapy. In particular, 3D microniche-based therapy with 105 hMSCs realized better ischemic limb salvage than treatment with 106 free-injected hMSCs, the minimum dosage with therapeutic effects for treating CLI in literature. To the best of our knowledge, this is the first convincing demonstration of injectable and primed cell delivery strategy realizing superior therapeutic efficacy for treating CLI with the lowest cell dosage in mouse models. This study offers a widely applicable cell delivery platform technology to boost the healing power of cell regenerative therapy.

### Immune Response to Poly(Propylene Fumarate) and Pericardium ECM-Based Biohybrid Material

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The goal of this work is to design an ECM-based biohybrid material as a lasting replacement for cardiovascular prosthetics. ECM-based biohybrid materials are hypothesized to promote a prohealing immune response over chemically cross linked alternatives. An estimated half of all heart valve replacements use tissue based prosthetics, which are

widely manufactured with glutaraldehyde (GA) treated bovine pericardium despite detrimental calcification and unnatural healing of the implant. We have shown that a thin layer of the biocompatible polymer poly(propylene fumarate) (PPF) coated on pericardium protects against enzymatic degradation without significant alterations to the tissue. This current work investigates the inflammatory response and remodeling activity initiated by the biohybrid material in comparison to the GA-treated alternative. The biohybrid material was implanted in a subcutaneous model in Sprague Dawley rats, and evaluated at 3 and 6 weeks for matrix organization and cellular inhabitation. Local immune cell populations were characterized and quantified with immunohistochemical staining, and recruiting cytokines were evaluated with an ELISA. These metrics were compared to GA-treated and untreated pericardium. To isolate the significance of an adapted immune reaction to the xenographic tissue, the experiment was repeated in athymic rats. *In vitro* experiments were also conducted to assess activation of immune cells in response to the tissue implants. The moderate macrophage activation, cell infiltration, and matrix remodeling of PPF-reinforced tissue (compared to controls) suggests that the polymer acts to mitigate an intense acute inflammatory response so as to promote controlled remodeling and viable cell ingrowth, which would contribute to a sustainable implant.

### Preclinical Study of Grafted Human Adipose Substitutes using Magnetic Resonance Imaging

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Soft tissues are needed for reconstructive surgery procedures and tissue engineering represents a promising approach for the production of readily available human adipose tissue (AT) substitutes. Preclinical studies are needed to investigate the behavior of *in vitro* reconstructed adipose tissues (hrAT). Magnetic resonance imaging (MRI) is one of the most powerful modality to image fat depots non-invasively. We hypothesized that the volume and blood perfusion of hrAT engineered *in vitro* and then grafted subcutaneously to athymic mice could be precisely determined by MRI. Grafted hrAT were clearly visualized by MRI (1 Tesla, Aspect Imaging), enabling volume delineation. Their mean initial volume was  $160.2 \pm 34.4 \text{ mm}^3$  for a mean surface of  $1.41 \pm 0.36 \text{ cm}^2$ . The graft volume retention was followed weekly, revealing a gradual resorption rate averaging at 65% and 44% of initial substitute's volumes at three and six weeks after grafting, respectively. Upon harvest, the grafted tissues revealed an abundance of adipocytes and the presence of blood vessels, without evidence of cysts or necrosis. Vascular perfusion measured by dynamic contrast-enhanced (DCE)-MRI using the Gadomer 17 contrast agent confirmed the graft's vascularization 14 and 21 days post-implantation. Preliminary results indicate that at those time-points, the perfusion of hrAT possessing an *in vitro* preformed network of capillaries was observed but was not significantly different from the hrAT that were not enriched in endothelial cells. These studies establish that MRI is a powerful non-invasive modality that would benefit preclinical studies of grafted human AT substitutes. Supported by CIHR.

### Artificial Microenvironment of Decellularized Bone Marrow Induced Hematopoiesis

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Clinical use of blood donations benefits many patients. The constructing and utilizing an artificial blood producing system *ex vivo* for

alternative blood donation is one of paramount goals. For realizing this system, the reconstruction of hematopoietic stem cell (HSC) niche is important for controlling the activity of HSCs. The niche is consisted of several supporting cells and extracellular matrix (ECM). Here, we focused on the ECM as a scaffold for hematopoiesis and constructed the microenvironment using the decellularized cancellous bone (DCB) that was prepared by our original decellularization method. In this study, we investigated the hematopoiesis in the DCB implanted into mouse ectopically. We hypothesized that the appropriate matrix for hematopoiesis would induce HSCs homing and blood producing even though they were located ectopically. In order to investigate the HSCs homing and blood producing in DCB, we evaluated the neovascularization and the localization of HSCs and hematopoietic progenitor cells in DCB. From the results of flow cytometry and colony forming cell assay, the homing of long term-HSCs was confirmed. It was also suggested that the hematopoietic cells produced by homed HSCs were migrate to peripheral blood and mice original bone marrow. In addition to the long term-HSCs homing, the histology of reconstructed bone marrow was similar to original bone marrow. It suggested that artificial microenvironment of DCB navigated the behavior of infiltrated cell in order to provide the HSC niche. The combination of this bone marrow ECM and cells *ex vivo* would be applied artificial blood producing system.

#### **Biocompatibility and Functional Rat Femoral Bone Fracture Healing Response of Modified Resorbable ZK40 Alloy**

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Ti or stainless steel (SS) pins are widely used for small bone fracture fixation often to augment external fixation hardware requiring replacement due to complication related loosening. Magnesium (Mg), naturally present in bone, has been investigated as a resorbable metal along with new alloys displaying higher mechanical strength than degradable polymers. In the current study, the ZK series following our publication has been modified (M-ZK40) to satisfy the unmet clinical needs in small animal bone fixation model. To demonstrate the biocompatibility and load bearing functional bone response M-ZK40, pins were inserted into the rat intramedullary space to repair the femoral defect using Ti-alloy pins as the negative control. After 2, 8, and 14 weeks,  $\mu$ CT imaging of harvested femurs showed 87, 34, and 18 percent of the original volume of the M-ZK40 pins, respectively. Degradation of M-ZK 40 pins was significantly accelerated in the load-bearing implantation site compared to *in vitro* results. Histology of embedded femurs, using trichrome and toluidine staining method, still exhibited normal bone healing response, and no significant difference was observed between M-ZK40 and Ti-alloy group. Elemental analysis of liver and kidney, using an inductively coupled plasma (ICP-OES), presented no accumulation of degradation products in liver and kidney of the M-ZK 40 group compared to the Ti-alloy group. No sign of any inflammatory response was also observed in H&E staining of liver and kidney sections. Results thus demonstrate that resorbable M-ZK40 Mg alloy maintained good biocompatibility and bone response when exposed to load-bearing environments.

#### **A Macrophage-Fibroblast Co-Culture, as an *In-Vitro* Tool to Predict the *In-Vivo* Behavior of a Degradable Vascular Graft**

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**Aims:** Despite of the intensive research in vascular graft development, an efficient small diameter vascular graft is still unavailable for clinical use. A synthesized thermoplastic polyurethane (TPU) vascular grafts were fabricated via electrospinning. Our aim was to develop a macrophage/fibroblast co-culture model as a predictive tool to elucidate the inflammatory responses of the host to the graft prior to implantation.

**Methods:** The structural and mechanical properties and viability of the cells were studied. The inflammatory behavior of the TPU grafts was compared to those of expanded polytetrafluoroethylene (ePTFE) grafts *in-vitro* and *in-vivo* (after 1 week and 1 month implantation into the infrarenal aorta of rat models). Gene expression of M1 and M2 marker of Macrophages (CD68, CD80, CCR7 and CD163) and pro- and anti-inflammatory cytokines (TNF $\alpha$ , IL-1 $\alpha$ , IL-10) were investigated via PCR, *in-vivo* and *in-vitro*. cell distribution/confocal microscopy studies were carried out *in-vitro* and *in-vivo*.

**Results:** The porous structure of the TPU grafts supported the cell attachment, viability and growth more than the ePTFE grafts. The expression level of anti-inflammatory, CD163 macrophages and IL-10 cytokine were significantly higher in TPU compared to ePTFE grafts *in-vitro* and after one week and one month implantation *in-vivo*. The expression of pro-inflammatory cytokines (TNF $\alpha$  and IL-1 $\alpha$ ) was higher in ePTFE grafts in *in-vitro* and *in-vivo* studies. TPU grafts revealed notable early cellular ingrowth *in-vivo* compared to ePTFE.

**Conclusions:** Results confirmed the superior healing and anti-inflammatory characteristics of TPU grafts compared with ePTFE grafts in the *in-vivo* and *in-vitro* experiments.

#### **Interplay between Macrophages and Human Endometrial Stromal Cells on Mesh Performance in a Mouse Model**

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**Introduction:** Pelvic Organ Prolapse (POP) is the herniation of pelvic organs into the vagina. Despite broad acceptance of mesh use in POP surgical repair, the complication rate is unacceptable. Human endometrial mesenchymal stem cells (eMSC) are a new source of MSC which can be purified using SUSD2 antibodies. The anti-inflammatory properties of eMSC are poorly understood. In this study we aimed to develop a method for tracking eMSC delivered on novel polyamide/gelatin (PA+G) mesh to examine their modulatory role on the macrophage responses in immunocompromised (NSG) mice.

**Methods:** SUSD2+eMSCs were isolated from endometrial biopsy by magnetic beads sorting. A mCherry lentiviral plasmid was used to permanently label eMSC. Transduction efficiency was determined by FACS and mCherry/SUSD2+eMSC were seeded onto PA+G mesh and implanted in a defect made between the skin and abdominal fascial layer in mice. Tissues were harvested at 7 and 14 days. Immunohistochemical assessment of the macrophage response used F4/80 (Pan Marker) and Arginase (M2).

**Results:** More than 50% of eMSC expressed mCherry. A small number of mCherry+eMSC were identified in mouse tissue after 7, but not 14 days. Large numbers of macrophages was seen round mesh filaments at both time-points but <5% were arginase+.

**Conclusion:** This study shows that human eMSC can be transduced with a lentiviral construct for *in-vivo* tracking. The low percentage of M2 macrophages around mesh filaments suggests that most were of the M1 inflammatory phenotype. Ongoing studies will evaluate the interaction between eMSCs and macrophages associated with the foreign body response.

### Physiologically Trained Cardiac Adipose Tissue Derived Progenitor Cells within Fibrin Scaffolds to Improve Cardiac Function

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Cardiac cells are subjected to mechanical and electrical forces. Therefore, *in vitro* electromechanical stimuli could benefit further integration of therapeutic cells into the myocardium. Our goals: study the viability of a tissue engineered construct with cardiac adipose tissue-derived progenitor cells (cardiacATDPCs); and examine the effect of electromechanically stimulated cardiacATDPCs within a myocardial infarction model.

CardiacATDPCs were electromechanically stimulated, harvested and labelled to generate the 3D fibrin construct. The stimulation protocol: 2 ms pulses of 50 mV/cm at 1 Hz and 10% stretching during 7 days. The construct was implanted in the murine heart and animals were sacrificed at 3 weeks. 40 animals were randomly distributed: without cells, and with stimulated or non-stimulated cells. Echocardiography, gene and protein analysis were also carried out.

*In vitro* electromechanical stimulation on cardiacATDPCs showed increased expression of main cardiac markers. After 3 weeks of 3D culture, cells exhibited high viability and remained labelled. Cell treatment resulted in functional improvement of left ventricular ejection fraction relative to post-infarction values; indeed, stimulated cardiacATDPCs produced a 4.7% average increment compared to non-stimulated cells. Moreover, treated animals evidenced a 10% higher vessel cell density than non-treated animals. Finally, histology showed cell proliferation and key cardiac markers expression of implanted cardiacATDPCs, but also scarce migration to the myocardium.

The electromechanical stimulation protocol designed enhances cardiac properties of therapeutic cells. The construct used confers a suitable environment for cell viability, proliferation, cardiac maturation and migration to infarcted myocardium. All together, electromechanical stimulation of therapeutic cells previous implantation could be a valuable tool for cardiac regeneration approaches.

### The Effect of Terminal Sterilization on the Material Properties and *In Vivo* Remodeling of a Porcine Dermal Biologic Scaffold

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Biologic scaffold materials are regulated by the FDA as medical devices and are therefore subject to established sterilization methods and guidelines prior to clinical use. Although device processing methods including the method of terminal sterilization are known to dramatically affect the host tissue response, there is a paucity of quantitative data upon which to make educated decisions. Common methods of terminal sterilization of biologic scaffolds include electron beam (e-beam) and gamma ( $\gamma$ ) irradiation and ethylene oxide (ETO); however each method will alter material properties and ultimately the *in vivo* remodeling response. Currently, no consensus exists for an effective yet minimally-destructive sterilization protocol for porcine dermal biologic scaffolds which can preserve structure, mechanical properties, and ability to facilitate constructive tissue remodeling. The objective of the present study was to characterize the effect of several types (ETO, e-beam, &  $\gamma$ ) and doses (10, 25, & 40 kGy) of terminal sterilization on the material properties and *in vivo* remodeling response of porcine dermal biologic scaffolds *in vitro* and in an *in vivo* rodent model. Higher doses of either e-beam &  $\gamma$  irradiation were found to elicit marked ultrastructural changes in

the collagen fiber architecture, decrease mechanical properties, augment the macrophage polarization response and increase the rate of degradation. However, lower doses of terminal sterilization can be used with minimal effects upon the structure and function of the materials. These findings provide insight for selection of an appropriate type and dose of sterilization for biologic scaffold materials to optimize clinical outcomes.

### Induced to Cure: Engineering iPS Cell Derived RPE Scaffolds to Treat Degenerative Eye Diseases

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The recent success with embryonic stem (ES) cell derived retinal pigment epithelium (RPE) has provided hope for a cure for degenerative eye diseases. Induced pluripotent stem (iPS) cells are an autologous source of stem cells potentially with fewer immune-challenges as compared to ES cells. Using a developmentally guided differentiation protocol we have developed fully polarized RPE tissue from iPS cells. The RPE monolayer along with its secreted ECM and a degradable scaffold form a tissue that mimics the native tissue in structural and functional properties. This tissue performs several key RPE functions like phagocytosis of photoreceptor outer segments, ability to transport water from apical towards basal sides, and the ability to secrete cytokines in a polarized fashion. Currently, we are testing the safety and the efficacy of this tissue in animal models. We have begun Phase I Investigational New Drug (IND) enabling studies with the goal to transplant autologous iPS cell derived RPE in patients in advanced age-related macular degeneration (AMD), one of the leading blinding diseases in the US. We propose to develop National Institutes of Health Clinical Center as an "alpha-stem cell clinic" with the capability to recruit patients, manufacture clinical-grade autologous iPS cell derived RPE tissue, perform transplantation, do patient care, and be able to transfer technology to academic and private sectors. We suggest that our open access model with complete access to the entire IND package will reduce redundant efforts in the field, foster public-private partnerships, and help move ocular regenerative medicine field forward.

### Evaluation of Poly (carbonate-urethane) Urea (pcuu) as a Urinary Bladder Tissue Engineering Scaffold

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Despite early promises of bladder tissue engineering, a recent report of unsuccessful clinical trials (Joseph DB, 2014) suggests that the technology needs further improvement and evaluation through animal models of bladder dysfunction (Sloff M, 2014). The goal of the present study was to evaluate electrospun fibrous PCUU scaffolds in a rat model of bladder outlet obstruction (BOO). Briefly, the PCUU scaffolds were prepared via conventional electrospinning and coated layer-by-layer with fibronectin and gelatin to aid cell adhesion before seeding with rat adipose-derived stem cells. Female Sprague-Dawley rats were subjected to partial outlet obstruction following established methods and maintained under normal conditions for 10 days. Cystectomy was performed on the dome of bladders and repaired with PCUU scaffolds (1 cm x 1 cm). The urinary voiding volume and frequency was recorded weekly and the rats were sacrificed to harvest bladders at the end of the three week period. The results to date demonstrated that PCUU scaffolds exhibited mechanical behavior similar to that of native tissues and coating of the scaffolds with ECM proteins enhanced cell attachment *in vitro*. In addition, augmentation of the bladder with PCUU scaffolds improved the survival rate of the model rats and that the bladder capacity improved with time. The histological analysis of the explanted scaffold indicated smooth muscle cell and connective tissue

infiltration. Overall, the present study has made a contribution to the field of bladder tissue engineering with a novel synthetic scaffold and a clinically relevant bladder dysfunction model for evaluation of the engineered tissue constructs.

#### Mir-646 in Clear Cell Renal Carcinoma Correlated with Tumour Metastasis by Targeting the Nin One Binding Protein

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**Introduction:** Renal cell carcinoma (RCC) is a common urologic malignancy and accounts for 3% of adult malignancies and causes 90 000 deaths worldwide annually. Previous studies have found that nin one binding protein (NOB1) was identified as a potential oncogene in human glioma and miR-646 plays an important role in human growth and development. However, NOB1 in tumorigenicity and its correlation with miR-646 in renal cell carcinoma (RCC) have not been investigated.

**Methods:** The expression of NOB1 and miR-646 from 100 cases of clear cell RCC (ccRCC) and 30 cases of adjacent non-tumour tissues were detected by quantitative real-time PCR. The expression of miR-646 was correlated with NOB1 expression, tumour features and patient metastasis-free survival. Using a xenograft tumour model, we observed the *in vivo* tumorigenesis effect of miR-646 and NOB1.

**Results:** miR-646 negatively regulated NOB1 and inhibited the proliferation and migration of renal cancer cells. There was a significant upregulation of NOB1 in ccRCC and it was further increased in metastatic cases, while miR-646 was downregulated in tumour tissues and further decreased in metastatic ccRCC. The down-regulation of miR-646 also indicated a higher probability of developing metastasis. miR-646 expression was an independent predictor of ccRCC metastasis by the univariate analysis and binary logistic regression model ( $P < 0.05$ ). Furthermore, suppression of NOB1 increased the phosphorylation of several proteins in MAPK pathway.

**Conclusions:** Our findings suggest that NOB1 as a novel target of miR-646. Overexpression of miR-646 decreased tumorigenesis of renal cancer through the modulation of MAPK pathway.

#### Intensified Decellularization and Coating with CCN1 of Equine Carotid Arteries Generates Non-cytotoxic Scaffolds with Reduced Inflammatory Potential Measured by Cytokine Expression in the Monocytic Cell Line THP-1

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Decellularized equine carotid arteries (dEAC) are alternatives for alloplastic materials like PTFE in vascular replacement therapy. The residual immunogenicity of decellularized scaffolds however may compromise endothelialisation and evoke macrophage activation which is pivotal in wound healing and graft acceptance by the expression of inflammatory and wound healing cytokines. We recently achieved a complete removal of cellular molecules of dEAC by an intensified decellularization protocol (dEAC-intens). Moreover, coating with the matricellular protein CCN1 has improved healing and remodeling properties of conventionally dEAC *in vivo*. Here we aimed to determine reseeding capability and macrophage activation of CCN1-coated dEAC-intens *in vitro*.

EAC were decellularized by detergents for 40 h (dEAC-con) or 72 h (dEAC-intens) and coated with or without 100 ng/ml CCN1. Endothelial cells or THP-1 cells representing a monocyte/macrophage cell line were seeded onto the scaffolds. Endothelialization was determined immunohistologically and expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-8, IL-1 $\beta$ ), monocyte-recruiting cytokines (MIP-1 $\alpha$ , MCP-1) and anti-inflammatory cytokines (IL-1ra, IL-10) in THP-1 cells was determined using quantitative RT-PCR.

Both dEAC showed an intact endothelial layer indicating a non-cytotoxic surface of either scaffold. In THP-1 cells dEAC-con and dEAC-intens did not change expression of either cytokine compared to plastic. CCN1-coating of dEAC-intens however decreased MIP1 $\alpha$  and MCP expression significantly to 42.6% and 45.1%. CCN1 induced IL-8 expression on dEAC-con 4.5-fold but significantly less on dEAC-intens (1.9-fold).

In conclusion, intensified decellularization of EAC results in biocompatible grafts and after CCN1-coating reduced monocyte-recruiting cytokine expression and inflammatory IL-8 expression in macrophages. Thus dEAC-intens may present an improved biomaterial for vascular grafts.

#### Differentiation of Human Mesenchymal Stromal Cells Correlates with Electrophysiological Competence Similar to Bladder Smooth Muscle Cells

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Greater than 15–30% of the elderly suffer from stress urinary incontinence which may be caused by loss/degeneration of the urethral (US) sphincter muscle. Myogenically differentiated mesenchymal stromal cells (MSC) may restore and enhance sphincter muscle function. Human bone marrow-derived MSC were expanded under GMP conditions in human plasma- and platelet lysate-containing medium, differentiated with TGF- $\beta$ 1, PDGF-AB and ascorbic acid and compared to primary human bladder SMC since these cells form the internal US, which supports continence. The expression of early-to-late contractile myogenic markers was measured and compared to electrophysiological properties for functional maturity. After 7–14 days of myogenic differentiation cells significantly expressed  $\alpha$ SMA, transgelin, calponin and SM-MHC according to qRT-PCR, immunofluorescence and/or western blot. Myogenic gene levels decreased at day 21 and increased again at day 28. Consistent with the contractile gene expression observed during myogenic differentiation of MSC, changes in voltage-gated Na<sup>+</sup> current levels followed a similar pattern with the current size increasing at days 7–14 following myogenic differentiation, and then decreasing thereafter. The expression of Na<sup>+</sup>-channels (identified as the Nav1.4 subtype) was restricted to differentiated MSC and bladder SMC, indicating functional maturation of differentiated MSC. Differentiated MSC exhibited elevated cytosolic Ca<sup>2+</sup> which was suppressed by Cd<sup>2+</sup>, an inhibitor of voltage-gated Ca<sup>2+</sup>-channels, whereas no increase in intracellular Ca<sup>2+</sup> occurred in undifferentiated MSC. This protocol may be used to differentiate adult MSC into SMC with an intermediate-to-late SMC contractile phenotype exhibiting voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels comparable to bladder SMC which may be important for urological regenerative medicine applications.

#### Characterization of Tendon Stem/Progenitor Cells and In Vitro Comparison with Adipose Derived Stem Cells

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Adipose-derived stem cells (ASCs) have been deeply characterized for their usefulness in musculoskeletal tissue regeneration; recently, other mesenchymal stem cell (MSCs) sources have also been proposed. This study compares for the first time human tendon stem/progenitor cells (TSPCs) isolated from hamstring tendons and human ASCs with the main aim to provide further knowledge regarding the potential use of TSPCs in musculoskeletal tissue regeneration approaches.

Human TSPCs and ASCs were isolated from healthy donors undergoing ACL reconstruction or liposuction, respectively (n=7). Proliferation and CFU-F ability were assessed. The typical stem cells marker expression was evaluated by FACS and RT-PCR analysis. TSPCs and ASCs multi-differentiation potential was assessed after induction with specific differentiation media.

Both populations showed similar morphology, doubling time and clonogenic ability and they exhibited the typical MSC surface markers and the expression of stemness-specific transcription factors. After osteogenic induction, alkaline phosphatase activity and extracellular calcified matrix production were higher in both cell populations with respect to controls. However, ASCs showed a greater ability to form calcified matrix and expressed higher RUNX2 levels than TSPCs. ASCs also differentiated more efficiently than TSPCs towards adipogenic lineage. Interestingly, after chondrogenic induction, TSPCs produced more abundant glycosaminoglycans and expressed higher levels of aggrecan with regards to ASCs.

In conclusion, TSPCs are a subpopulation of cells with peculiar features of stem cells able to differentiate efficiently toward osteogenic and chondrogenic lineages, and thus, due to the large number of available waste hamstring fragments, they represent a convenient cell source for musculoskeletal regenerative medicine.

### Development of a Transport Tool for Regenerative Medicine

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Cell processing procedures are managed using good manufacturing practices, and transplantation and infusion are performed with highly optimized clinical techniques. Thus, a high-quality transportation system is essential for connecting cell processing and transplantation steps. In terms of logistics, the most difficult factor is that the cells to be transported are living. Sterility, temperature stability, and shock resistance are essential. Furthermore, oxygen control is important when mesenchymal stem cells are transported in an unfrozen state. In 2013, we established a consortium to gather the latest Japanese technology and create a prototype transport box. The box contains a bag to hold the cells; this bag is permeable only to gas, not to viruses or microbes. An atmosphere regulator stabilizes the concentrations of oxygen and carbon dioxide. The outer box has superior heat-insulation capacity, shock resistance, and UV-shielding effects and is light weight. This is one of many possible options for cell transport. Additional simulations are needed to examine the possibility of other transport patterns. Continuous development and construction of novel, more optimized systems are essential, and high-quality transport boxes are expected to contribute to the further development of regenerative medicine.

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### Chemotherapeutic Drug-containing Microspheres for Tumor Suppressing Adipose Regeneration

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**Background:** Fat grafting to the oncologic breast is still in question because fat component may promote breast cancer cells (BCCs). Thus, a fat graft incorporating chemotherapeutic drugs at concentration to inhibit BCC growth while also allowing for tissue preservation would be ideal after breast cancer surgery. The aim of this study was to

manufacture doxorubicin, paclitaxel, and 4-OH tamoxifen-containing microspheres that suppress BCCs growth while also maintaining normal cellular growth of adipose tissue components, including adipocytes and adipose-derived stromal cells (ASCs).

**Materials and methods:** Microspheres encapsulating chemotherapeutic drugs were manufactured. Drug loading and release kinetics were determined from chemotherapeutic microspheres. Bioactivity of chemotherapeutic-loaded microspheres and drug in media alone was determined by assessing death of BCCs (BT-474, MDA-MB-231, MCF-7) after 72 hours of incubation in culture.

**Results:** Doxorubicin, paclitaxel, and 4-OH tamoxifen microspheres contained  $4.4 \pm 0.9 \mu\text{g}$ ,  $9.2 \pm 0.8 \mu\text{g}$ , and  $5.4 \pm 0.4 \mu\text{g}$ , respectively. A dose response of chemotherapeutic microspheres was determined for both ASCs and BCCs. For each drug, a dose of microspheres could be determined that allowed for normal stromal cell proliferation while also providing toxic effects to BCCs. A toxic effect of doxorubicin, paclitaxel, and 4OH-tamoxifen was found on BCCs with a dose of 10, 1, and 10 mg, respectively. These doses were also found to allow for normal ASC proliferation.

**Discussion:** Considering IC50 of doxorubicin, paclitaxel, and 4-OH tamoxifen is higher on ASCs than BCCs, certain drug levels should exist to kill BCCs not ASCs. Future work consists of completing studies to confirm these dose effects *in vivo*.

### The Application of Injectable Allogeneic Osteogenic Micro-tissue in Repair of Large Segmental Bone Defect

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**Purpose:** To investigate the feasibility of the application of injectable bone tissue engineering based on osteogenic micro-tissue in the large segmental bone defect.

**Method:** We first harvested a dense osteogenesis cell sheet from the fetal rabbit bone marrow mesenchymal stem cells, which were induced in osteogenesis induced culture for 4 weeks. Then, we cut the cell sheet into fragments, and mixed the fragments with 1.5% sodium alginate solution, adding CaCl<sub>2</sub> solution to prepare the osteogenic micro-tissue. After that, the osteogenic micro-tissue was injected subcutaneously to test its ectopic osteogenesis potential. Finally, we injected the osteogenic micro-tissue into the scaffolds, which were used for the repair of the critical bone defects, as to demonstrate its *in situ* osteogenesis potential. The calcium alginate gel without the cell fragments as the control group. After the X-ray examination, specimens were harvested in the fourth week. Computed tomography scanning and histological examinations were performed. The results were analyzed by paired Student's t-test with SPSS 13.0 soft package.

**Results:** The fetal rabbit BMSCs showed good ability of proliferation and differentiation. The osteogenesis cell sheets showed a good characteristic of osteogenic differentiation. Computed tomography scanning and histological examinations confirmed new bone formation in the ectopic and *in situ* of the osteogenic micro-tissue group.

**Conclusion:** The study indicates that osteogenic micro-tissue has a good osteogenesis potential, and can be used in the repair of segmental bone defect. It will simplify the process of bone tissue engineering, and shorten the waiting time before the BTE (bone tissue engineering) treatment.

### Three Dimensional Spheroid Formation Improves Beta-cell Differentiation Potential of Adipose-derived Stem Cells

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Pancreatic islet transplantation is a promising treatment for insulin dependent diabetes. Because of shortage of pancreas donors and poor survival rate of allogenic islets, there are limitations for clinical applications of islet transplantation. Therefore, generation of insulin producing beta cells from stem cells has a great potential for the replacement of islet transplantation. Patient-derived adipose tissue-derived stem/stromal cells (ADSCs) are an attractive cell sources. This study explored the potential of three dimensional spheroid formations for enhanced beta-cell differentiation because of. In this study we introduced pancreatic and duodenal homeobox gene 1 (PDX-1) gene into ADSCs and differentiated under 3D spheroid culture system. Pdx-1 plays a key role in normal pancreas development and is required for maintaining the normal function of islets. Insulin transcript levels and expression of key transcription factors required development were significantly increased by spheroid formations. In addition, we observed that spheroid cultured cells showed higher insulin secretion in response to glucose change than control groups. These results demonstrate that formation of 3D cell spheroids can promote differentiation of mesenchymal stem cells into insulin producing cells because of their biomimetic structures.

### Biomechanical and Microstructural Characterization of Partially Ligated Common Carotid Arteries from S129 Wild Type and Fibulin-5 Knockout Mice

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Arterial stiffening is an age-dependent medical problem that doubles ones' risk of cardiovascular events. Arterial stiffness is associated with loss of functional elastic fibers and increased collagen content. However the importance of arterial stiffness to arterial remodeling is not well understood. Since altered wall shear stress impacts arterial remodeling, we used a partial carotid artery ligation model of disturbed flow (d-flow=low and oscillatory wall shear stress) on one side of both wild type (S129) and the stiffened fibulin-5 knockout mice (KO). KO mice lack an extracellular protein essential for normal elastin formation. We hypothesize that stiffened arteries *in vivo* undergo a more pathologic arterial remodeling compared to that of normal arteries in response to d-flow.

We performed biaxial biomechanical testing, confocal imaging, opening angles and histological studies 4 weeks post-partial ligation. Pressure-diameter (P-d) data were collected at constant axial extension levels. Compliance was calculated at the *in-vivo* axial stretch level. Intima media thickness was analyzed from hematoxylin and eosin stained histological slides. Results were compared with the unpaired, two-tailed t-test and significance was taken at  $p < 0.05$ .

We have linked *in vivo* mechanical and fluid mechanics to discover that stiffened carotid arteries from the KO mouse, in response to d-flow, undergo significantly greater subsequent arterial stiffness and inward remodeling compared to wild type control mice. These results are important to the design and testing of translational experiments that more closely mimic human disease.

### ASC Vascular Assembly in Hypoxia is Mediated by the Extent of Normoxic Preambly

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Adipose-derived stem cells (ASCs) contain an endothelial progenitor cell (EPCs) population that can self-assemble into vascular networks. Recently, we demonstrated that this process is inhibited in hypoxia (<2% O<sub>2</sub>). However, if vascular pre-assembly occurs for 6

days in normoxia (20% O<sub>2</sub>), exposing the networks to hypoxia enhances subsequent vascular growth. This suggests that hypoxia may affect EPCs differently depending on whether they exist as individual cells or as pre-assembled vascular structures. This study seeks to determine the minimum amount of normoxic pre-assembly time needed to promote vascular growth in hypoxia and to identify a possible mechanism for the 'switch' from inhibition to promotion of EPC growth following vascular assembly.

Human ASC aggregates were encapsulated in fibrin gels at 20,000 cells/ $\mu$ L and cultured in endothelial basal medium with 10% serum. Vascular structures were allowed to pre-assemble in 20% O<sub>2</sub> conditions for 0, 2, 4, or 6 days. Following each pre-assembly period, gels were transferred to either 2% or 20% O<sub>2</sub> conditions for a further 6 days and then stained for CD31 and  $\alpha$ -SMA. Proliferation was assessed via BrdU incorporation into DNA.

Vascular assembly in groups with 0 and 2 days of pre-assembly were significantly inhibited in hypoxia, while groups with 4 and 6 days of pre-assembly were equal to or greater than normoxic control groups. Vascular assembly was closely associated with increased proliferation in CD31+ cells and increased pericyte coverage of networks. Thus, 4 days of pre-assembly is sufficient for a 'switch' to promote vascular growth in hypoxia.

### A New Alginate-Sulfate/Nanocellulose Bioink for 3D Printing

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Bioprinting is used to manufacture 3D constructs in a layer-by-layer fashion utilizing biologically relevant material such as biopolymers and cells. However there is a lack of materials that can be used as so called "bioinks". Nanocellulose mixed with alginate fulfills these requirements for a bioink as it is cytocompatible with chondrocytes and allows precise printing of various architectures. Unfortunately, chondrocytes do not proliferate in alginate. However, alginate sulfate has been shown to increase chondrocyte proliferation in a 3D environment compared to normal alginate while maintaining their chondrogenic phenotype. We therefore combined the good rheological and mechanical properties of nanocellulose with the proliferative capacity of alginate sulfate with different degrees of sulfation. The inks with the sulfated alginate were shear thinning and had a storage modulus of 7.28 kPa (highly sulfated) and 23.35 kPa (low sulfated) after 10 min of CaCl<sub>2</sub> gelation. Cells were viable up to 7 days when encapsulated in the ink. When cells were encapsulated in the ink and printed, cell viability was low at day 1 but recovered at day 7. The cell spreading associated with sulfated alginate was only visible for cells encapsulated in highly sulfated alginate and, surprisingly, only in the non-printed samples. In conclusion we can say that the alginate sulfate keeps its proliferative capacity in combination with nanocellulose, but is impaired when cells are exposed to the high shear during the printing process.

### Dendritic Cells in Contact with Dental Pulp Stem Cells Expand CD4+Foxp3+ T Cells

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Recently, studies have shown that mesenchymal stem cells derived from human exfoliated deciduous teeth (SHEDs) have prominent immune modulatory properties, which may have clinical applications, in both cell-based therapies and tissue engineering, especially for diabetes type-1, since such properties diminish the rejection risk. Parallel to this, dendritic cells (DCs) are of great importance in early immune responses, since these cells affect significantly the balance between helper and regulatory T cells. This study was designed, therefore, to evaluate the SHEDs influence on DCs

ability to activate T cells and to expand CD4+Foxp3+ T cells. After co-culture with SHEDs, monocyte derived-DCs (moDCs) presented lower expression of differentiation markers (such as BDCA-1) and maturation markers (such as CD40 - decreased in mature DCs (mDCs)). To assess the ability of SHEDs-exposed moDCs to modulate T cell responses, the former were separated from SHEDs, and co-cultured with peripheral blood lymphocytes (PBL). After 5 days, the proliferation of CD4+ and CD8+ T cells was evaluated and found to be lower than that induced by moDCs cultivated without SHEDs. In addition, an increase in the proportion of CD4+Foxp3+IL-10+ T cells was observed among cells stimulated by mature moDCs that were previously cultivated with SHEDs. Soluble factors released during co-cultures also showed a reduction in the pro-inflammatory cytokines and an increase in the anti-inflammatory molecules. This study shows that moDCs in contact with SHEDs acquired an immune regulatory phenotype, evidenced by changes in maturation and differentiation rates, inhibition of lymphocyte stimulation and ability to expand CD4+Foxp3+ T cells.

### Evaluating Age-Dependent Mechanosensitivity in Mesenchymal Stromal Cells to Identify Therapeutic Targets for Enhanced Bone Regeneration

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Bone tissue engineering is a promising strategy to enhance bone regeneration and scaffolds are increasingly used as delivery platforms for therapeutics to direct bone formation by mesenchymal stromal cells (MSCs) *in vivo*. Additionally, the role of mechanotransduction in osteoinduction as well as the age-dependent loss of differentiation potential in MSCs is now well recognised. However, there is limited information about age-dependent effects in MSCs mechanosensitivity and the genes controlling these effects. Therefore, this project aims to identify age-dependent mechanoresponsive targets in order to functionalize scaffolds for the induction of enhanced bone formation. Bone marrow MSCs from child (C-MSCs) and adult (A-MSCs) donors were seeded on 10 and 300 kPa polyacrylamide gels, coated with type I collagen, and cultured in growth medium (GM) or osteogenic medium (OM). In both GM and OM, stiffness-dependent increases in mineralization and alkaline phosphatase (ALP) activity were seen in C-MSCs and A-MSCs after 14 days in culture, with C-MSCs showing the highest levels of expression. Stiffness-mediated upregulation in the genetic expression of bone morphogenetic protein-2 (BMP2) and placental growth factor (PGF) was observed in C-MSCs but not in A-MSCs after 3 days culture in GM. These results not only show an enhanced propensity for osteogenic differentiation of C-MSCs compared to A-MSCs induced by the surface properties, but also the activation of angiogenic pathways (i.e. PGF stiffness-upregulation in GM) in C-MSCs. Future investigations into the age-dependent mechanisms underpinning the mechanoresponsiveness of MSCs may thus elucidate new therapeutic targets for improved bone and vascular regeneration.

### Differentiation Potential of Human Mesenchymal Stem Cells to Cornea Epithelium using Conditioning Medium

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**Objective:** Human mesenchymal stem cells (MSC) have differentiation potential to several stromal and connective cell lineages, but their capability to differentiate into cornea epithelial cells is not clear. In the present work, we have analyzed the *ex vivo* epithelial

differentiation potential of four MSC types cultured in inductive medium.

**Methods:** Primary cell cultures of human MSC were established from tissue biopsies of Wharton's jelly (HWJSC), bone marrow (BMSC), adipose tissue (ADSC) and dental pulp (DPSC). Cells were cultured in DMEM medium following standard culture conditions. Then, cells were subjected to epithelial differentiation induction by using conditioning medium (CM) containing insulin, triiodothyronine, cholera toxin, hydrocortisone and epidermal growth factor (EGF) for 28 days. Expression of genes with a role in epithelial differentiation was quantified by Affymetrix Human Genome U133 plus 2.0 oligonucleotide microarrays. Probe-sets showing increased expression (from "absent" or "marginal" to "present") were selected.

**Results:** 93 probe-sets showed increased expression in induced HWJSC, 63 in BMSC, 79 in ADSC and 44 in DPSC. Cluster analysis suggests that HWJSC and ADSC could have the highest differentiation potential. Genes related to the development of cell-cell junctions (GJC1, CDH4), cytokeratins (KRT78, KRT20, KRT25, KRTAP19-3) and basement membrane (LAMA3, LAMB2L, LAMC2) were upregulated in HWJSC.

**Conclusions:** Our findings suggest that HWJSC and ADSC show the highest *ex vivo* epithelial differentiation potential and could be used for human cornea tissue engineering.

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### Effect of Hyaluronic Acid on Brain Cancer Development

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Glioblastoma multiforme (GBM) is a common and aggressive form of brain cancer. Patients exhibit poor survival rates, tied both to the significant intra and interpatient heterogeneity of the tumor as well as the complex signaling pathways underlying malignancy. There is a significant clinical need for biomaterial-based platforms that recapitulate the complexity of the native tumor microenvironment. These tumor avatars could facilitate mechanistic investigation as well as for clinical applications aimed at biomarker discovery and improving therapeutic targeting. These devices need to reproducibly create environments to support malignant phenotype. Here, we present a series of gelatin-based hydrogels that offer tunable physicochemical properties in a three dimensional microenvironment. We report the relationship between local hyaluronic acid (HA) content and tumor progression. HA is a primary structural component of the brain and is thought to contribute to glioma invasion and malignant phenotype via its CD44 receptor. Xenograft cells derived from patient biopsies that maintain patient-specific morphologic/molecular characteristics were cultured in a series of HA-modified gelatin hydrogels, with their malignant phenotype determined as a function of HA content and exposure to model therapeutic (e.g., reversible tyrosine kinase inhibitor Erlotinib). Notably, matrix-immobilized HA content significantly impacted both the transcriptomic and proliferative signatures of xenograft cells. Matrix-immobilized HA also significantly impacted the therapeutic efficacy of the model EGFR-inhibitor Erlotinib. These results suggest a pathway towards decipher the possible mechanistic link between the CD44-HA interactions and EGFR, a receptor overexpressed or mutated in more than half GBM tumors.

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### Programming Cell Delivery by Single-cell Encapsulation in Microgels

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Improving techniques to encapsulate cells in hydrogels could lead to advances in programming *in vivo* delivery of stem cells and their



secreted factors for regenerative medicine. While there are currently over 250 clinical trials with mesenchymal stem cells (MSCs), it remains challenging to optimally leverage MSCs to achieve therapeutic benefits. Here, a novel high-yield microfluidic technique was developed to encapsulate single MSCs in a thin layer of alginate hydrogels to program the *in vivo* delivery of cells by enabling different routes of administration and microscale control of matrix mechanics. For the first time, singly encapsulated firefly luciferase-expressing mouse MSCs from BALB/c mice were injected intravenously into C57/BL6 mice to mimic allogeneic transplantation. Strikingly, the half-life of donor cell clearance is increased by ~10 fold when cells are encapsulated, but not when cells are mixed with empty microgels. Human MSCs encapsulated and injected in the immunocompromised mice yielded similar results, with hydrogel encapsulation even enhancing the bioluminescence signal after injection in this model. The prolonged delivery of cells leads to a significant increase in the total amount of systemic donor cell secretions in blood as determined by expressing the secreted form of luciferase in MSCs prior to injection. The efficacies of singly encapsulated MSCs in a number of disease models are currently being explored. Our approach represents a promising way to improve the delivery of MSCs and their secreted factors for therapeutic purposes.

#### Geometrically Controlled Micro-Vascularized Constructs based on Electrochemical Transfer of Endothelial Monolayer

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Angiogenesis is a vital process for tissue maintenance and repair. A major challenge in the development of functional and clinically relevant three-dimensional tissue constructs is the formation of vascular networks for oxygenation, nutrient supply and waste removal. Here we present a vascularization strategy that combines geometrically controlled microchannels, angiogenic sprouting and self-assembled microvascular networks. Specifically, human endothelial cells (ECs) were seeded on oligopeptide-functionalized gold-coated microrods to form a complete cell monolayer and to generate endothelium-lined microchannels by electrochemical transfer of ECs within fibrin hydrogels (7.5 mg/ml). Computational simulations identified an optimal geometrical three-channel configuration assuring a safe oxygen concentration throughout the constructs. Fibrin hydrogels were then incubated either statically or dynamically in a perfusion bioreactor for 7 days. 3D simplified models, composed of fibrin hydrogels lined by two endothelial monolayers, were developed to quantify the vascular network parameters. The distance between the two monolayers was set to reach an oxygen level comparable to the oxygen level in the three-microchannels configuration. Experimental groups consisted of fibrin hydrogels either without cells, or loaded with monolayer of mesenchymal stem cells (MSCs) or co-culture of MSCs/ECs with or without angiogenic growth factors (GFs). The co-culture system in the presence of angiogenic GFs showed significantly more pronounced endothelial sprouting ( $280 \pm 39 \mu\text{m}$ ), total length of vascular networks ( $9714 \pm 1636 \mu\text{m}$ ), branches number ( $7.6 \pm 0.9$ ), and expression of both  $\alpha$ -SMA and NG2 compared to all other conditions. These results suggest a promising biofabrication approach in which geometrically controlled microvascular networks can be developed within large tissue-engineered constructs.

#### Direct Reprogramming of Human Fetal Fibroblasts towards Cardiomyocytes

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A myocardial infarction leads to ischemia and subsequent death of cardiac tissue; as cardiomyocytes are unable to regenerate post in-

fraction, there is justification for novel cellular therapies to be developed that may require the derivation of functional cardiomyocytes by reprogramming. Differentiated mouse somatic cells have been directly reprogrammed into cardiomyocytes through overexpression of cardiomyocyte transcription factors (Ieda *et al.*, 2010). The aim of this study is to reprogramme human fetal fibroblast cells into cardiomyocytes through the non-viral overexpression of the cardiomyocyte-specific transcription factors: GATA4, MEF2C and TBX5 (GMT). Primary fibroblasts were isolated through explant migration of human fetal skin and heart tissue. Two non-viral vectors were generated containing the PIRE5 sequence to enable bicistronic expression of GATA4 with GFP and MEF2C with TBX5. Isolated fibroblasts were transfected with these vectors using nucleofection and stable integration was selected using G418. Flow cytometry measured increased expression of the early cardiomyocyte transcription factor NKX2.5 in GMT nucleofected cells at day 2 and 7 and was maintained at 3 weeks post-nucleofection, compared to non-transfected cells. No increase in the expression of the sarcomeric protein  $\alpha$ -MHC was observed in transfected cells at these time points. These results indicate that non-viral reprogramming of primary human fetal fibroblasts with GMT initiates early cardiomyogenic differentiation but is insufficient to induce the development of mature cardiomyocytes within this time scale.

Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142, 375–386.

#### Methods and Effects of Sterilization on Extracellular Matrix Hydrogels

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Preclinical studies have shown marked tissue repair benefits of extracellular matrix (ECM) cryptic peptides, which are concentrated in ECM hydrogels. However, terminal sterilization of ECM scaffolds by well-accepted methods prevents subsequent formation of an ECM derived hydrogel. It is critically important to identify a method for sterilization conducive to formation of ECM hydrogels prior to clinical translation. The objectives of the present study were: (1) identify a method for sterilization of ECM that allows for subsequent hydrogel formation, and (2) characterize the viscoelastic and biologic properties of the ECM hydrogel following sterilization.

Porcine urinary bladder was decellularized to form urinary bladder matrix (UBM) and subjected to the following sterilization methods: exposure to ethylene oxide gas, gamma and electron beam irradiation, or supercritical CO<sub>2</sub>. Hydrogel formation was tested following sterilization of (1) the UBM sheet, (2) mechanically comminuted UBM powder, and (3) following digestion and lyophilization. Qualitatively, supercritical CO<sub>2</sub> sterilized lyophilized digest formed the most rigid hydrogel. Sterilization of the UBM sheet by any method did not allow subsequent hydrogel formation. These differences were corroborated by rheological characterization. The chemotactic and immunomodulatory properties hallmarks of ECM hydrogels were also altered by sterilization. Together, these data highlight the importance of the ECM form (i.e., sheet vs. powder vs. lyophilized digest) that is exposed to terminal sterilization. The choice of sterilization methodology dramatically affects the viscoelastic and biologic properties of ECM hydrogels. Ongoing studies are focused on determining whether results shown in the present study are universal to ECM scaffolds derived from different tissues.

#### Building Blocks for a Multi-tissue Tracheal Replacement: Cartilaginous and Epithelial Constructs

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Tracheal cartilage supports the open airway, thus engineered cartilaginous structures of defined geometries may be valuable components for providing luminal patency in tracheal replacement strategies. Like the native airway, engineered tracheas must also contain an immunoprotective respiratory epithelium. Therefore, it is critical to engineer cartilage-epithelial composite tissues. In this work, high-density human mesenchymal stem cell (hMSC) constructs with and without growth-factor-delivering chondroinductive microspheres (MS) were engineered into cartilaginous rings and tubes. Wall thickness was modulated by varying hMSC number, and lumen diameter (2–12 mm) was tuned using custom tissue culture systems: annular wells for tissue rings and glass tubes for tissue tubes. After 3 weeks, chondrogenesis, quantified by glycosaminoglycan (a major cartilage ECM component) production normalized to DNA, was reduced in larger diameter cartilage tubes. Cartilage rings produced from fewer cells had significantly thinner walls but similar chondrogenesis. Microsphere-containing rings were significantly thicker and more chondrogenic than cell-only rings. Rings from all conditions exhibited uniaxial ultimate tensile stress values that were at least equal to that of rat trachea. Epithelialized cartilage was developed by establishing a human bronchial epithelial cell sheet on the surface of hMSC + MS disc-shaped constructs. Epithelial-cartilaginous constructs produced spatially-controlled, tissue-specific markers: cartilage contained glycosaminoglycan and collagen II (most prevalent collagen in tracheal cartilage), and epithelium stained for cytokeratin (intermediate filament in epithelium). In this work, (1) wall thickness and lumen diameter of engineered cartilage rings and tubes were controlled and (2) engineered cartilage tissues were epithelialized. These are promising milestones towards a multi-tissue type tracheal replacement.

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#### Translation of Novel Strategies for Mechanical Conditioning for Functional Bone Tissue Engineering

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We aim to develop a cell-based injectable solution for treatment of non-union bone fractures. Our approach is based on the use of functionalised magnetic particles (MNPs) targeted to the TREK-1 mechano-sensitive receptor on the mesenchymal stem cell (MSC) membrane. Attached MNPs respond to the application of an oscillating external magnetic field resulting in remote receptor activation and enhanced osteogenic differentiation. In this way, we can deliver functional mechanical stimuli to therapeutic cell populations *in vivo* following injection either systemically or to the repair site. This concept has been validated through a series of *in vitro*, *ex vivo* and small animal studies and is currently being tested in a pre-clinical sheep model for bone repair.

Five million autologous MNP-labelled STRO-4 positive MSCs were encapsulated within a naturally derived bone extracellular matrix gel and implanted within a critical sized defect (0.8 × 1.5 cm) in the medial femoral condyle of a sheep. Implanted cell populations were stimulated over 13 weeks by custom built magnetic array housed within a standard sheep harness. Bone fill was assessed by  $\mu$ CT and validated histologically. Preliminary data suggests enhanced targeted bone fill in MNP-magnet stimulated cell groups over non-stimulated cell groups. Variability in donor response was further evaluated *in vitro* in 3D hydrogel systems by encapsulating MNP-labelled MSCs from 17 donors within a 2.5 mg/ml collagen hydrogel,

magnetically stimulating (MICATM bioreactor) for 1 hr/day over 28 days and mineralisation levels analysed by  $\mu$ CT. Our novel results demonstrate the feasibility of a remote magnetic nanoparticle approach for cell therapy applications.

#### The Promotion of Bone Regeneration by the Chitosan-p24/hydroxyapatite Scaffold Via Modulation on Tgfb-1/bmp/smad Signaling Pathway

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In bone tissue engineering, a combination of biomimetic scaffolds with bioactive factors has emerged as a new strategy for the repair of critical sized bone defect. In this study, we developed a new biomimetic composite scaffold loaded with BMP2-derived peptide P24 by the chemical modification manner. The *in vitro* studies showed that the bioactivity of P24 was preserved in the CS-TBA/HA scaffold, and the P24 release lasted up to 90 days was achieved. The CS-P24/HA scaffolds with different proportion of P24 showed the good biocompatibility. Furthermore, the levels of the mRNA for Runx2, ALP and collagen I were significantly up-regulated on CS-P24/HA compared to CS/HA *in vitro*. Similarly, the cells exhibited higher ALP staining, ALP activity and calcium deposition levels on CS-P24/HA than CS/HA ( $P < 0.05$ ). *In vivo* osteoinductive studies revealed that the degrees of ectopic osteogenesis in the rat were significantly higher in CS-10%P24/HA than the CS/HA scaffolds. Finally, *in vivo*, CS-P24/HA had a superior ability of bone reconstruction for calvarial bone defects. CS-10%P24/HA scaffold manifested the best repair efficacy due to a synergistic effect of CS-P24 and HA. In addition, the critical proteins pSmad2/3 in BMP pathway showed clear nuclear localization and osteocalcin were significantly elevated on CS-P24/HA group. In conclusion, our results demonstrated that CS-P24/HA scaffold can induce bone formation and promote bone regeneration by activating BMP/Smad signaling pathway of BMSCs both *in vitro* and *in vivo*, therefore, CS-P24/HA scaffold is deemed as a strong potential candidate for the repair of bone defects in bone tissue engineering.

#### Regulation of Functional Vascular Networks using Cell-laden Gelatin Hydrogels

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Current approaches in vascular network bioengineering are largely carried out using natural hydrogels as embedding scaffolds, but most natural hydrogels present a poor mechanical stability and insufficient durability, which are critical limitations their widespread applicability. Here, the suitability of gelatin-tyramine (gelatin-tyr) hydrogels, which could be enzymatically crosslinked with tuning of storage modulus and proteolytic degradation rate, was explored as an injectable hydrogel for supporting the human progenitor cell-based formation of stable and mature vascular network. Gelatin-tyr hydrogels was cytocompatible with human blood-derived endothelial colony-forming cells (ECFCs) and white adipocyte tissue-derived mesenchymal stem cells (MSCs) (yielding high viability > 87%). Moreover, gelatin-tyr hydrogels was able to modulate proliferation and spreading of ECFCs in relation to the proteolytic degradability under the same storage modulus. Additionally, murine gelatin-tyr hydrogels were successfully extracted and synthesized from dermis of murine skin using the appropriate methods. Importantly, it is shown that implantation of cell-laden gelatin-tyr hydrogels into immunodeficient mice results in a rapid formation of functional anastomoses between the bioengineered human vascular network and the mouse vasculature. Furthermore, the degree of enzymatic cross-linking of the gelatin-tyr hydrogels can be used to modulate the

cellular behavior and the extent of vascular network formation *in vivo*. These data proposed the synthesis technique of gelatin-tyr hydrogels from allogeneic or xenographic dermal skin, and suggested these hydrogels can be used for biomedical applications that require the formation of microvascular networks, including the development of complex engineered tissues.

#### Development of Vascular Inducible Hydrogel with Functional Peptide Sequences for Angiogenic Therapy

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Several injectable hydrogels have been shown to preserve or improve post-infarction cardiac function, where thickening infarcted wall is a key factor of the treatment. However, the role of cell infiltration and neovascularization into the hydrogel is still unclear. Silk fibroin can be processed into gels and its gelation and mechanical properties can be adjusted by the concentration and/or pH of fibroin solution. Thus fibroin is a candidate for injectable material. However, cell infiltration and vascularization cannot be expected as fibroin has no cell adhesive sequences such as RGD. Here, we recombinantly produced a vascular-inducible peptide (VIP), which was then introduced into the fibroin gel to append cell infiltration and vascularization functions to the gel. The VIP is composed of fibroin H-chain repeat peptide, REDV tandem peptide, MMP-2 cleavage peptide, and VEGF mimic peptide. As the VIP showed enhancement effects on HUVEC proliferation *in vitro*, it was mixed with fibroin and citrate buffer (pH 3.0) to form a gel. The resultant gel was embedded subcutaneously in rats for one, four, and eight weeks. H&E staining and CD31 (vascular endothelial cell marker) immunostaining revealed that the VIP promoted cell infiltration and vessel formation into the fibroin gel *in vivo*. Hence, rat myocardial infarction models have been prepared, and effects of cell infiltration and vascularization into the injected gel on myocardial regeneration are now being investigated.

#### Development of a Bioengineered Corneal Endothelium for Use in Endothelial Keratoplasty

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Disease or injury to the corneal endothelium (CE) is responsible for approximately 40% of corneal transplants. Current options to restore CE function include a full cornea transplant and Descemet's Stripping Endothelial Keratoplasty (DSEK); however donor corneas are limited worldwide. To address this need, we have developed an engineered basement membrane (EBM) that can be used to bioengineer a CE cell monolayer suitable for use in a DSEK transplant.

To fabricate the EBM, sheets of laminin and collagen IV were created using a surface-initiated assembly process. These sheets were transferred onto a collagen type I gel to form a bilayer scaffold. The EBM structure and composition was validated using confocal laser scanning, multiphoton and atomic force microscopy. Both bovine and human CE cells were seeded onto the EBMs, cultured for up to 28 days and fixed and stained for nuclei, ZO-1 (tight junction protein), laminin, and F-actin.

Microscopy results showed that the EBMs had well defined protein layers. The human and bovine CE cells seeded on the EBMs formed confluent monolayers, expressing ZO-1 at the cell borders and achieved densities of 1600–1700 cells/mm<sup>2</sup> over 14 and 28 days in culture. The integrated EBM and CE monolayer was similar in structure, thickness and handling characteristics to that of a corneal graft prepared for a DSEK procedure.

These results suggest that our high-density bioengineered CE could be used in a DSEK transplant where a donor CE is unavailable, and future studies will investigate preclinical performance using an *in vivo* animal model.

#### Using 3D Bioprinting Technology to Spatially Distribute Chondrocytes and Induced Pluripotent Stem Cells in Nanocellulose Bioink

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**Objectives:** The ability to create 3D cartilage tissue on demand would enable scientific and technological advances in tissue engineering as well as in basic research and drug screening. Here, we aim to develop a bioink suitable for 3D bioprinting and chondrogenic differentiation of induced pluripotent stem cells (iPS).

**Materials and methods:** A bioink of nanocellulose and alginate was developed for 3D bioprinting. Different fractions of nanocellulose and alginate were prepared for cell encapsulation, and viability of the cells was evaluated after printing. Proliferation was analyzed using MTS. Cell distribution was analyzed with non-linear microscopy.

**Results:** After bioprinting, viable cells were found after up to 35 days and a 3-fold increase in metabolic activity was seen after four days, indicating a suitable environment for growing cells. Using a lower concentration of nanocellulose yielded a higher number of viable cells, whereas a higher amount of nanocellulose yielded more stable constructs. The iPS cells and the chondrocytes were thereafter printed in separate strands in an overlapping grid structure. The production of GAGs was increased in the intersections, indicating a crosstalk between chondrocytes and iPS cells.

**Significance:** We conclude that the developed nanocellulose bioink is well suited for bioprinting with iPS cells and chondrocytes, sustaining cell viability over time. By controlling the positioning of the cells in a 3D distribution, the chondrogenic differentiation of iPS cells can be enhanced through cell-cell crosstalk.

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#### Induction of Hemogenic Endothelium and Production of Functional Blood Components from Human Pluripotent Stem Cells

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Induced pluripotent stem cells (iPSCs) have created opportunities for the scalable manufacture of blood products. Advancing iPSC technologies into the clinic and designing bioreactors for the production of blood cells from iPSCs, require an understanding of the hierarchy of hematopoietic progenitors in hPSC cultures, the identification of chemically defined conditions and matrix proteins supporting induction of hematopoiesis. We found that definitive myelolymphoid hematopoiesis in human iPSC cultures is initiated at the hemogenic endothelium (HE) stage, which can be identified by a VE-cadherin + CD73-CD235a/CD43<sup>-</sup> phenotype. VE-cadherin + CD73<sup>+</sup> cells were found to be non-HE progenitors. HE gives rise to CD235a + CD41a + erythromegakaryocytic and lin-CD34 + CD43 + CD45 +/- myelolymphoid progenitors. Based on the molecular profiling of OP9 and other stromal cell lines with different hematopoiesis-inducing activity, we discovered that Tenascin C, an extracellular matrix protein associated with HSC niches, strongly promotes HE and blood development from hPSCs. Using Tenascin C, we were able to develop completely defined serum-free conditions for the efficient generation of HE and myelolymphoid cells from hPSCs. In addition, we identified two groups of transcriptional regulators that are capable of inducing HE with pan-myeloid (GATA2 and ETV2) and erythromegakaryocytic (GATA2 and TAL1) potentials from hPSCs. Using selective expansion and directed differentiation of either CD235a + CD41a + erythromegakaryocytic or lin-CD34 + CD43 + CD45 +/- multipotent progenitors, we were able to produce red blood cells, neutrophils, eosinophils, macrophages,

osteoclasts, dendritic, Langerhans, and T cells from iPSCs in large quantities. Overall, these studies provided a platform for the development of bioreactor technology for blood production from iPSCs, and engineering tissue models for infectious diseases.

### Interaction between Macrophages and Decellularized Scaffold: a Triggering Event

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Macrophages play a crucial role in the remodeling process of tissue engineering [1]. These cells can exhibit pro (M1) or anti-inflammatory (M2) phenotypes dramatically influencing the resolution of the engineered graft [2], in particular for vascular tissue engineering, macrophages can furthermore promote the re-endothelialization of the graft [3]. Since decellularized scaffold could positively modulate the macrophage polarization towards anti-inflammatory and pro-remodeling phenotype, the aim of this study was to investigate the expression of pro and anti inflammatory markers involved in the inflammatory and re-endothelialization processes on arterial decellularized scaffolds.

Murine macrophages RAW 264.7 were cultured for 24 h and 96 h on decellularized arteries and on electrospun silk fibroin as control. The expression of pro- and anti-inflammatory genes and VEGFA was evaluated, since VEGF is involved in the recruitment of endothelial cells but at the same time it can promote intimal hyperplasia phenomena. Results showed that macrophages on decellularized scaffolds have a lower expression of IL-1 $\beta$ , IL-6, MMP9 and TNF- $\alpha$ , especially after 24 hours in comparison to silk scaffolds, while the expression of Arg1 and CD206 was higher for macrophages on decellularized arteries. VEGF-A expression showed different trends, decreasing on decellularized and increasing on silk.

These results suggest that decellularized scaffold promotes low inflammation with fast resolution, beside this high VEGF at 24 h promotes endothelialization while lower VEGF at 96 h could avoid intimal hyperplasia onset.

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### Production of a Biological Scaffold from Swine Aortic Valves and its Repopulation with Human Dental Pulp Stem Cells

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The heart is a vital organ that pumps blood allowing its circulation through the body. The aortic valve and their ventricular support structures form its centerpiece. All chambers of the heart are directly related to the valve, and its leaflets are directly incorporated into the heart skeleton. Failures in the aortic leaflets are the most common heart valves diseases, leading to negative impact on the patient's life, and increasing costs of health systems worldwide. The development of materials effectively capable of replacing aortic valve damaged tissue is of great interest in regenerative medicine and tissue engineering. The mechanical and biological valves have been widely studied, but several issues related to the integration to the host have not yet been resolved. Promising decellularization protocols for such tissue have been investigated as an alternative for the preparation of replacement material and to serve as a substrate for the repopulation of the matrix. However, some of the decellularization protocols used today have some limitations, since there is still no method that can achieve a complete decellularization while maintaining the structure of extracellular matrix for later repopulation, avoiding rejection after implantation and future replacement of the prosthesis. This study aims to evaluate the achievement of a biological matrix by decellularization of swine aortic valve and its repopulation with stem cells isolated from human dental pulp. So far, we tested three decellularization protocols. Protocols based on trypsin yielded a smaller amount of DNA when compared to the protocol where only detergent was employed.

### Tissue Engineered Trachea *In Vitro* Characterization

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The decellularized matrices obtained from the extracellular matrix (ECM) of cartilage, which contain the natural elements of the cartilage present good biocompatibility for the growth of chondrocytes (1). Theoretically, these scaffolds can be ideal for use in cartilage engineering, however, the process used to decellularization of this tissue is crucial, since all cellular material must be removed to avoid any type of immune response post-implantation, either way, the structural components from the ECM should be preserved to provide a scaffold with good biomechanical features and promote efficient invasion of tissue cells. For obtain a tracheal tissue decellularized scaffold, porcine tracheas were used, there was treated with physical-enzymatic methodology with ultrasonic treatment and enzymatic detergent with Ethylene-diaminetetraacetic Acid (EDTA), Trypsin, Sodium Deoxycholate, Sodium Dodecyl Sulfate and Tributyl Phosphate. This treatment was qualitatively evaluated by Scanning Electron Microscopy (SEM) and Histological techniques (DAPI, Sfranine-O, H&E). The Collagen type II distribution and alteration was following by immunofluorescence by anti-MHC-I and anti-MHC-II in frozen section. The scaffolding capacity of decellularized matrices was following during two weeks by human adipose tissue extracted MSCs seeding for *in vitro* behavior of the matrices trough immunofluorescence by anti-FAK, anti-SOX9, anti-Col II and anti-RUNX2. The thermal characterization of the decellularized scaffold was made using Termogravimetric Analysis (TGA) and Differential Scanning Calorimetric (DSC).

The microscopic evaluation and immunofluorescence showed that the decellularization process was effective. It was not alteration of the matrix structure after the process and the matrix is a good scaffold for cell adhesion and differentiation.

### New Approach of Methoxy Polyethylene Glycol (mPEG) Grafting for Islets of Langerhans Xenotransplantation

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Rejection of transplanted Langerhans islet by host immune system is a major problem in islet transplantation for diabetes mellitus, especially for diabetes mellitus, type 1. A proposed method to solve this problem is coating the surface of Langerhans islets by functionalized molecules. The purpose of this study was to obtain the best composition for mixture of two mPEGs with molecular weights (Mw) of 5 and 20, 5 and 10 kDa activated with succinimidyl valerate (mPEG-SVA) and succinimidyl carbonate (mPEG-SC), separately. The mixture consisted of two mPEGs with Mw of 5 and 20 kDa or 10 kDa. The amount of IL-2 secretion by host immune system, viability of Langerhans islets (Wistar rat) before and after reaction with host immune system (C57BL/6) and percent of reacted mPEG were considered as criteria for quantitative evaluation of islet surface PEGylation. In addition, the effect of hydrolysis half-life of mPEG-SVA and mPEG-SC on response of immune system at three stage of reaction with lymphocytes and viability of Langerhans islets before and after coating at each stage were evaluated. The result of this study

indicated that a mixture of two mPEGs of different Mw is better than a single polymer and PEGylation of islets at a composition of 75 and 25% of activated mPEGs with Mw of 10 and 5 kDa, respectively for camouflaging Langerhans islets against host immune system is the best choice. Also, coating of islet with mPEG-SVA, due to higher hydrolysis half-life with respect to mPEG-SC was more stable.

### Extracellular Matrix Remodeling During Retina Development *In Vitro*

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Tissue/organ development in human is a highly orchestrated complex process, regulated in part by the surrounding extracellular matrix (ECM) proteins and cell surface integrins. Every complex tissue in our body including retina has its own ECM configuration that plays critical role in cellular differentiation, adhesion, migration, and maturation. In this study, we applied a 3 D *in vitro* suspension culture system to direct differentiation of human induced pluripotent stem cells into primitive optic vesicles and studied the ECM remodeling during early events of retinogenesis.

Embryoid bodies (EBs) were formed from dissociated iPS cells and directed differentiation towards neuroretina development. EBs undergoing 3D *in vitro* differentiation started expressing different neural and eye field markers, including Otx2, Sox2, Rx, LHX2, Six6, and PAX6 at different time points of differentiation. Retinal progenitor marker, CHX10 was observed on day 16 onwards. Several cell adhesion family members like E-cadherin, ICAM1, CD11A, CD11B, VLA-6 were downregulated while neural and retina specific cell adhesion molecules NCAM1, NPRAP, VLA-1 and CD49D were upregulated. Several glycoproteins that were previously shown to play key role during retinogenesis namely Tenascin C, CD44, and fibronectin I were upregulated. Developing EBs were rich in laminin, collagen, and vitronectin.

Our findings show that developing optic vesicles in EBs expressed several ECM and adhesion molecules known to play critical role during retinogenesis *in vivo*. Stem cell derived *in vitro* model can help for further functional characterization of ECM proteins during development and identify the key matrix components required for retina engineering *in vitro*.

### Developing a Degradable Polymer Scaffold for Vascular Graft Tissue Engineering using a Novel Photocurable Poly(glycerol Sebacate)

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Poly(glycerol sebacate) (PGS) has been successfully used to produce polymer scaffolds for various soft tissue engineering applications. In the *in vitro* production of tissue engineered blood vessels, for use as vascular grafts, the elasticity and rapid degradation rate of this scaffold material has been associated with improved extracellular matrix deposition rates and quality, particularly relating to elastin fibres. However, manufacturing scaffolds from PGS requires the application of high temperatures and extended reaction times to crosslink and cure the polymer, limiting its utility.

Here, we have developed a novel photocurable form of PGS with improved processing capabilities: PGS-M. By methacrylating the secondary hydroxyl group of the glycerol units in the PGS polymer chain, the material is rendered photocurable and can be crosslinked rapidly on exposure to UV light at ambient temperatures. Our results have shown that both the polymer's molecular weight and the degree of methacrylation can be controlled independently and that the mechanical properties of the crosslinked material are critically dependent on these two parameters. The polymer has also demonstrated acceptable biocompatibility and rapid degradation under physiological conditions.

Using a mould-based porogen leaching method, porous tubular scaffolds have been produced from PGS-M with suitable structure, porosity and mechanical properties for use in vascular graft tissue engineering. There is also the additional potential to generate bespoke scaffolds using an additive manufacturing process. Future work is set to examine these scaffolds in vascular graft tissue engineering using a pulsatile flow bioreactor system.

### Effect of DC/PLGA Scaffolds on the Attachment and Proliferation of Costal Cartilage Cells

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Knee cartilage can easily occur functional damage as cartilage depend on aging and exercise. As well as cartilage is easily damaged from injury and cartilage regeneration has its limitation. Thus, tissue engineering approach is needed for cartilage regeneration. Biomaterial is classified as synthetic and natural polymer, widely used as drug delivery materials for tissue regeneration. Typically poly (lactic-co-glycolic acid) (PLGA) as synthetic polymer has attracted a lot of interests. But PLGA have the low rate of cell adhesion and causing the inflammation reaction. In this study, we carried out the possibility of the application of duck's feet derived collagen from livestock waste for cartilage regeneration and tissue engineering. Here in, we fabricated pore size of 90~180 um, 180~250 um, 255~350 um and 350~425 um using PLGA and duck feet derived collagen powder (DC) having loaded (80%) PLGA scaffolds. Three-dimensional scaffolds were prepared using solvent casting/salt leaching method. To analyze physical properties and morphology of DC/PLGA scaffolds, FT-IR and SEM were conducted. Assay of MTT are carried out to measured the attachment and proliferation of costal cartilage cells (CCs) in pore size of DC/PLGA composite scaffolds. RT-PCR was performed to confirm the cartilage specific genetic marker. To confirm extracellular matrix secretion on pore size of DC/PLGA scaffolds by histological staining. As a result, costal cartilage cells attachment and proliferation showed excellent in pore size of 255~350 um for 80 wt% DC/PLGA scaffolds. This research was supported by the Brain Korea 21 PLUS Project, NRF and Technology Commercialization Support Program (KMFAFF 814005-03-1-HD020).

### Expansion of Hematopoietic Progenitor Cells in a New Dynamic Co-culture System

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Several studies have now shown improved expansion of hematopoietic progenitor cells (HPC) in co-culture with mesenchymal stem cells (MSCs) or in cultures done on extracellular matrix protein (ECM)-coated surfaces. However, the majority of these expansion protocols remain static in nature, which partially limits the efficacy of expansion. The main objective of this work was to develop a dynamic culture system for UCB CD34+ cells that would include MSCs, an ECM proteins mix and dynamic medium flow. We first designed and validated a three dimensional two chambers flow circulation system connected together by a porous membrane. This set up was designed so that factors secreted by MSCs could reach and stimulate the growth of the hematopoietic cells. Next, we compared the efficiency of expansion of UCB CD34+ cells in static (control) or in the new dynamic flow chamber. No significant growth advantages were observed with the dynamic culture chamber when the ECM mix was placed between the porous membrane and HPCs, or when MSCs

were left out. However, a significant rise (2.3-fold) in total nuclear cell expansion was obtained when the ECM mix was positioned in the chamber containing the MSC. Similarly, the expansion of myeloid clonogenic progenitors was increased by 2.6- and 4.2-fold vs. static and dynamic control, respectively ( $p \leq 0.05$ ). The rise in progenitor expansion was most notable for the CFU-G/M/GM (4.7-fold) and CFU-GEMM (4.8-fold) subpopulations ( $p \leq 0.05$ ). In conclusion, we developed a dynamic co-culture system that can improve the expansion of HPC under flow.

#### Investigation of Proper Transplantation Site of Islet Cell Sheets for Treating Type 1 Diabetes Mellitus

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**Background:** We have previously reported an approach to create neo islet tissues under the subcutaneous site using islet cell sheets. However, the subcutaneous site was not optimal because of the poor supply of blood flow, thus requiring a large amount of islet cells. Here we investigate a more appropriate site for transplanting islet sheets, where lesser islet cells can result in normoglycemia.

**Method:** Dispersed rat islet cells ( $0.8 \times 10^6$  cells) were cultured on laminin-5 coated poly(N-isopropylacrylamid)-engrafted dishes. After 2 days of cultivation, islet cells were harvested as a uniformly connected tissue sheet simply by lowering the culture temperature, using a support membrane with a gelatin layer. Streptozocin was injected into SCID mice to induce diabetes, and then two islet cell sheets were transplanted into the subcutaneous site (subcutaneous group) or on the liver surface (liver surface group). Non-fasting blood sample was obtained periodically by tail snipping.

**Results:** The non-fasting blood glucose level of the liver surface group was decreased gradually within 7 days. In contrast, the subcutaneous group maintained the state of hyperglycemia. In the liver surface group, rat c-peptide was detected in serum, and a large number of insulin or glucagon positive cells were engrafted on the liver surface compared with the subcutaneous group, which was confirmed by immunohistological analysis.

**Conclusion:** This study demonstrated that the transplantation of islet cell sheets on the liver surface has a potential to become a novel therapeutic approach for type 1 diabetes mellitus.

#### Production of a Biological Scaffold Originated from Bovine Bone Tissue and Subsequent Repopulation with SHEDs

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According to the World Health Organization, there are around 150 illnesses, conditions and syndromes related to bone abnormalities. Skeletal insufficiencies linked to orofacial conditions are a public health issue. During the quadrennium 2008–2011, 20,985 ambulatory care procedures were performed in Brazil. The most common treatment options for severe bone loss are the grafting techniques, which may be classified as autogenous, xenogenous or alogenous according to its origin. Over the last decade, there has been growing interest in biological scaffolds composed by extracellular matrix derived from tissue decellularization, considering that the two main immune response generating antigens are DNA and the membrane oligosaccharide,  $\alpha$ -gal. Thus, our goal was to obtain demineralized and decellularized bovine bone tissue in order to produce hydrogels that would serve as biological scaffolds, and then repopulate it with stem cells isolated from human exfoliated deciduous teeth (SHEDs). The trabecular part of the bovine femur obtained from an abattoir was ground, demineralized and decel-

lularized. After such procedures, we obtained a fine powder, with DNA concentration below 50 ng per mg of dry weight of the matrix, which is recommended by the literature. Also, the agarose gel showed a smear, indicating that the remaining DNA in extracellular matrix was degraded. This way, demineralization and decellularization were successfully performed and the amount of DNA remaining in the matrix was inferior to what the literature recommends, as cited above. After the obtainment of hydrogels, we will recellularize it with (SHEDs).

#### Implantable Engineered Tissue Microenvironments for Studying Metastasis

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Circulating tumor cells are known to preferentially metastasize to certain organ sites. A large number of studies have outlined the metastasis mechanism, however there still remain questions regarding tissue specific tropism. We hypothesize that vascular anatomy, ensuing hemodynamics, and near tissue microenvironment are critical to the attraction of metastasis cells. To address this, we have engineered implantable tissue microenvironments using tunable porous hydrogel scaffolds that reproducibly form vascularized tissue analogues *in vivo*. Specifically, we subdermally implanted four different scaffolds with varying pore diameters (53–75, 150–180, 250–300, and 425–500  $\mu$ m) and polymer contents (5, 15, 30, and 50%) in a single host mouse. After 5 weeks, histological characterization of the implant revealed that scaffolds accommodated distinct vasculatures as a function of pore diameter and mechanical strength. We next simulated circulating tumor cells by intravenously injecting 1–2 million cancer cells. Six to twelve hours later we retrieved the implanted scaffolds and determined the correlation between vasculature anatomy and systemic migration of cancer cells. Our results indicate there could be a connection between tissue dependent vascular anatomy and the recruitment of specific types of circulating tumor cells. Engineered tissue microenvironments are expected to become a valuable platform for dissecting the metastasis mechanism and uncovering important parameters of the pro-metastatic niche.

#### *In Vitro* Cell Based Responses on New Mesh Knitted Scaffolds for Pelvic Organ Prolapse (POP) Treatment - Evaluation of Endometrial Mesenchymal Stem Cell (emsc) and Macrophage Behaviour

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Surgical intervention is required in 19% of women suffering from POP, often with mesh augmentation. Unfortunately, mesh-related problems and re-operations have necessitated new approaches to POP treatment. We are designing new mesh types with better mechanical properties, combining this with a new source of human stem cells isolated from endometrium (eMSC). We have animal models to evaluate this new tissue engineering approach, but we can also screen cell responses and behaviour *in vitro* as a rapid method to predict the host tissue response as well as the influence of the eMSC.

A number of mesh types were developed, primarily knitted from polyamide (PA) coated with a stabilised gelatin coating (PA+G). Uncoated PA meshes and commercial polypropylene meshes were used as controls. eMSC proliferated on the meshes, and could be differentiated to SMCs (with TGF $\beta$ 1 and PDGFB), or fibroblasts (with CTGF), appropriate for tissue elasticity and mechanical integrity. Human macrophages were derived from PMA (phorbol 12-myristate 13-acetate)-treated THP-1 monocytic cells and used either as un-activated M0 or M1 (pro-inflammatory) macrophages that were

activated with cytokines (20 ng/ml IFN $\gamma$  and 1  $\mu$ g/ml LPS). All meshes induced transient TNF $\alpha$  production indicative of M1 macrophages with lower levels of IL-10 indicative of a regenerative M2 macrophage. The balance of M1/M2 responses was further tested by modulation in the presence of eMSC. The results demonstrate that our new mesh types are highly suitable as scaffolds for seeding eMSC, for controlling stem cell behaviour and for examining immunomodulatory effects associated with putative host tissue responses.

#### Microfluidic Single Cell Encapsulated Microgels as Building blocks for 3D assembly of Cellularized Colloidal Gels

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Colloidal gels, a novel class of hydrogels that allow for a “bottom-up” approach for the design of biomaterials by employing biopolymer particles as building blocks, have recently emerged as an intriguing concept in regenerative medicine. These gels exhibit properties superior to traditional bulk scaffolds, including i) accurate control over scaffold’s properties, ii) strong capacity for controlled delivery of biomolecules, and iii) physically crosslinking particulate network. Importantly, the micro-architecture of colloidal gels is in line with the construction of human tissues that can be regarded as hierarchically organized cellular constructs, characterized by a complex microstructure based on assembly of building blocks made of cells and extracellular matrix. Inspired by this concept, the current research aims to develop a class of innovative, biomimetic colloidal gels by employing mesenchymal stem cells and alginate microgels as building blocks, to create 3D cellular constructs with complex structure and tissue-specific function. Hereby, single cell-encapsulated microgels are prepared by microfluidic technique, which allows for high cell viability, high throughput production of cell-laden microgels, and preparation of multicompartiment microgels as multifunctional cell carriers. By introducing strong interparticle bindings using click-chemistry, self-assembly and gel formation based on single cell contained microgels. Such cellular structures show great potential for engineering 3D tissue constructs that can steer cell fate and induce tissue regeneration.

#### Endothelialization of Engineered Coronary Artery Bypass Grafts using Bone Marrow-Derived Mesenchymal Stem Cells

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Tissue engineered vascular grafts (TEVG) have the potential to overcome many of the major limitations of coronary artery bypass grafting (CABG). However, current vascular implants do not efficiently form a confluent endothelium spontaneously. Sourcing of autologous endothelial cells (EC) for pre-seeding grafts may be feasible by use mesenchymal stem cells (MSC), which can differentiate into many cell lineages, including EC. Isolating MSC from the patient’s bone marrow is clinically feasible, but it does not yield enough MSC to create a monolayer. Therefore, sparse MSC seeding of the graft will be required for urgent CABG patients. We have successfully maintained cell retention of sparsely-seeded human MSC (purchased from Lonza and shown to exhibit classic tri-lineage differentiation) onto our completely-biological TEVG after 24 hr exposure to laminar shear stress (SS) of 12 dynes/cm<sup>2</sup> but not after 96 hr, indicating a slower ramping period is needed. Densely-seeded MSC were retained after exposure to SS for 24 hr and 96 hr. Expression of  $\alpha$ -smooth muscle actin in MSC evaluated by immunocytochemistry was decreased after 24 hr of SS in densely-seeded MSC, consistent with endothelial differentiation. This was not observed in sparsely-seeded MSC, implying cell-to-cell contact or longer SS exposure is required. Static culture in Lonza’s EGM-2 bulletkit and exposure to SS in endothelial basal medium (lacking EC growth factors) increased VEGFR2 and CD31 markers but not VE-

cadherin, suggesting a longer stimulus exposure time is needed for extensive MSC differentiation to EC.

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#### Application of a VEGF-binding Heparan Sulphate in a Murine Hindlimb Ischaemia Model

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Ischaemic vascular disease is a circulatory disorder where the blockage or narrowing of arteries reduces blood flow to major organs, accounting for more than 30% of all deaths annually. Current standard of care involves lifestyle changes or more dramatic surgical interventions, including angioplasty and stenting, bypass grafting or atherectomy; but, patients require long-term medication to minimise the risk of recurrence. Emerging strategies involving cell- or large molecule-based treatment have been developed, aimed at modulating the levels of vascular endothelial growth factor-165 (VEGF<sub>165</sub>), a potent blood vessel-forming factor. However, these strategies have not been approved for clinical use due to potential side effects. To address this shortcoming, we have engineered a heparan sulphate (HS) variant from porcine mucosal HS that increased VEGF<sub>165</sub> bioactivity resulting in enhanced blood vessel formation when applied to embryonic chorioallantoic membranes (Wang *et al.*, 2014). In this study the efficacy of HS was investigated in a murine femoral artery ligation model of hindlimb ischaemia. Recovery was assessed by time-of-flight magnetic resonance angiography and laser Doppler imaging. The data show a dose-dependent increase in blood volume in the effected limb following treatment with HS compared to control. This is particularly evident 8-days post-injury, highlighting the beneficial effects of HS administration immediately post-injury. These results help to demonstrate the clinical potential of modulating the activity of endogenous growth factors using glycosaminoglycan agents like HS, and further stress the importance of harnessing the effects of pro-healing factors generated at injury sites.

#### PCL/USC for Bladder Reconstruction

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The aim of this study was to investigate the synergic effect of human urine-derived stem cell (USC) and the heparin-immobilized basic fibroblast growth factor (bFGF)-loaded scaffold (composed with Polycaprolactone/Pluronic F127/3 wt% bladder submucosa matrix) for regeneration of the bladder smooth muscle and urothelial layer in a rat model. The surface modified scaffold (scaffoldheparin-bFGF) was analyzed for heparin immobilization efficacy, amount of released bFGF, biocompatibility and differentiation rate into smooth muscle and urothelial cells with urine-derived stem cells (USCs)

*in vitro*. For *in vivo* study, partial bladder wall cystectomy (about 40%) group, unmodified scaffold implanted group after cystectomy (scaffold), scaffoldheparin-bFGF implanted group and USC combined scaffoldheparin-bFGF group were tested. The cultured cell on the scaffoldheparin-bFGF showed significantly high biocompatibility compared to the unmodified scaffold. At *in vivo* study, USC-scaffoldheparin-bFGF group showed significantly increased bladder capacity and compliance compared to other groups. With histology and immunohistochemical analysis, regeneration of smooth muscle, multilayered urothelium, condensed submucosa layer and no host CD8 lymphocyte aggregation were observed at the USC-scaffoldheparin-bFGF group. The scaffoldheparin-bFGF allowed significant amount of heparin immobilization and bFGF loading resulting enhanced biocompatibility and differentiation rate *in vitro*. The USC combined scaffoldheparin-bFGF induced synergic effect, such as increase bladder capacity, compliance, histological reconstruction and reduced inflammation in a partial cystectomy rat model. Therefore, we proposed that USC-scaffoldheparin-bFGF could be an effective strategy for bladder reconstruction. (2014R1A1A3049460)

### Regulatory Considerations and Testing Strategies for Cell and Tissue Based Products During Product Development

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Recent trends in medical product development include many new medical products for which tissues and cells are the basis or components of novel therapies. This exciting technology has created new challenges for regulators to classify and enforce these products during the regulatory approval process. Classical device or biologic categorization often is no longer applicable for these therapies. New types of testing requirements and study design for regulated studies are often necessary during the non-clinical and clinical phases of testing. Once approved for market, additional challenges for product testing following manufacturing are also required. This presentation will review and explore recent trends and considerations for testing requirements for tissue based and combination products for regulated studies.

### Msc Mediated Endochondral Ossification Utilising Micropellets and Brief Chondrogenic Priming, Towards Scale-up

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With limited autologous and donor bone graft availability, there is an increasing need for alternative graft sources. We have previously shown chondrogenically priming mesenchymal stem cell (MSC) pellets for 28 days *in vitro* will reproducibly result in endochondral bone formation *in vivo*. However, the priming time is extensive for clinical applications and the resulting bone quantity is insufficient to fill large defects. In this manuscript we present a micropellet ( $\mu$ pellet)-fibrin construct aimed at the scale-up of bone formation quantity while decreasing the *in vitro* priming duration required. *In vitro* data showed expression of chondrogenic genes and matrix after 7 days chondrogenic priming, leading us to hypothesize that a brief priming phase could be used to induce bone formation *in vivo*. 7 day and 28 day cultured pellet, pellet-fibrin and  $\mu$ pellet-fibrin constructs were implanted subcutaneously into nude mice for 8 weeks.  $\mu$ pellet-fibrin constructs cultured *in vitro* for 7 or 28 days showed signs of advanced hypertrophy and formed comparable bone to standard pellets *in vivo*. Here we have demonstrated MSC mediated bone formation following only 7 days *in vitro* priming. Further shortening of this priming time coupled with an injectable system could lead to the development of a minimally invasive system to augment large bone defect healing.

### Multilayered Hollow Tubes for Cardiovascular Tissue Engineering

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One of the key concepts in tissue engineering (TE) strategies is the need for mass transport of nutrients and oxygen supply. In this work, we propose the creation of hollow tubes, as blood vessel substitutes, able to support endothelial and smooth muscle cells adhesion and proliferation. Hollow tubes will be obtained by building-up multilayers on sacrificial tubular templates using layer-by-layer technology and template leaching [1]. Tubes of chitosan and alginate multilayers were prepared with crosslinking and their physicochemical characterization was performed using different techniques. To optimize cell culture conditions, human endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were separately seeded on tubular structures. Since multicellular systems of ECs and SMCs are the main constituents of blood vessels, cell culture studies were then performed using a co-culture of both cell types. To perform the culture of HUVECs on the inner wall of the tubes and the HASMCs on the outer layer, we develop an home-made apparatus that was able to feed cells with their respective culture medium. Extensive biological characterization was assessed to evaluate the potential of these structures. The hollow tubes produced showed to be a suitable structure to promote cell adhesion and spreading, maintenance of phenotype, opening a new research field in order to develop innovative tubular structures for cardiovascular TE applications.

**References:** 1. Silva, J.M., *et al.* Advanced Healthcare Materials, 2014, 3(3): p. 433–440.

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### Functional Vascular Scaffolding System for Reconstructing Small Diameter Blood Vessels

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We have previously reported the development of vascular scaffolds fabricated by electrospinning of poly( $\epsilon$ -caprolactone) (PCL) combined with collagen, which could maintain standards such as high burst pressure strength, resistance to material fatigue, ease of suturing, and reliability and consistency of manufacturing. This vascular scaffold can be produced in a variety of dimensions with exceptional consistency in composition and physical properties. In this study, we hypothesized that the use of multiple bioconjugation of endothelial progenitor cells (EPC)/endothelial cell (EC) specific antibodies and anti-thrombogenic agents (e.g. heparin) onto the vascular scaffold can facilitate *in situ* endothelialization while preventing blood clotting. Toward this goal, we optimized bioconjugation parameters on a vascular scaffold to provide proper biological properties and structural configuration that enhanced cellular interactions of ECs on the lumen. To examine the functionalized vascular scaffold, the scaffolds were examined by flow chamber study using a parallel rectangular flow chamber system for cell capturing capability and platelet adhesion assay for anti-thrombogenic effect. The results showed that the functionalized scaffolds, bioconjugated with EPC/EC-specific antibodies (CD133, Flk-1, VE-cadherin) and heparin, were able to achieve effective EPC/EC capturing and anti-thrombogenesis. It has been demonstrated that the generation of the functional vascular scaffold system, along with existing tools for selection and directed immobilization of EPC/EC-specific antibodies and anti-thrombogenic agent, could provide the necessary components for successful tissue engineering of small diameter blood vessels.



### The Development of a Novel Device for Transplantation of Cell Sheets in the Field of the Oropharynx and Hypopharynx

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**Background:** With the development of image enhanced endoscopy and endoscopic diagnosis, the number of superficial cancer in the oropharynx and hypopharynx is increasing. The endoscopic submucosal dissection (ESD), endoscopic mucosal resection, or endoscopic laryngopharyngeal surgery is performed for these superficial squamous cell carcinomas. Because of complicated structure of pharynx, when ESD was performed for the lesion near the pyriform sinus, postoperative adhesion was observed. It impairs swallowing function and becomes the cause of aspiration pneumonia.

And similar to the esophageal ESD, postoperative stricture is observed when a large area of mucosa was removed. Some study showed the endoscopic transplantation of fabricated autologous epithelial cell sheets prevent postoperative stricture. It is expected same effect after the ESD of the oropharynx and hypopharynx. However, because of the narrow and complicated 3D structure of pharynx, we can transplant the cell sheets on only a limited area by using existing transplant method.

**Objective:** To develop a brand-new method for transplantation of the cell sheets after the pharyngeal ESD.

**Result:** Using the 3D printer, we developed a novel delivery device that was similar to a blow ball pipe. It could deliver the cell sheets without contacting the mouth and laryngopharyngeal mucosa, and pushed out by the air. It was smaller than the existing balloon device, so it could deliver the cell sheets on any areas of the pharynx. We performed pharyngeal ESD with this device in a porcine, and investigated the feasibility of cell sheet transplantation in the field of the oropharynx and hypopharynx.

### Nanosecond Pulsed Electric Fields (nsPEFs) Enhance Chondrogenesis of Mesenchymal Stem Cells (MSCs)

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Biophysical signals play a critical role in differentiation of mesenchymal stem cells (MSCs), however, they have not been routinely adopted in current differentiation medium. In previous study, we found that nanosecond pulsed electric fields (nsPEFs) regulated phenotype of chondrocytes via activation of Wnt/ $\beta$ -catenin signaling pathways[1]. In the current study, we have found that nsPEFs enhanced chondrogenesis of MSCs, partially through phosphorylation of P38-MAPK signaling pathways.

Primary Porcine bone marrow-derived stem cells (bMSCs) were subjected to 5 pulses of electric field (10 kV/cm or 20 kV/cm, 100 ns duration), before gene expression of COL I/II/X, aggrecan (AGG) and Sox9 were evaluated at day 3 and 14 respectively. Histology and quantification of glycosaminoglycan (GAG) was carried out after 21 days in culture. The protein of P38-MAPK and phosphorylation of P38-MAPK were evaluated by western blotting at 0.5 h after nsPEFs treatment.

Both nsPEFs at 10 kV/cm and 20 kV/cm significantly increased gene expression of COL II and Sox9 at day 3, however had little effect on the gene expression of COL I/X. 10 kV/cm nsPEFs increased COL II at day 3 (1.9-fold,  $p=0.01$ ). Interestingly, 10 kV/cm nsPEFs, increased AGG expression to 2.5-fold at day 3 ( $p=0.001$ ), however 20 kV/cm nsPEF did not. Inhibitors of P38 partially inhibited this effect.

nsPEFs significantly enhanced chondrogenic differentiation of MSCs with dose-dependent effects. Moreover, nsPEFs provide a biophysical stimulus independent of ligand-receptor response, which may potentially be used for clinical applications.

### Reference

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### Cathepsin-K Targeted Nanoparticles for Regenerative Repair of Elastic Matrix in Proteolytic Disease

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Abdominal Aortic Aneurysms (AAA) involve dilation and weakening of the aortic wall due to chronic breakdown of matrix proteins, such as elastin, via an overexpression of matrix metalloproteases (MMPs). Auto-regenerative repair of disrupted elastic matrix by smooth muscle cells (SMCs) at the AAA site is intrinsically poor and together with chronic proteolysis prevents buildup of new, intact elastic matrix to enable AAA growth arrest or regression to a healthy state. Oral doxycycline (DOX) therapy has been shown to inhibit MMPs to slow AAA growth, but has systemic effects and inhibits new elastin deposition within AAA tissue. We have thus developed cationic (DMAB)-modified poly(lactic-co-glycolic acid) (PLGA) NPs with pro-elastogenic and anti-proteolytic properties for localized, sustained DOX delivery within AAA tissue at a much lower dose that also augments elastin regenerative repair. To provide greater specificity of NP targeting, in this study we conjugated the DOX-NP surface with an antibody against cathepsin-K, a lysosomal protease that is highly overexpressed within AAA tissue. In TNF- $\alpha$ -stimulated cultures of rat AAA SMCs (EaRASMCS), we showed using western blot and immunofluorescence imaging that Cathepsin-K is highly overexpressed relative to unstimulated EaRASMCS and healthy rat aortic SMCs (RASMCs). A monoclonal cathepsin-K antibody (Abs) was conjugated to NPs using a) a covalent binding chemistry and b) physical adsorption. Spectrophotometric analysis of Alexa 546-tagged cathepsin-K conjugated NPs showed highest conjugation efficiency for a 5 h-covalent tagging. Release studies indicated an initial burst of DOX followed by a steady state release, while *In-Vitro* analysis indicated AAA marker decline.

### What Increases Firms' Dependence on External Organization in R&D? : The Case of the Regenerative Medicine Industry

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This research focuses on firms' decision makings in the regenerative medicine industry about how much they depend on external organizations in R&D. Dependence on external organizations enables for firms to reduce cost and time. However, they might face a difficulty to build competitive advantages, because competitors can also use them. The case analyses show that two factors influence their externalization in R&D: the speed of technology change and scale of economy in demand.

Firstly, technology change in the regenerative medicine is too fast for firms to judge which cells will generate sales the most in the future, because researchers might find a way to create a new pluripotent cell. Secondly, even after firms decide which cell to invest in, they face another difficulty to attain economy of scale, because they can't predict a probability accurately that injury and disease, which needs the cells to cure, happens. Therefore, firms tend to depend on external knowledge in basic research and to make alliance with external organization which provides customers with final products in business development.

In the present regenerative medicine industry, these two factors increase uncertainty for firms to get return from investment in R&D, and they use external organization to avoid useless investment in R&D more than competitors following. In order to promote for new entrants to start business in this industry, public research institutions are demanded to generate advanced knowledge to compensate for these private firms' decisions, and public investment in R&D is necessary to grow this industry.

### Aluminum Chloride as a Crucial Factor for Enhancing Specific Gene-Recombinant Adenovirus Transfer to Primary Stem Cells

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Various studies in gene therapy technology have developed in the decades. A common problem in the use of gene delivery is the limited factor of gene transfer by viral and nonviral vectors to targeted cells. Adenovirus (Ad)-mediated gene transfer to airway epithelia is inefficient because the apical membrane lacks the receptor activity to bind adenovirus fiber protein. Tat (Transcriptional activator) protein and calcium phosphate precipitates were known as a factor for delivery of exogenously human immunodeficiency virus to cells. On the other hand, other studies are reported their side effects in that Tat has cytotoxicity in high concentration and CaPi are not effective on many primary cells in gene transfer. To overcome these problems, we study the transgene expression of recombinant Ad (rAD) in the presence of AIC13 on mesenchymal stem cells (MSCs), adipose stem cells and periodontal ligament stem cells. In particular, AIC13 at lower value, 10 MOI in the optimal concentration of 10  $\mu$ M increased transduction efficiency at rAd-EGFP as well as showed low cytotoxicity on MSCs. MSCs transduced of rAd expressing specific genes, BDNF and BMP-2 were evaluated to confirm the stable expressions of both genes. MSCs transduced of BDNF released higher BDNF protein in medium and BMP-2 transduced MSC promoted significantly alkaline phosphatase activity higher than controls. We imply that these results demonstrated AIC13 to provide an innovative tool for MSC-based gene therapy in clinical use. Now, we're testing the influence of the later on bone healing with the gene-level tests and calvarial defect rat model.

#### Bio-Functionalized Alginate Hydrogels for Improved Cell-matrix Interactions and Growth Factor Sequestration Kinetics

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Alginate is a commonly used polysaccharide biomaterial for microencapsulation cultures due to its inherent ion exchange gelation characteristics. However, alginate does not contain motifs supporting cell adherence, limiting its use in cell-based applications. Here we describe a gelatin- and heparin- modified alginate with increased utility. First, the available carboxylic acid groups on the alginate chains were increased by carboxymethylation of hydroxyl groups. Next, gelatin terminal amines were conjugated to alginate carboxyl groups through EDCI coupling, and heparin carboxyl groups were conjugated to alginate carboxyl groups via PEG-diamine linkers, yielding alginate-gelatin-heparin (Alg-GH). Alg-GH retained gelation characteristics, supporting rapid formation of hydrogels. Three formulations were tested for cell adherence, with different alginate to gelatin ratios (4:1, 3:1, and 2:1 starting material), as well as alginate only. MSCs showed an increasing level of adherence, cell spreading, and presence of filopodia with increasing gelatin content. Additionally, cells seeded on or encapsulated within Alg-GH (2:1 formulation) showed increased presence of viable cells compared to alginate-only in both conditions, and proliferated over time. Lastly, Alg-GH hydrogels supported consistent growth factor retention and release levels, which were significantly greater than those in alginate only, demonstrating efficacy of the heparin modification. Finally, we found that we could tune the degradation rate of the hydrogel by controlling the concentration of calcium in the external milieu, allowing for recapturing of cells from culture. This work suggests that functionalization of alginate with bioactive components may yield more versatile materials for regenerative medicine applications.

#### Mechanism of BMP- and CaP-induced Bone Formation by Human Periosteum Derived Cells

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In skeletal tissue engineering, researchers aim to engineer constructs using the developmental biomimetic approach to heal critical bone defects. The combined interplay of osteoinductive biomolecules (BMPs), osteochondroprogenitor cells (human periosteum derived cells (hPDCs)) and osteoconductive carriers (CaP-scaffolds) represent a promising strategy. The aim of the current study was to investigate the mechanism of BMP-induced skeletal tissue formation by different BMP-ligands combined with hPDCs and CaP-scaffolds.

Based on the BMP receptor profile expressed by hPDCs we selected specific ligands for incubation in 2D cultures. mRNA transcript analysis displayed robust osteochondrogenic differentiation in the presence of BMP-2, -4 and -9. These were therefore coated onto CaP-scaffolds, BioOss<sup>®</sup> and CopiOs<sup>®</sup>, followed by hPDC-seeding. After 24 h, protein lysates were investigated for activation of BMP signaling pathways. Activated canonical BMP-signaling was induced by BMP-2, whereas BMP-4 mainly activated the non-canonical pathways. Upon *in vivo* implantation for 5 weeks, the most abundant ossicle formation was found by BMP-2-coated CopiOs<sup>®</sup>. Remnants of cartilage tissue could be observed in BMP-coated CopiOs<sup>®</sup> constructs suggesting endochondral bone formation and implanted cells actively contributed to the newly formed bone.

As a final step, hierarchical clustering displayed that distinct BMP ligands demonstrate different potencies and mechanisms in inducing osteochondrogenic differentiation both *in vitro* and *in vivo*. Subsequently, these differences can also be reflected in the *in vivo* setting, where both the choice of BMP as well as the CaP-scaffold affects cartilage and bone formation in a quantitative as well as qualitative manner.

#### 3D Bioprinting of Spatially Graded PCL/Hydrogel Constructs for Cartilage Tissue Engineering

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Mesenchymal stem cell (MSC) loaded hydrogels offer a promising route for treating focal cartilage defects. While such hydrogels have been shown to support chondrogenesis of MSCs, their mechanical properties are generally poor, which is a major limitation when attempting to regenerate large osteoarthritic joint surfaces. Developments in bioprinting technology have enabled the co-deposition of hydrogels with a PCL backbone to engineer mechanically reinforced constructs, although it is still unclear as to what the optimal hydrogel is in terms of both printability and MSC chondro-inductivity. The aim of this study was to compare the chondro-inductivity and printability of a number of hydrogels (Alginate, BioINK<sup>™</sup>, Gelatin methacrylamide (GelMa) and Agarose). We then sought to spatially modulate the PCL fiber spacing through the depth of 3D printed PCL/hydrogel constructs in an attempt to recapitulate the depth-dependent mechanical properties of native articular cartilage.

After 4 weeks in chondrogenic conditions alginate and agarose supported enhanced chondrogenesis (sGAG, Collagen II deposition). GelMa supported the highest level of MSC proliferation, however the resulting tissue was more fibro-cartilaginous in phenotype (Collagen I deposition). Hydrogel printability was assessed by plotting a square grid and measuring the post-printing line spreading, with alginate demonstrating superior printability. Finally an anatomically accurate (tibial condyle) construct was printed with a spatially varying PCL backbone. The depth-dependent mechanical properties of the resulting composite was comparable to native articular cartilage (Deep-Middle-Superficial Zone: 1514 KPa-1007 KPa-589 KPa).

This study demonstrates that alginate is a suitable hydrogel for 3D Bioprinting reinforced MSC-laden grafts with mechanical properties compatible with putative joint resurfacing strategies.

#### A Replenishable Cell Delivery System for the Heart

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Cell delivery to the infarcted heart is an exciting and promising therapy, but poor cell survival and retention necessitates multiple, invasive administrations of cells. In the present study we developed an approach to enable non-invasive multiple replenishments of biological therapy to the heart, and demonstrate its function in a pre-clinical rodent model. A biomaterial-based reservoir, a gelatin cryogel initially seeded with 1 million luciferase expressing mouse mesenchymal stem cells, was attached to the epicardial surface of the infarcted rat heart. An implantable catheter was used as a conduit between this reservoir and a subcutaneous port. The biomaterial reservoir could be refilled with cells through the port at defined time points, increasing the resident cell number 10-fold, and we demonstrated that this system can achieve multiple dosings. The ability of the system to allow targeted injection of molecular therapies directly to the biomaterial reservoir in contact with the heart was also demonstrated via delivery of the imaging substrate D-Luciferin through the subcutaneous port. In summary, we present a delivery system that allows targeted, replenishable and sustained presentation of cellular therapy to the heart. This system provides a platform for other therapeutic strategies (small molecules, proteins) and delivery to other diseased tissues. This delivery system applies dosage form design to cell therapy, and will simplify cell dosage analysis for the treatment of cardiac disease.

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#### Marrow-Isolated Adult Multilineage Inducible (MIAMI) Cells Embedded within Gelatin-Based Electrospun Nanofiber Scaffolds for the Treatment of Peripheral Arterial Disease

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Peripheral arterial disease (PAD) is one of the major vascular complications that affects elderly. Current treatments involve high risk invasive procedures such as bypass surgery or angioplasty. In this study we focused on the use of MIAMI cells, which are a highly homogenous sub-population of multipotent mesenchymal stromal cells (MSCs) [1]. The MIAMI cells are seeded on electrospun gelatin nano-fiber scaffolds with tunable architecture (random/aligned orientation) which we believe will promote angiogenesis and aid in treating PAD [2, 3].

Metabolic activity and oxygen consumption were assessed. Electrospun gelatin scaffolds were fabricated using a custom made apparatus. Each scaffold (area of 0.8 cm<sup>2</sup>) was seeded with 1 × 10<sup>5</sup> MIAMI cells and cultured in 21% O<sub>2</sub> for 3 weeks in endothelial differentiation medium. Calcein AM staining was assessed at 10 and 21 days to assess cell morphology and viability. At 21 days, the cellular scaffolds were fixed in formaldehyde and stained for CD31 to assess endothelial differentiation.

We were able to confirm that MIAMI cells from older donors lose their stem cell like behavior as they consume more oxygen and are more metabolically active. Analysis of CD markers confirmed a reduction in embryonic stem cell markers as MIAMI cells age. Seeded MIAMI cells on scaffolds remained viable for up to 21 days and they differentiated to endothelial lineage.

These data reinforces the use of electrospun scaffolds as a delivery vehicle for stem cell therapies and highlights the importance of fiber orientation to enhance angiogenic potential.

#### Collagen Scaffolds with Adaptable 3D Topographical Features made by Lyophilization

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Regenerative medicine aims to restore damaged tissues. Therefore, construction of scaffolds and incorporation of cells and biologicals have been widely investigated. Scaffolds provide templates guiding tissue formation and have considerably impact on cellular behavior. Intrinsic surface topographies, i.e., have been shown to direct cellular differentiation. Lyophilization allows construction of scaffolds with tunable pore architectures (size and orientation) by adapting freezing conditions. In this study, it was investigated whether 3D topographical features in collagen scaffolds could be influenced by freezing using various diluted acids.

Insoluble type I collagen fibrils (0.7% w/v) were suspended in different concentrations (0.05, 0.25, 0.5, 1 and 2 M) of monocarboxylic acids (methanoic, ethanoic and propanoic acid) and dicarboxylic acids (ethanedioic, propanedioic, butanedioic and pentanedioic acid). Collagen suspensions were frozen at -20°C and -80°C and subsequently lyophilized. Scaffold structures were characterized using scanning electron microscopy and pore sizes were measured.

Scaffolds showed variations in scaffold morphologies including pore size, wall thickness and wall structure. The use of dicarboxylic acids resulted in acid specific differences in pore structures, whereas monocarboxylic acids did not result in remarkable structural differences, taking diluted acetic acid results as a reference. Dicarboxylic acids with an even or uneven number of C-atoms resulted in frayed or smooth wall structures, respectively, although with different appearances. Ice crystal formation is key to the specific morphologies observed and modulation of crystal growth by dicarboxylic- or other acids allows generation of micrometer-defined topographies.

Biological responses of cells to scaffolds with distinct morphologies are currently being investigated.

#### Enabling the Visualization of an Engineered Endothelium on ePTFE Vascular Grafts via Magnetic Resonance Imaging

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**Objective:** Super-paramagnetic iron oxide nanoparticles (SPIONs) have been used as a contrast agent to enable magnetic resonance imaging (MRI) of many cell types. It is a powerful tool to non-invasively monitor cell distribution in tissue engineering and regenerative medicine. However, SPIONs can induce the formation of reactive oxygen species (ROS), changing the redox state in the cell and contributing to deleterious changes in cell function. The objective of this research was to assess whether the use of antioxidant biomaterials will inhibit SPION-induced ROS generation and enable the visualization of an EC monolayer on a vascular graft.

**Methods:** Chitosan and poly(1,8-octamethylene citrate) (POC) were used to modify the surfaces of SPIONs and ePTFE, respectively. ECs were labeled with chitosan-coated SPIONs (CSPION) or SPIONs and cultured on POC or tissue culture polystyrene. The

following processes were assessed: The effects of CSPION and POC on ECs, including cell iron uptake, morphology, functions and ROS formation. T2-weighted MRI was used to image EC-seeded POC-ePTFE vascular grafts with CSPION labeling.

**Results:** CSPION-labeled ECs cultured on POC-coated surfaces mitigated SPION-induced ROS formation and maintained EC morphology, phenotype, viability and functions. Moreover, the hydrophilic POC coating of the ePTFE allows water infiltration within the porous walls of hydrophobic ePTFE, and reduces imaging artifacts caused by air. A monolayer of labeled ECs seeded on POC-ePTFE exhibited sufficient contrast with T2-weighted MR imaging to enable visualization.

**Conclusion:** The combination of CSPION and POC modification of ePTFE grafts enables the successful visualization of EC-seeded ePTFE grafts with T2 contrast enhancement.

### Ocular Surface Regeneration using Bioengineered Cell Construct

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Cornea functions as a protective barrier of the eye that contributes to clear vision. Corneal epithelium is maintained by stem cells in limbus located at the corneo-scleral junction. The loss of limbal stem cells due to variety of reasons lead to limbal stem cell deficiency (LSCD), leading to corneal opacity and loss of vision. Conventional mode of treatment by allogeneic transplantation of cornea is limited due to immune rejection, shortage of organ and possibility of infection. This necessitates the development of bioengineered corneal constructs for LSCD. This study investigates the use of a thermoresponsive copolymer poly (N-Isopropylacrylamide-Co-Glycidylmethacrylate) [NGMA] as a cell culture substrate for developing bioengineered corneal construct using different cells and assessing *in vivo* efficacy using a rabbit model. The NGMA exhibits phase transition at 28–30°C by shifting from hydrophilic to hydrophobic. NGMA dishes were used to culture limbal stem cells and the cell sheet construct spontaneously lifted up by incubation below 28°C. Rabbit limbal stem cell deficiency model was created by 360° excision of limbus. Bioengineered corneal construct developed from limbal stem cells was transplanted to LSCD eye and followed upto 1 year. The regeneration was evaluated by histological and immunohistochemical methods in comparison with control. The treated eye expressed markers such as  $\alpha$ Np63, ABCG2, CK3/12 and vimentin indicating proper regeneration of tissue. The results have proved that cell sheets can be synthesized by growing the limbal cells on the novel NGMA and can be used in LSCD condition for ocular surface regeneration.

### Design of Disease Models using Tissue Engineering Principles

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Tissue engineering technology platforms have resulted in innovative strategies to re-create a cell's naturally occurring niche to study normal and pathological cell-cell and cell-niche interactions. Biofabrication is used to design and produce scaffolds that feature tissue-like structures. Biomaterials such as hydrogels mimic crucial properties of the extracellular matrix that provide mechanical support

and anchorage to cells. Hence, cells can be seeded onto biofabricated scaffolds and within biomimetic hydrogels to replicate a tissue-like architecture observed in real life, providing physiologically relevant 3D culture conditions and cell behaviors.

The focus of our research is to engineer a species-specific niche to investigate processes promoting disease progression and drug responses, with particular emphasis on prostate and ovarian cancer. Biofabricated scaffolds are seeded with human osteoblast and human mesothelial cells to model bone-like and peritoneal microenvironments respectively. To re-construct an *in vivo* disease model, these humanized niches are implanted into an animal host together with human cancer cells to allow homing of these tumor cells to their disease-specific niche. We developed a humanized xenograft model of prostate cancer bone colonization in tissue-engineered bone. We also used a hydrogel-based ovarian cancer model to assess protease-mediated tumor growth and peritoneal spread.

Tissue engineering principles have allowed us to establish pre-clinical cancer models harboring the complexity of the disease seen in patients in order to advance our understanding of the underlying cancer cell biology and the contribution of the tumor's surrounding milieu. By incorporating patient-derived cells, we will be able to screen drugs in a personalized medicine approach.

### Spheroidal Culture of Tonsil-derived Mesenchymal Stem Cells for Parathyroid Regeneration

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Tonsil-derived mesenchymal stem cells (TMSC) have been reported to differentiate into parathyroid-like cells releasing parathyroid hormone (PTH) using activin A and sonic hedgehog. Here, we found that the implantation of three dimensional (3D-) spheroidal culture of differentiated TMSC (dTMSC) into parathyroidectomized (PTX) animal model increased survival rates and restored serum PTH levels compared with that of undifferentiated, control TMSC (cTMSC). Four sets of 3D spheroidal cultures performed in the concave microwell plates made of poly dimethylsiloxane (PDMS) for 14 days immediately after 2D-culture for 7 days were as follows: 1) SP1, cTMSC 2D-cultured for 7 days and 3D for 14 days; 2) SP2, cTMSC 2D-cultured for 7 days and then differentiated into dTMSC in 3D-culture for 14 days; 3) SP3, cTMSC 2D-cultured for 7 days and 3D for the first half of 7 days and then differentiated into dTMSC for the second half; 4) SP4, dTMSC 2D-cultured for 7 days and 3D for 14 days. Although spheroids obtained from 4 experimental sets were comparable to each other in the size and viability, the highest levels of PTH expression were found in groups SP2 and SP3, which were indistinguishable, and thus SP3 were taken in this study. Among PTX rats subcutaneously injected with SP1, SP3 or SP4, we found that those of SP3 showed the highest survival rates of 50% over 3 month periods, and restored physiological range of serum PTH and ionized calcium levels. Our results represent the feasibility for the use of spheroidal culture of TMSC in efficiently curing hypoparathyroidism.

### Blood Compatibility of Poly(vinyl Alcohol) Hydrogel with Topographical and Biochemical Modifications

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The lack of hemocompatibility, mechanical properties matching native arteries, and ability to induce *in situ* endothelialization cause small diameter vascular graft failure. Thus, there is a need for new synthetic vascular grafts that are compliant, hemocompatible and endothelialize. Poly(vinyl alcohol) hydrogel (PVA) is a good candidate as a vascular graft due to its hemocompatible and tunable

mechanical properties, yet its bio-inertness must be addressed to improve endothelialization. Both biochemical and topographical modifications are used to stimulate specific endothelial cell behaviours by mimicking the cellular niche. Thus, we hypothesize that the modification of PVA with biochemical and topographical modifications can provide a microenvironment that stimulates endothelialization without compromising hemocompatibility. By modifying PVA surface with cyclic RGD (cRGD), we achieved an increase in the adhesion of human umbilical vein endothelial cells (HUVECs). The cRGD modified PVA did not exhibit any significant change in platelet adhesion, activation and morphology when compared with unmodified PVA using *in vitro* hemocompatibility assays. Unmodified and cRGD-modified PVA were patterned with microgratings or microlens topography. Combination of cRGD and micro-topography on PVA showed significant increase in HUVEC viability compared with unpatterned and unmodified PVA. Platelet adhesion on all PVA with microgratings was comparable with unpatterned and unmodified PVA. Platelets also changed from the fibrous, activated phenotype on PVA with topography to round and less active phenotype on PVA with both cRGD and topography. The *in vitro* HUVEC adhesion tests and hemocompatibility tests demonstrated that modification of PVA with both biochemical and topographical cues to improve endothelialization retains hemocompatibility.

### Improvement of Decellularization Efficiency of Porcine Aorta by using Dimethyl Sulfoxide as Penetration Enhancer

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A number of artificial vascular graft materials have been in clinical field for the replacement of failed blood vessels. In addition to synthetic graft materials, decellularized vascular grafts have attracted great attention due to their innate high biocompatibility, especially the composition and architecture of extracellular matrix (ECM). Currently, most approaches to tissue decellularization are based on chemical treatment methods where ionic, non-ionic detergents or enzymatic agents are applied to remove cellular components. Nearly all-chemical decellularization protocols are based on excessive and time consuming procedures, which can take up to several weeks for thick tissues, e.g. aorta due to the dense ECM which prevents the agents' penetration. It was reported that the ionic detergent SDS (Sodium Dodecyl Sulfate) effectively removes cells and nuclear remnants but generates harsh effects on ECM structure when prolonged exposure time. In this study, we decellularized porcine aorta by using DMSO (Dimethyl sulfoxide) as a penetration enhancer aiming to shorten the decellularization time. Our results show that this new protocol considerably reduced the exposure time of the SDS treatment while preserving important ECM components such as glycosaminoglycan and collagen compared to just SDS treated aortas. DNA assay and DAPI stain clearly revealed the effect of the DMSO on cell removal. In addition, biocompatibility tests performed by Alamar Blue and Live/Dead Assays on seeded valve interstitial cells on the aorta indicated high cell attachment to the scaffolds. As conclusion, the new decellularization process not only reduces exposure time of harsh chemicals but also protects the crucial ECM components.

### In Situ Oxygen Delivery to Islets within an Implantable Immunisolating Device *In Vivo* Enhances their Viability and Function

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Allogeneic islet transplantation is emerging as a promising treatment option for Type 1 Diabetic patients. Large-scale clinical islet transplantation is currently restricted by the limited supply of human islets and need for systemic immunosuppression. The use of immunoisolation devices may address both limitations by allowing the use of allogeneic stem cell derived islets or xenogeneic porcine islets with little to no immunosuppression. Devices, such as the TheraCyte, have been extensively tested and demonstrate allo-protection without immunosuppression in animal models and humans.

However, efficacy in humans becomes an issue when the devices are densely loaded to achieve a reasonable size (credit card as opposed to a large flat screen TV). In this study, we utilize a modified TheraCyte device to test the effects of enhanced oxygen delivery on islet function and viability post subcutaneous transplant of densely loaded devices in a rat model. Devices were transplanted containing human (20,000–30,000IE) or rat (allogeneic model, 2500IE or 625IE) islets for 30 days.

Results demonstrate that without oxygen supplementation, devices transplanted with human and rat islets were unable to reverse diabetes and had abnormal intraperitoneal glucose tolerance tests (IPGTT). However, under the same conditions when devices were supplemented with electrochemically generated oxygen (pO<sub>2</sub>=300 Hg), diabetes was reversed and IPGTTs were normal. In the allogeneic model, IPGTTs were normal with a sub-marginal dose (625 IE), demonstrating the critical need for oxygen supply to densely loaded devices *in vivo*; suggesting that a fraction of the dose of islets can be used with oxygen supplementation.

### Identification and Characterization of Neurotrophic Factors in Porcine Small Intestinal Submucosa

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Small intestinal submucosa(SIS) is a natural degradable biomaterial derived from the small intestine of vertebrates. Porcine SIS has been widely used in repairing various tissues and organs, especially in nervous system. In this study, we proposed the isolation method to get an acellular SIS then major growth factors which are able to regulate neuronal development within SIS accompanying with decellularization was quantitatively assessed by ELISA.

Fresh porcine small intestines were obtained from healthy pigs. SIS was submerged in degrease solution and decellurization was done. The components of SIS were extracted with PBS and sterilized. The contents of representative growth factors including BDNF, erythropoietin, GDNF, NGF, PDGF were quantified by ELISA method.

The mean concentration of BDNF was 20±9.5 pg/ml, erythropoietin (EPO) was 56.0±19.2 pg/ml, GDNF was 195.3±60.9 pg/ml, NGF was 22.6±15.1 pg/ml, PDGF was 119.6±39.0 pg/ml.

The effect of SIS on neurite transformation was evaluated by embedding nerve cells (PC-12), a model system for neuronal differentiation, in the presence or absent of the SIS extract or PBS alone, time dependently. Compared to PBS control, the response of the cells to extracted SIS was quite distinctive showing an increased neurite formation in 3 and 4 day.

Decellularized SIS was obtained by sequential procedures; mechanical disassociation, degrease, enzyme digestion, freeze-drying, freezer-mill, extraction, concentration and filtration. BDNF erythropoietin, GDNF and PDGF was identified in the extract of SIS. Furthermore, extracted SIS was functionally working in neurite outgrowth of PC12 cells.

### Ectopic Reconstruction of Endometrium-like Tissue using Cell Sheet Technology

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**Introduction:** Endometrial defect lead to female infertility disturbing embryo implantation and development. Therefore, the endometrial regeneration is essential in the field of fertility treatment. The present study aimed to examine the function of ectopically reconstructed endometrial tissue using cell sheet technology.

**Materials and methods:** Endometrial cells were isolated from 4-week-old female SD-Tg(CAG-EGFP) rats and cultured for 2 days using a temperature-responsive culture dish. Endometrial cell sheets harvested by lowering temperature were transplanted subcutaneously into ovariectomized nude rats preparing estrogen-administrated and non-administrated groups.

**Results:** At 6 weeks after transplantation, GFP-positive tissues were observed in transplant sites. In the estrogen-administrated group, immunohistochemical images of endometrium-like tissues showed estrogen receptor  $\beta$ -positive epithelial layer and progesterone receptor-positive stromal layer. Furthermore, the surface of epithelium was calcitonin positive, which affected the implantation.

**Conclusion:** This study demonstrated that functional endometrium-like tissues in the estrogen-administration group were fabricated by endometrial cell sheets. Therefore, the endometrial cell sheet was speculated to be useful for the regeneration of functional endometrium.

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**Disclosures:** Teruo Okano is a director of the board of CellSeed Inc. Tatsuya Shimizu is scientific consultant for CellSeed Inc. These 2 authors are the stakeholders of the company and are inventors of cell-sheet-related patents. Tokyo Women's Medical University is receiving research fund from CellSeed Inc.

### Novel Cornea-mimetic, Collagen Based, Tissue Engineered Biomaterial

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There is an increasing clinical need for biomaterials suitable for corneal replacement, in lieu of donor tissue. A novel collagen I based biomimetic biomaterial has been developed in our lab, which exhibits high transparency, suturability and cornea-mimetic collagen fiber alignment. Molecular interactions between collagen and cyclodextrins (CDs) were utilized in order to achieve biomimetic ultrastructure and desirable macroscopic physical properties. Additionally, the properties of the membranes were modulated by functionally modified CDs.

Vitrigel membranes were prepared as previously described (Biomaterials 34 (2013) 9365–9372) with minor modifications to incorporate CDs as well as modified CDs with varying functional moieties and hydrophobicity. Membranes were processed and stained with uranyl acetate for TEM imaging. Membrane transparency was measured in the visible spectrum from 300–700 nm. Tensile testing on membranes was performed to evaluate Young's modulus and suturability.

Biomimetic lamellar structure and collagen fiber alignment was achieved through process modulation and use of functionalized CDs. Membrane transparency was similar to healthy cornea. Stress-strain curves indicate tunability of Young's moduli of the membranes as well as high values of strain at break, corresponding to increased suturability. *In vitro* Keratocyte and epithelial cell cultures on membranes and short term *in vivo* study demonstrate membrane biocompatibility and ease of suturability.

CD modified Collagen membranes demonstrate unique cornea-mimetic properties, and higher order self-assembly.

### Corneal Collagen Matrix Coated with Poly(p-xylylene) (PPX) for Keratocyte Orientation

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Corneal diseases are a worldwide problem and amongst treatment methods is the use of human donor tissues, artificial, non-biological cornea transplants, both of which are deemed insufficient solutions [1]. Furthermore, due to the limitations associated with allografts, collagen-based matrices have been prepared. Criteria that such scaffolds are expected to fulfill include: biocompatibility, mimicry of natural tissue structure, transparency, resilience toward culturing conditions, allow for fixation via suturing, exhibit flexibility suitable for the replication of eye curvature and structurally allow for the transfer of oxygen and nutrients. Transparency is known to be associated with the alignment of keratocytes and collagen fibres [2].

The aim of this study was to develop a method by which the alignment of keratocytes could be directed, thereby reducing the opacity of the prepared synthetic scaffolds. The collagen matrices were firstly coated with varying widths of a biocompatible and hydrophobic polymer, Poly(p-xylylene) (PPX). A pre-study conducted by our group found that proteins displayed attachment to PPX coated areas. Fluorescent microscopy analysis revealed an increase in the concentration and alignment of proteins at PPX zones. Based on these preliminary results, keratocytes were implanted on collagen-PPX. This approach proposes a facile and effective route for corneal cell alignment and it has potential for similar tissue engineering applications.

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### Therapeutic Angiogenesis by Short-Term Co-Delivery of Fibrin-Bound VEGF and PDGF-BB Proteins

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Therapeutic angiogenesis by Vascular endothelial growth factor (VEGF) delivery suffers from 2 major limitations: at least 4 weeks of delivery are required to avoid vessel regression, but sustained and uncontrolled expression can cause angioma growth. We found that the maturation factor Platelet-Derived Growth Factor-BB (PDGF-BB) can prevent aberrant angiogenesis by VEGF gene delivery. To overcome safety concerns of gene transfer, here we investigated the delivery of VEGF and PDGF-BB proteins from a state-of-the-art matrix-bound system, based on the transglutaminase (TG) reaction to bind the modified factors into fibrin hydrogels.

TG-engineered VEGF164 and PDGF-BB were cross-linked into fibrin hydrogels and different doses of each, both together or no factors (control) were injected in limb muscles of SCID mice. We found that: 1) gels were degraded in 10 days in all conditions; 2) by 2 weeks, TG-PDGF-BB completely normalized aberrant angiogenesis by high TG-VEGF doses, yielding only mature and functionally perfused capillary networks, with PDGF:VEGF ratios as low as 1:20; 3) 10 days of TG-PDGF-BB co-delivery with both low and high VEGF doses caused stabilization and long-term persistence of new vessels, whereas >90% regressed with VEGF alone; 4) in a mouse

hindlimb ischemia model, co-delivery at 1:20 ratio (50 µg/ml VEGF), but not either factor alone, caused 2-fold increases in both microvessel density and collateral arteries after 4 weeks, fully restoring blood perfusion.

Therefore, controlled co-delivery of TG-VEGF and TG-PDGF-BB proteins provides a convenient (off-the-shelf), safe (no genetic modification) and clinically applicable approach for therapeutic angiogenesis with a short-term 10 days' treatment.

#### Rapamycin enhances Sox9 Expression during Chondrogenic Differentiation of Amniotic Fluid Stem Cells

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**Purpose:** Human amniotic fluid stem cells are a suitable resource as donor cells for articular cartilage repair. Here we show that AFS differentiation to chondrocytes is enhanced via rapamycin treatment and we aimed at elucidating the mechanism of this process.

**Methodology:** We performed three dimensional pellet culturing of AFS cells in the presence of chondrogenic factors and treated cells with the mTORC1 inhibitor rapamycin for 14 days. We analyzed the generated tissue by real-time PCR, western blotting and immunohistochemistry. To elucidate the beneficial effects of rapamycin, we combined treatment with chemical hypoxia inducers and with siRNA mediated silencing of potential effector genes.

**Results:** Rapamycin treatment during AFS cell differentiation to chondrocytes enhanced Sox9 protein abundance and increased Sox9 target gene expression. Importantly, rapamycin treatment also induced Hif2a, but not Hif1a protein amounts. Finally knockdown of Hif2a reversed the effect of rapamycin treatment during chondrogenic differentiation of AFS cells.

**Conclusion:** Rapamycin has been shown to elicit a cytoprotective function in chondrocytes and to reduce the development of osteoarthritis. Here we have shown that rapamycin evokes an effect similar to hypoxia inducing agents during chondrogenic differentiation of AFS cells. Moreover we have shown that HIF2a is crucial for the rapamycin mediated effect. Therefore we suggest that mTORC1 inhibition contributes to chondrogenic differentiation via mimicking a hypoxia like effect during AFS cell differentiation.

#### Cell Sheet Transplantation Ameliorates Cardiac Dysfunction of Infarcted Infant Hearts by Cardiomyocyte Proliferation and Angiogenesis

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**Background:** For adult hearts, cardiac regenerative effect of cell sheet transplantation has been reported in animal experiments and clinical trials. But there have been no animal studies on cell sheet transplantation for infant hearts. The aims of the present study were to clarify the effect of cell sheet transplantation on infant heart, to expand the options for clinical therapy in infant heart failure.

**Methods and Results:** Infant rats of 2-week-old and adult rats of 12-week-old were used, and myocardial infarction (MI) was induced as the heart impairment model. Infant hearts after MI had better self-regenerative ability in wall thickness and fibrosis; and cardiac function was better compared to adult hearts; and we observed greater numbers of proliferating cardiomyocytes than in adults. Next, infant MI rats were treated with tissue-engineered myoblast sheet transplantation. The effects of the therapy on infant hearts with MI were thickened wall, inhibited fibrosis, improved cardiac function, prolonged period of proliferating cardiomyocytes, increased vasculature network and accumulated c-kit positive cells. On the other

hand, there was little effect of cardiomyocyte proliferation on adult MI hearts.

**Conclusions:** Cell sheet transplantation ameliorates cardiac dysfunction of infarcted infant heart; moreover, the cell sheet transplantation applied to infant heart may have more therapeutic effect than to adults, because of their cardiomyocyte proliferation potential. The present study provides essential new data for clinical therapy in infant patients.

#### Ex vivo Platelet Production from Hematopoietic Stem Cells: Molecular Signaling Directing Proplatelet Formation

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Producing platelets *ex vivo* from hematopoietic stem cells (HSCs) is not yet therapeutically viable due to low platelet yields per input HSC. HSC-derived megakaryocytes (Mks) are the precursors to platelets. During maturation Mks become polyploid and extend long, branched cytoplasmic protrusions termed proplatelets, which are sheared by blood flow to produce platelets. Typically less than 30% of Mks in culture display proplatelet formation (PPF), greatly limiting the number of platelets produced.

Mature Mks in the bone marrow vascular niche that extend proplatelets into sinusoids experience a variety of input signals from the extracellular matrix and cell-cell interactions. To better understand the molecular mechanisms driving PPF, we are using a novel assay, TRACER (Transcriptional Activity Cell aRays), which utilizes luciferase reporters to non-invasively provide dynamic readouts of transcription factor (TF) activity in Mk cultures. We are using the CHRF megakaryocytic cell line as a model system. CHRF cells cultured on ultra-low adhesion surfaces that inhibit protein adsorption do not exhibit PPF until transfer to a tissue culture (TC) surface where PPF occurs within 12–24 hours. We have evaluated activity of several TFs upon transfer to TC surfaces, and found that NF-E2 activity is consistently upregulated on the TC surface. This is consistent with the established importance of NF-E2 for Mk maturation and PPF. We are extending this assay to TFs associated with cytoskeletal organization and integrin modulation. Identification of key TFs will guide design of *ex vivo* culture systems to improve PPF and ultimately platelet yield.

#### Mesenchymal Stromal Cells in Bone Defect and Diabetic Wound Treatment: Preclinical Evaluation

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Mesenchymal stromal cells (MSCs) represent a useful therapeutic approach for treating various diseases, their safety and efficacy having been demonstrated in numerous clinical trials. In this study, human MSCs were prepared under GMP conditions and evaluated preclinically in order to be used in autologous manner for treatment of bone defects and diabetic wounds.

The capacity of hMSCs to heal bone was examined in a clinically relevant model of temporal bone defects in pigs. The defect was filled with an osteoconductive material alone or in combination with hMSCs (2 × 10<sup>6</sup> cells). 30 days after implantation, the temporal bone was examined by histological, immunohistochemical and CT scan analysis.

The potential of hMSCs to heal full-thickness wounds was examined on streptozocin-induced diabetic rats. Three types of cells: hMSCs, isolated from bone marrow (2 × 10<sup>6</sup> cells), adipose tissue (ASCs; 2 × 10<sup>6</sup> cells) or bone marrow mononuclear fraction (BMC;

2×10<sup>5</sup> progenitor cells) were transplanted into the wound area and histologically and immunohistochemically evaluated after 14 days.

No adverse reactions occurred in any animal following MSC implantation. Newly formed bone occupied a significantly larger surface of the original defect size in pigs implanted with MSCs. In the wound healing model, wound size was significantly reduced in all groups, including controls. However, rats implanted with MSCs, ASCs and BMC displayed accelerated and complete wound closure compared to controls.

From a clinical perspective, MSCs represent a safe and efficient method of treating bone defects and diabetic wounds.

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### Effects of Epigallocatechin-gallate, an Antimicrobial Crosslinking Agent, on Proliferation and Differentiation of Human Dental Pulp Cells Cultured in Collagen Scaffold

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**Objectives:** The purpose of the study was to evaluate the efficacy of epigallocatechin-gallate (EGCG), an antimicrobial crosslinking agent, on proliferation and differentiation of human dental pulp cells (hDPCs) cultured in collagen scaffold.

**Methods:** The differentiation or proliferation was analyzed by alkaline phosphatase (ALP) activity, real time-polymerase chain reaction (PCR), alizarin red staining, and confocal laser scanning microscope. The morphological feature of hDPCs cultured in EGCG-treated collagen was evaluated by scanning electron microscopy (SEM). The antimicrobial effect of EGCG was assessed by disc diffusion assay. For the assessment of mechanical properties of collagen treated with EGCG, the surface roughness and compressive strength were measured.

**Results:** The proliferation and differentiation of hDPCs cultured in collagen scaffold increased significantly in the presence of EGCG. Antimicrobial activity of EGCG on several bacterial species was similar with that of glutaraldehyde although cytotoxicity of EGCG was significantly lower. The mechanical properties such as surface roughness and compressive strength of crosslinked collagen were higher than those of uncrosslinked collagen.

**Conclusions:** Our results showed that EGCG promotes proliferation and differentiation of hDPCs. Furthermore, the enhanced mechanical properties of collagen scaffold induced by EGCG may play important roles in cell fate. Consequently, the application of EGCG might be new strategies for regenerative endodontic treatment.

### Mimicking the Human Trabecular Bone Niche to Promote Spinal Fusion

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Currently, the most effective surgical approach for spinal fusion requires the use of BMP-2-infused materials. However, the massive doses of BMP-2 generally utilized are associated with severe side effects to the healthy tissues surrounding the implant. Herein, we propose to bypass these limitations through a new class of biomaterials able to reproduce the chemical, physical and topographical osteogenic stimuli of the human osteogenic niche. These materials are synthesized through a biologically inspired mineralization process which recapitulates the chemical and spatial mechanisms that the organic matrix of bone uses to direct the nucleation of the mineral phase. The resulting composites are used to fabricate scaffolds that not only promoted a more efficient differentiation of human mesenchymal stem cells, but also enabled the *in vivo* formation of bone

tissue, regardless of the site of implantation. When implanted in a rabbit ectopic site, the entire volume of the scaffolds generated trabecular bone in only 2 weeks, without the use of heterologous cytokines or cells. If implanted in an orthotopic site (rabbit spinal fusion model), the composites promoted new bone deposition, formed trabecular bone, completely integrated with the vertebral processes, and initiated the remodeling into cortical bone, in only 6 weeks. These results were achieved in about a third of the time usually required by the classes of biomaterials currently available. Our study represents a significant step forward in the development of acellular, growth factors-free, off-the-shelf substitutes for bone augmentation.

### Feasibility Study on the Use of Quantum Cell Expansion System to Treat Limb Non-Unions

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Mesenchymal stromal cells (MSCs) are an appealing source to manage fracture non-unions. However, *ex vivo* expansion and osteogenic differentiation must be optimized. New bioreactors have been developed to generate a large number of MSCs in good manufacturing practice (GMP) conditions. The Quantum Cell Expansion System (Terumo BCT) is a closed and automated system that releases a human grade product [1]. This bioreactor has 11,500 hollow fibers giving a 2.1 square meter surface area. We previously tested a pilot cell therapy for limb non-unions [2]. We aim at applying this therapy to a larger number of orthopedic patients, using the Quantum System. We investigated possible alternatives to fetal bovine serum (FBS) that allowed both sufficient MSC expansion and good capability of terminal osteogenic differentiation. Platelet lysate and pooled human sera were selected comparing traditional flask-based methods with the bioreactor cultures. Cytofluorimetric analysis and multilineage differentiation capability were assessed. The highest MSC yield was obtained in the platelet lysate samples. However, the MSC multilineage differentiation capability was reduced. So far, we hypothesize that the human sera could be a valid alternative to FBS for MSC expansion to be used in orthopedics.

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### The Use of Innovative Scaffolds in the Development of Corneal Stroma-Derived Stem Cell Therapies for Future Corneal Regeneration Strategies

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There are 1.5 million cases of corneal blindness diagnosed annually, but only 100,000 corneal transplants performed. This is predominantly due to poor access to quality donor tissue. Therefore, alternative effective regenerative therapies are required. We have



developed a bankable corneal stroma-derived stem cell (CSSC) source, for use in corneal regeneration. This follow-on work compares the feasibility of substrates to act in combination with CSSC, for the development of ocular surface regenerative strategies.

CSSC extracted from human corneoscleral rims were expanded on three substrates: (i) natural amniotic membrane-derived matrix (OmnigenTE™, NuVision™ Ophthalmics); (ii) Ologen Collagen Matrix (OCM; Aeon Astron B.V.); and (iii) Dual nano- and micro-fiber electrospun poly(lactic-co glycolic) acid (PLGA) scaffolds. CSSC expansion, proliferation, phenotypic marker profile and structural remodelling on the different substrates were assessed.

CSSC on OmnigenTE formed a monolayer, and maintained a mesenchymal stem cell phenotype (CD73+, CD90+, CD105+, CD34-), when cultured on the basement membrane. CSSC infiltrated the pores within the OCM and PLGA microfiber scaffolds, and a keratocyte phenotype was induced (CD34+, ALDH+, ACTA2-) leading to deposition of collagen and proteoglycans on the scaffolds. Addition of corneal epithelial cells onto the pseudo-basement membrane nanofiber layer of PLGA further restored normal epithelial-stromal interactions.

This work demonstrates the ability of different substrates to induce phenotypic changes in CSSC. CSSC-OmnigenTE has potential to become a topical stem cell therapy (biological stem cell bandage) for acute trauma medicine. OCM and electrospun PLGA induce a keratocyte phenotype and thus are more suitable for long-term partial replacement strategies for corneal transplantation.

#### Effect of Mesenchymal Stem Cell (MSC) Aggregation on Large Bone Defect Repair

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Engineering delivered cells to secrete paracrine factors necessary for the healing process might enhance the potency of cell-based therapeutics for large bone defect repair. Recent work has shown that the secretion of immunomodulatory factors by human mesenchymal stem cells (hMSCs), including interleukin-6 (IL-6) and prostaglandin E2 (PGE2), is augmented by three-dimensional cell aggregation. Our objective was to investigate the effect of MSC aggregation on cell-based bone regeneration within a critically-sized rat femoral defect, hypothesizing that MSC spheroid delivery would improve functional bone repair.

hMSC aggregates transplanted subcutaneously into an immunocompromised rodent model (Nude rats) showed similar cell viability and construct vascularity compared to the implantation of single cells. When challenged within a bone defect model, all treatments (MSC aggregates, single cells, and hydrogel vehicle alone) exhibited comparable regenerated bone tissue volume and mechanical properties. Interestingly, biological variability was identified as a significant predictor of regenerated tissue metrics and motivated the decision to evaluate MSC aggregates within a syngeneic model (Lewis rats). Rat MSC (rMSC) spheroids exhibited immunomodulatory factor secretion *in vitro* consistent with hMSC spheroids. Delivery of rMSC aggregates attenuated bone defect repair at 4 weeks *in vivo*. Ongoing work will analyze regenerated bone tissue metrics at 12 weeks and perform complementary studies to better understand this effect.

MSC aggregation alters immunomodulatory function, positioning spheroid delivery as an alternative cell-based platform for critically-sized bone defect repair.

#### Pre-vascularization of Natural Extracellular Matrix Scaffold

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Vascularization is one of the critical obstacles facing tissue engineering. A functional vascular network with well-formed capillaries is critically important in supporting three-dimensional (3D) tissue survival *in vivo*. We have created a completely biological and highly aligned nanofibrous extracellular matrix (ECM) scaffold that was derived from a human dermal fibroblast cell sheet. The prevascularized ECM scaffold holds great potential in creating prevascularized 3D tissues when multiple scaffolds are layered or combined with other cells and/or biomaterials. Our objective was to create a prevascularized ECM scaffold by cocultivating endothelial cells (ECs) and human mesenchymal stem cells (hMSCs) in ECM scaffold. It was expected that the ECs formed microvessels, while hMSCs functioned as pericytes to stabilize the microvessels. In the present study, a physiologically low oxygen tension (2% O<sub>2</sub>) was applied to pretreat hMSCs to stimulate their secretion of proangiogenic factors. Human umbilical vein endothelial cells (HUVECs), a model type ECs, were then cocultured with hMSCs in the scaffold under the normal oxygen (20% O<sub>2</sub>) condition for up to 7 days. Results showed that more branched and mature vasculature networks were formed in the low oxygen pretreated samples. Also, when compared to the untreated samples, the proangiogenic factors VEGF, bFGF, and Ang1 were 1.7, 1.2, and 1.1 fold higher, respectively. Moreover, more hMSCs around the microvasculature were found in the proliferation stage for the low oxygen pretreated samples, suggesting that the low oxygen pretreated hMSCs can better stabilize the microvessels. This prevascularized ECM scaffold holds great promise for engineering 3D prevascularized tissues.

#### Functional Characterization of a Novel Protocol for Encapsulation of Pancreatic Islets within a Conformal Layer of Poly(Ethylene Glycol)-Based Hydrogel

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Type 1 Diabetes is an autoimmune disease with no currently available therapies for a definitive reversal of the pathological phenotype. Among available therapies, pancreatic islet transplantation is a promising strategy, although the requirement of life-long systemic immunosuppression and the limited number of islet donors reduces its widespread application. In this context, islet encapsulation is a promising strategy to create a physical barrier to prevent islet destruction by the recipient immune system. Furthermore, the encapsulation material can be exploited as a substrate to conjugate immunomodulatory antibodies or bioactive molecules in order to enhance the immunoprotection activity and to drive targeted immunosuppression. In this study we developed a protocol for coating murine islets with a conformal layer of poly(ethylene glycol) (PEG)-based hydrogel by employing a photopolymerization method that leads to single islet encapsulation and provides a suitable substrate for targeted functionalization. To validate the biocompatibility of our approach, we assessed encapsulated islet viability and functionality *in vitro*, and we analysed possible gene expression variations. Our encapsulation protocol preserved islet viability and did not affect significantly expression of apoptotic and hypoxic marker genes. The physiological responsiveness of encapsulated islets to glucose-stimulated insulin secretion was confirmed both at mRNA and protein level. Moreover, effects of the coating on islet architecture were investigated by analysing cytoskeletal morphology and expression of cell-cell and cell-matrix interaction marker genes, observing no substantial differences between encapsulated and control islets. Concluding, our encapsulation protocol provides a safe and biocompatible approach to encapsulate pancreatic islets, thus suitable for targeted functionalization.

### Mesenchymal Stem Cells (MSCs) Improve Beta Cell Function within Biomimetic PEG Hydrogels

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Cell transplantation is a promising strategy in clinics for severe diseases of organs, such as pancreas. However, limited graft survival and serious immunosuppression requirement remain as the major challenges for this approach. These limitations could be mitigated through encapsulation of insulin secreting cells in 3D biomimetic scaffolds that incorporate MSCs. In this study, we first developed pseudo-islets from individual min6 cells, next coencapsulated pseudoislets with mesenchymal stem cells (MSCs) in biomimetic PEG hydrogel scaffolds that include native extracellular matrix proteins. MSCs promoted glucose dependent insulin release significantly. Our results demonstrated that cell survival and insulin release from pseudoislets were both improved significantly. PEG hydrogel scaffolds around pseudoislets would function as a mechanical barrier against immune proteins and immune cells, where MSCs may provide additional protection from small radical species due to their antiapoptotic properties. This improvement in coencapsulation of islets and stem cells would be significant *in vivo* and could further contribute to graft tolerance. Our studies are underway to further characterize the immune suppressive properties of MSCs in this biomimetic platform.

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### Toward “Rainbow Retinas” that Dynamically Report on Retinal Cell Differentiation in Patient-Specific Stem Cells

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Efficient generation of high-quality retinal cells from patient derived induced pluripotent stem cells is a major challenge in the use of these tissues for regenerative therapy. Here we aim to improve directed differentiation protocols for neuroretinal tissues via the use of fluorescent reporter constructs that faithfully report the dynamic expression of important neuroretinal cell fate markers. Specifically, we employ CRISPR/Cas9 gene editing and homology directed repair to introduce a fluorescent reporter construct at the orthodenticle homeobox 2 (OTX2) locus. OTX2 is a key regulatory transcription factor that plays an important role in retinal cell fate determination. Our techniques used to increase the efficiency of cellular delivery of CRISPR/Cas9 machinery and to increase donor construct integration will enable the creation of a library of cell fate marker reporter lines. These reporter lines provide an advantage over fixed cell immunostaining as they can be employed in high throughput screening of directed differentiation protocols to increase yields and purity of clinically-relevant retinal tissues.

### Fluid Shear Stress Effects on Three-dimensional Culture and Drug Sensitivity of Ewing Sarcoma

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By replicating key aspects of the tumor niche, three-dimensional (3D) tumor models provide a way to delineate the effects of the microenvironment upon tumor progression. Most such models overlook the effect of mechanical stimulation, of proven importance in mechanically-stressed tissues like bones and sarcomas from which they derive.

In this study, we cultured Ewing Sarcoma (ES) cells from a pediatric bone tumor on a porous electrospun poly( $\epsilon$ -caprolactone) 3D scaffold within a flow perfusion bioreactor. ES cells were cultured under flow perfusion in media with different viscosities to isolate the effects of fluid shear stress while minimizing effects on mass transport. We hypothesized that flow perfusion would model physiological levels of shear stress present *in vivo* and influence ES cell phenotype and drug sensitivity.

Cells were analyzed for DNA content, protein expression, and sensitivity to insulin-like growth factor-1 receptor (IGF-1R) targeted therapies, given the crucial role this receptor plays in ES tumorigenesis and progression. The shear stress induced by flow perfusion stimulated IGF-1 ligand production and enhanced ES sensitivity to IGF-1R inhibition. Further, it downregulated the c-KIT and HER2 oncoproteins when compared to static conditions, and induced a more physiologically relevant ES phenotype. A mechanistic interpretation for shear stress-mediated regulation of IGF-1/IGF-1R axis is provided.

The mechanotransductive forces within this tissue-engineered tumor model led to profound changes in drug sensitivity and cell phenotype. By modeling the interplay between architectural and mechanical cues, and deciphering their relevance in acquired drug resistance, we may identify new therapeutic strategies that target the tumor niche.

### A Minced Tissue Model for Tissue Expansion with Minced Autologous Tissue for Urological Reconstructive Surgery

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In previous studies we created a urinary conduit in an *in vivo* animal model for bladder emptying with minced autologous urothelium. To further develop the conduit, we compared cell regeneration and tissue expansion with biografts of urothelium alone or urothelium together with detrusor muscle in *in vitro* and *in vivo* studies. Porcine minced tissue, 0.3×0.3 mm, of urothelium only or urothelium and detrusor muscle was seeded by plastic compression in collagen-biografts for *in vitro* cultivation of 2–3 weeks. *In vivo*, we transplanted the same type of cells on 3D cylinder moulds into the subcutaneous fat of the pig abdominal wall in a one-step procedure for harvesting the urinary bladder tissue and transplanting back, terminating after 4–5 weeks. The expansion rate was 1:3. Outcome was measured by histology of the luminal surface and underlying tissue. Shams without minced tissue were used as controls. *In vitro* cultivation demonstrated a single cell-layer of urothelium after 2 weeks. No other cells could be detected. There was no morphological difference between samples when comparing urothelium only to urothelium with detrusor. In *in vivo* transplantation studies, minced urothelium only demonstrated a multi-layered transitional urothelium but not when transplanting minced urothelium together with detrusor muscle nor in shams. These samples demonstrated that angiogenesis was greater with higher grade of inflammation. Minced tissue models can be used to expand urothelium. It is easy and fast to perform *in vitro* or *in vivo*. *In vivo*, there was no improvement on urothelial outcome when seeding together with detrusor muscle.

### Chitosan Methacrylate Coated 3D Printed Alginate Hydrogels for Tissue Engineering

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Bioprinting is the state of the art technology in biology, engineering and medicine. Designing and fabricating complex constructs by printing living cells is the decisive step in tissue engineering. Furthermore, bioprinting allows to create multi-cellular constructs where cell-cell and cell-material interaction mimic the physiological environment [1].

In this study a new approach to biofabricate 3D construct loaded with HMSCs suitable for cartilage and bone tissue engineering is proposed. The 3D construct is composed of chitosan-methacrylate (ChiMA) - coated - alginate fibers. ChiMA coating confers an higher stability to alginate fibers due to electrostatic interactions between the two oppositely charged polysaccharides [2] and to the photocrosslinking of its vinyl moieties [3]. This approach allowed to form stable 3D biocompatible and bioprintable scaffolds. The fabricated materials were characterized in terms of mechanical and physical properties. To further highlight the advantages of 3D bioprinting approach, the variations in cellular response between conventional scaffold seeding and bioplotting techniques were investigated.

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### A 3D Stratified Colon Model for Colorectal Cancer Progression

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Recent investigation of colorectal cancer (CRC) metastasis has identified the tumor microenvironment as a large proponent of metastasis. Factors such as tissue stiffness and cell-cell interactions have been targeted as affecting cancer cell phenotype, proliferation and drug susceptibility. To study these effects *in vitro*, we aim to develop a micro-facsimile of colonic microstructure using cellularized hydrogel (collagen type I (ColI), hyaluronan (HA)) strata that mimic colonic tissue layers. Strata are layered into a polydimethylsiloxane (PDMS) microfluidic device to generate an intestine-on-a-chip model for CRC progression. Submucosal tissue is fabricated using rabbit colonic smooth muscle cells (RCSMCs) suspended in ColI/HA hydrogel. RCSMCs show increased proliferation, propensity for extracellular matrix (ECM) remodeling, and ability to align fibrillar ECM components in softer ColI hydrogels with lower concentrations. RCSMCs also demonstrated normal phenotype in fibrillar-material containing hydrogels, such as ColI/HA, in comparison to digested hydrogel formulations, such as gelatin/HA, where RCSMCs appear less viable. Colonic epithelium is fabricated using confluent CACO-2 cell monolayers grown on HA hydrogels. CACO-2 cells display characteristic cuboidal morphology and increased proliferation on stiffer HA. Tumorigenesis is simulated by seeding HCT116 cells in the epithelial or submucosal strata (representing different cancer initiating sites). HCT116 cells undergo epithelial-to-mesenchymal transition when grown in high stiffness tissue, demonstrating a highly migratory phenotype. Our CRC model can further be tested by modulating the ECM and cellular content of each tissue strata to further replicate *in vivo* conditions, and used to explore the effects of the microenvironment on tumor phenotype and response to anti-cancer drugs.

### A Comparison of Degradable Hydrogels for Endochondral Bone Tissue Engineering

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Cartilaginous tissues engineered using mesenchymal stem cell (MSC) seeded hydrogels have been shown to form bone *in-vivo* by executing an endochondral programme offering a promising route for bone regeneration. However undegraded hydrogel material can impede vascularisation and bone formation in central regions of larger constructs, potentially limiting the capacity of such tissue engineered constructs to regenerate large bone defects. The objective of this study was to compare the capacity of a number of hydrogels including alginate, a low molecular weight gamma-irradiated alginate ( $\gamma$ -alginate), gelatin-methacrylamide (GelMa) and BioINK<sup>TM</sup> to support endochondral bone formation *in-vivo*. MSC laden hydrogels were chondrogenically primed *in vitro* and implanted subcutaneously in nude mice for 6 weeks to assess their capacity to support conversion of cartilaginous templates into bone *in vivo*.

After 4 weeks *in vitro*, all hydrogels were found to support differing degrees of chondrogenesis. After 6 weeks *in vivo* all hydrogels supported some levels of endochondral bone formation, with both alginate hydrogels supporting significantly higher levels of mineralization compared to GelMa & BioINK ( $\mu$ CT). Accelerating the degradation rate of the alginate hydrogel via gamma-irradiation enhanced endochondral bone formation compared to unmodified alginate (Col I&X, H&E). Although the total level of mineralization was lower, GelMA supported more diffuse bone formation through the depth of the constructs ( $\mu$ CT). In conclusion, the success of endochondral bone tissue engineering strategies will depend on the specific hydrogel or scaffold used to engineer the hypertrophic cartilage precursor.

### Antimicrobial Biologic Scaffold for Pelvic Tissue Function Reconstruction

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The propylene scaffold used in clinic femal pelvic organ prolapse (POP) carry an inherent risk of sepsis formation, erosion related to their biomechanical properties and low biocompatibility. Biologic scaffold materials composed of mammalian extracellular matrix (ECM) are thought to be a better strategy for the POP repair and functional reconstruction. However, bacterial adhesion to biomaterials remains a major problem in the implant site. This study developed a novel multiple biological scaffold materials with excellent antimicrobial property for pelvic tissue function reconstruction. The ECM membrane was produced by Grandhope Biotech Co., Ltd. Then the antimicrobial biologic scaffold was prepared by immobilizing of chitosan/tigecycline nanoparticles onto ECM micro/nano-surfaces. The antibacterial test *in vitro* showed that antimicrobial biologic scaffold had long-acting antibacterial effect with more than 15 days. The antimicrobial biologic scaffold was proved to good cell compatibility by evaluation of cell toxicity test and cell proliferation assay. Using propylene scaffold as control, the antimicrobial biologic scaffold of biocompatibility evaluation *in vivo* was implanted in the goat abdominal wall for 6 months. The scaffold and the tissue surrounded were harvested for HE staining and immunohistochemistry. The slight inflame reaction viewed through the total experiment period. The MMP9 expressing level of the antimicrobial biologic scaffold was obviously higher than the propylene scaffold. It was indicated that the new collagen organization was fast reconstruction in the antimicrobial biologic scaffold. The novel antimicrobial biologic scaffold would be hope for a new generation of implant materials of pelvic tissue function reconstruction.

### Sizing Up Genetic Modulators of Contact-Inhibition of Locomotion using a Micropattern-Based Model of the Fibrillar Breast Tumor Microenvironment

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One hallmark of cancer that enables tumor cells to metastasize is the ability to circumnavigate other cells within the tumor

microenvironment (TMEN), a feature known as the loss of contact inhibition of locomotion (CIL). Recent evidence implicates the organization of the fibrillar TMEN as a physical regulator of metastasis during breast cancer progression. Although the interaction between migrating cancer cells and fibers is well studied, it is unclear whether cells confined in a fibrillar TMEN will exhibit CIL and how physical and molecular perturbations modulate this behavior. To quantitatively understand how CIL is regulated in a fibrillar context, we utilized high aspect ratio micropatterned surfaces because cells confined on these patterns exhibit striking similarities to cells migrating on fibers *in vivo*. We examined the influence of PARD3 expression, E-cadherin expression, ErbB2 activation, and TGF $\beta$ -induced EMT on the ability of a cell to slide past a neighboring cell. We quantified the effect of these various perturbations by defining a simple metric, the characteristic fibrillar-like dimension (CFD), as the width at which intermediate sliding is achieved. Our results demonstrate metastasis-inducing perturbations reduce the CFD, with multiple perturbations having a more profound influence than individual perturbations. Additionally, these findings suggest the CFD is an effective metric to understand the effects of various perturbations on the loss of CIL. Taken together, these findings provide insight into the interplay between the physical effects of the dynamic breast TMEN and the molecular effects of metastasis-promoting genes.

#### Low Adhesive Scaffold Collagen, Inducing Spheroid Formation, Promotes the Osteogenic Differentiation

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**Background:** Collagen has biocompatibility and biodegradability with tissue or organ, therefore, collagen is the most promising material for tissue engineering. In particular, the binding of collagen to specific cells is considered an essential function to develop scaffolds. However, in some cases the binding inhibits the cell motility. We succeeded in developing low adhesive scaffold type I collagen (LASCOL) (patent pending). In this study, we report that LASCOL markedly facilitates osteogenic differentiation of rat marrow mesenchymal stem cells (rMSCs). Furthermore, we investigated the effects of bone wound healing by implanting LASCOL in a defect of rat tibia.

**Methods:** The culture dish was coated with LASCOL or atelocollagen. Subsequently, rMSCs were cultured on each coated-dish with osteogenic basal medium. To evaluate osteogenic differentiation, we quantified the mineralization by SEM-EDX. Furthermore, we transplanted collagen materials into the defect of rat tibiae ( $\phi$ 2.5 mm critical-sized defect).

**Results:** Rat MSCs formed spheroid bodies by culturing on the LASCOL coated dish. We observed that spheroids adhered to LASCOL scaffold. Each cell of spheroid highly expressed alkaline phosphatase activity. The mineralization of rMSCs was significantly promoted by culturing on LASCOL. Only spheroid cultured on LASCOL showed the activity of mineralization by staining with Alizarin red S reagent. We demonstrated that LASCOL implant would accelerate bone regeneration.

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#### Culture Methods for Primary Adult Rat Cardiomyocytes

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Heart failure (HF) is a leading cause of death among adults and occurs when the cardiovascular system is unable to meet the meta-

bolic and respiratory demands of the body. It has a complex disease progression, incorporating cellular, molecular, neurohumoral, and physiological changes aimed at maintaining perfusion and function. If prolonged, these compensatory mechanisms cause maladaptive remodeling in the heart. Cardiac fibroblasts, cardiomyocytes, and extracellular matrix (ECM) all contribute to this process. An accurate *in vitro* model for HF should integrate these components to understand the mechanisms leading to disease pathology. Established protocols for long-term culture of cardiac fibroblasts and neonatal myocytes exist. However, primary isolated adult myocytes have demonstrated low adherence, low survival, and altered morphology and function in culture. The addition of certain supplements in the media, such as EGTA, creatine, carnitine, and taurine, have shown potential for overcoming these challenges. Considerations include preventing infection, buffering, ionic composition, and nutritional supplementation. Particularly important is optimizing calcium concentration, as cultured myocytes are ideally quiescent to prevent terminal contraction, gross morphological changes, and cell death. By experimenting with such media components, we have identified a media type which, thus far, best preserves morphology and function. Cell viability, morphology, and functionality is analyzed using live/dead assays, immunocytochemical staining, and isoproterenol treatment. After 24 hours in culture, 65% of myocytes were viable, a majority showed characteristic morphology and were functionally contractile. Further research of media components and manipulation of culture conditions could provide a viable option for long term cardiomyocyte culture.

#### Live Cell-scaffold Printing using Biodegradable PDLA-PEG/Hyaluronic Acid Copolymer for Cartilage Tissue Engineering

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**Objectives:** We have recently applied projection stereolithography (PSL) in a one-step live cell-scaffold fabrication (1), but it is currently limited by the nature of the PEG material which is not biodegradable and does not supply cell binding ligands. In this study, we tested the combination of poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLA-PEG), as the structure polymer, and hyaluronic acid (HA), as the co-polymer to supply cell binding ligands, for PSL fabrication of human adipose stem cell (hASC)-laden cartilage scaffold.

**Methodology:** hASCs were isolated from lipoaspirate with Institutional Review Board approval (University of Pittsburgh and University of Washington). Synthesis of methacrylated PDLA-PEG and HA were performed by reacting with methacrylic anhydride. The fabricated scaffolds were cultured in control medium or TGF- $\beta$ 3-containing chondrogenic medium for 4 weeks. Cell viability was examined at different time points and chondrogenesis of hASCs within the scaffolds was assessed by mechanical testing, real time RT-PCR and histological staining.

**Results:** After fabrication, hASCs showed high viability throughout the constructs, which possessed high mechanical property with a compressive modulus of 780 kPa. Without chondroinductive supplements (Control group), cell viability decreased with time and concomitant poor cartilage matrix deposition, as revealed by Alcian blue and Safranin O staining. In chondrogenic medium treated group (TGF- $\beta$ 3 group), hASCs maintained viability, proliferated, expressed chondrogenic genes and deposited collagen and glycosaminoglycan (GAG) into the scaffolds, indicating robust chondrogenesis within the scaffolds.

**Significance:** Our findings suggest a promising approach for customizing repair of cartilage defect using PDLA-PEG/HA copolymer.

**Reference:** (1) Lin *et al.* Biomaterials, 2013, 34(2):331–9.

#### Engineering the Microvasculature for *In Vivo* Liver Tissue Engineering

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Providing sufficient microvascularization to support new tissues is a major hurdle in *in vivo* tissue engineering (TE). This project aimed to implant mouse liver sinusoidal endothelial cells (LSECs: LYVE1+/CD31-) in *in vivo* TE chambers to enhance the vasculature in the chamber for future liver TE projects. LSECs were isolated from C57BL/6 mice. After 3 days in culture LSECs were implanted as either single cell suspensions (SCS) of 500,000 cells or as 100 multicellular spheroids of 5000 LSECs in bilateral TE chambers created on the epigastric vascular pedicle in SCID mice. SCS LSECs were implanted in one chamber and spheroids in the opposite chamber with GFR Matrigel. Control chambers, without cells were also established, and chambers assessed by immunohistochemistry and morphometry. At 2 weeks implanted LSECs formed liver sinusoidal-like capillaries (LYVE1+/CD31-) in SCS and spheroid chambers. Morphometric LYVE1+percent vascular volume (PVV) was increased non-significantly in LSEC implanted chambers compared to control. LSEC implanted chambers also demonstrated a significant increase in the PVV of CD31+host vessels (p=0.0129) with the LSEC spheroid implanted chambers showing a near doubling of CD31+PVV. The particularly positive effect of implanted LSEC spheroids on the host CD31+ vessel growth correlates with a significant increase in VEGF-A production of LSEC spheroids in culture compared to 2D LSEC culture (p=0.0013). This study illustrates the progenerative influence of liver sinusoidal endothelial cell capillaries, and although difficult to culture their inclusion in future liver TE studies could have an extremely positive impact on liver tissue growth.

### 3D Silk Bone Marrow Model: A Promising Tool to Test Efficacy of New Biomimetic Drugs On Platelet Production

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Platelets are released into the bloodstream by bone marrow megakaryocytes, large multinucleated cells that differentiate from hematopoietic stem cells under the control of thrombopoietin. Inherited thrombocytopenia are a heterogeneous group of rare disorders that occurs when a patient suffers from an abnormally low peripheral blood platelet count due to genetic mutations in genes relevant for control of megakaryocyte function. Great effort has been invested to generate drugs able to increase platelet production in these patients. Among these there is Eltrombopag, a small non-peptide thrombopoietin bio-mimetic, currently approved by many regulatory agencies worldwide for increasing platelet count in patients with different forms of thrombocytopenia. Despite the clinical practice, the mechanisms underlying Eltrombopag impact on megakaryocyte function are unknown. Here, we investigate the effects of Eltrombopag taking advantage of our well-established model for *in vitro* megakaryocyte differentiation by human hematopoietic progenitor cells and our recently-established 3D silk-based bone marrow model for studying *ex vivo* thrombopoiesis. Results demonstrated that Eltrombopag promotes normal megakaryocyte differentiation and platelet production, with a significant dose-dependent increment. Of note, Eltrombopag induced the activation of AKT and ERK1/2, two signaling molecules that together have been demonstrated to be crucial for the regulation of physiologic platelet production. Finally, megakaryocyte cultured in the 3D silk bone

marrow system showed similar platelet release number and function in thrombopoietin or Eltrombopag-treated samples.

These data demonstrated that modeling human thrombopoiesis into an engineered silk-based bone marrow system may represent a promising tool to develop personalized tissue engineering strategies to predict efficacy of new drugs.

### International Collaboration Research Between Thailand and Japan to Realize a Clinical Application of Regenerative Medicine using an Automatic Cell Processing Machine R-CPX

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Thailand and Japan have been conducting international collaboration research to realize practical application of regenerative medicine. For its realization, facilities enabling high quality cell culture and culturing experts are necessary. With support from the Japanese government, we decided to use our automatic cell processing machine, R-CPX, to substitute them. R-CPX uses two clean robots and automates entire process of culture operation, from primary culture through to cell harvest, in conformity with GMP. R-CPX also incorporates automatic decontamination unit with vaporized hydrogen peroxide, which eliminates the need for it being installed in highly clean environment. What is more, R-CPX can culture cells from different donors simultaneously. For the research, we decided to target treatment of cartilage defects in knees using mesenchymal stem cells cultured from bone marrow. Then we developed its protocols in Japan and installed them into R-CPX. We modified R-CPX that was originally developed for domestic use in Japan to enable overseas use, as well as remote monitoring and operation which enable safe and reliable operations even if R-CPX's technical experts are off site. Chulalongkorn University has been using the R-CPX installed there and conducting clinical research in accordance with regulations in Thailand. In this presentation, we will introduce development of the R-CPX and progress of the clinical research in Thailand.

I. Nakashima K, Hasunuma H, Nakamura M, Wakitani S, Hashimoto Y, Saito M, Development of the automatic cell processing machine for the clinical application of the cartilage regeneration, TERMIS-WC 2012.

### Treatment of Pediatric Esophageal Disease with a Tissue Engineered Biomimetic Construct

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Esophageal atresia occurs in 1:3,000 to 1:5,000 live births in the United States. In the most severe form, long-gap esophageal atresia, esophageal replacement by gastrointestinal transposition may be necessary. Current reconstructive options are associated with complications such as stenosis and dysmotility. A tissue-engineered (TE) esophageal construct may therefore offer a viable alternative to conventional treatments for severe esophageal disease.

Donor rat esophageal tissue was physically and enzymatically digested to isolate organoid units (OUs). OUs were seeded onto biomimetic electrospun synthetic PLGA/PCL scaffolds and cultured in a physiologic bioreactor system. After 2 weeks of culture, TE constructs were orthotopically transplanted in Sprague-Dawley rats and extracted after 2 weeks for analysis.

After 2 weeks of *in vitro* culture, immunofluorescence and RT-PCR demonstrated the presence of smooth muscle, fibroblast, and neural cell types which had migrated and layered in an esophagus-like conduit. When implanted, TE constructs integrated well with host tissue with normal GI, urinary, and feeding habits.

We conclude that OUs can be seeded onto electrospun scaffolds and cultured in a hollow organ bioreactor to create a biomimetic esophageal construct. Although allogeneic OUs were used in this study, we believe that autologous OUs could be isolated from human patients following esophageal biopsy. Further studies will be needed to evaluate human cell sources, large-animal models as well as the immune response to these esophageal scaffolds.

#### Tacrolimus Co-administration Suppresses Anti-donor Immune Response to Allogeneic Mesenchymal Stem Cells but does not Influence Disease Severity in a Mouse Model of Hind Limb Ischemia

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Mesenchymal stem cells (MSCs) are adult stem cells that inducible secrete paracrine factors with anti-inflammatory, immunomodulatory and pro-angiogenic effects. Many studies have reported hypo-immunogenic and anti-inflammatory/immunosuppressive properties of MSCs *in vitro* as well as *in vivo*. However, recent reports also suggest that MHC-mismatched MSCs (allo-MSCs) may trigger donor-specific cellular (T cell) and humoral (B cell/antibody) immune responses *in vivo*, which could limit therapeutic potential. Co-administration of clinically-approved immune-suppressive drugs such as the calcineurin inhibitor tacrolimus may, therefore, suppress the anti-donor immune response to allo-MSCs and enhance their therapeutic efficacy *in vivo*. In this study, anti-donor immune responses and therapeutic efficacy were examined following single intra-muscular injection of fully MHC-mismatched (C57BL/6) allo-MSCs with co-administration of a short course (14 days) of tacrolimus or vehicle in BALB/C mice with surgically-induced HLI. The results indicated that tacrolimus co-administration suppressed allo-MSC-associated expansion of myeloid cells but not of CD4+ and CD8+ memory T-cells in the spleen when compared with vehicle-treated allo-MSCs recipients. Tacrolimus co-administration with allo-MSCs did not affect the subsequent proliferation of splenic T-cell in a T-cell "re-call" assay stimulated by donor (B6) or third-party (FvB) dendritic cells. In vehicle-treated mice, intramuscular allo-MSCs induced readily-detectable anti-donor antibodies of predominantly IgG1 isotype. The induction of anti-donor antibodies was almost entirely prevented in mice receiving allo-MSCs with tacrolimus co-administration. Despite its effects on elements of the anti-donor immune response to allo-MSCs, tacrolimus co-administration did not influence the severity of HLI in experimental mice, as determined by laser Doppler flowmetry, limb functionality and capillary density.

#### Isolating and Optimizing the Biological Performance of Immuno-protected Pseudoislets

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Type 1 diabetes is an autoimmune disease characterized by loss of functional beta-cell mass and subsequent insulin insufficiency that is currently being managed by insulin injections. Unfortunately, insulin injections have long-term side effects and cannot fully recapitulate the endogenous function of native pancreatic islets. Replacement of insulin-producing cells through islet transplantation has been successful in achieving insulin independence for type 1 diabetics. However widespread adoption is limited by the need for immunosuppression, limited cell source, and poor viability and performance of transplanted cells. We envision overcoming these challenges through combining cell and tissue reconstitution technologies along with biomaterial device engineering. To characterize the self-organization process that directs reconstitution of islets *in vitro*, we dissociated and patterned primary islet cells or stem-cell derived cells in geometrically and structurally defined 3D microtissues fully embedded in different microenvironments of varying physiochemical properties. We then relate the positioning of the two principal components of the Islet (i.e. alpha and beta cells) to the functional performance of the

reconstituted tissues using microscopy techniques, and established assays for insulin secretion. Furthermore, we engineered an immune-protective cell encapsulation device with stringently controlled pore sizes down to 20 nm and demonstrated the ability to limit penetration and diffusion of immune cells, immunoglobulin species, and inflammatory cytokines. We show encapsulated cells are both functional and viable after 4 weeks in culture. These studies demonstrate proof-of-concept for optimizing the viability and performance of transplantable immune-protected insulin secreting microtissues that can potentially offer a viable cure for type 1 diabetics.

#### Tissue Engineering of Corneal Endothelial Grafts

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**Purpose:** Production of artificial endothelial grafts using human tissues processed in a clinical tissue bank.

**Methods:** Eye tissues and fresh human cancellous bone were obtained from cadaveric donors. Both tissues were stored and processed in Asturias Regional tissue bank according to Spanish laws.

Peripheral endothelium was obtained from corneas during posterior endothelial preparation. Peripheral Descemet membrane was cut and the explants were seeded and cultured on a dish previously treated with fibronectine. Once confluent, endothelial cells were detached and subcultured on a collagen type I membrane.

Collagen I was isolated from scleral tissue or cancellous bone. Briefly, both tissues were degreased, demineralized and lyophilized. For collagen solubilization an acid digestion technique was employed in the presence of pepsin (1 mg/ml).

Collagen type I was cast into a silicone mold and air-dried at room temperature and crosslinked using UV light.

Human corneal endothelial cells growing on the collagen membranes were analyzed by phase contrast microscopy, scanning electron microscopy and immunocytochemistry.

**Results:** Type I collagen was effectively isolated from human bone and scleral tissue. Human endothelial cells were able to attach and grow onto collagen type I membranes. Cells maintained their morphology and cellular markers, showing positive Na+/K+ and ZO-1 stain.

**Conclusions:** We have successfully obtained artificial lamellar endothelial grafts using cells from Descemet peripheral rings and collagen membranes from bone or scleral tissue of the same donor. This strategy could supply extra endothelial tissue and compensate the deficit of cadaveric graft for endothelial transplantation.

#### Blood Derived Angiogenic Cells (BDAC): a Novel Cell Type for Autologous Therapeutic Angiogenesis

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Critical limb ischemia (CLI), a severe form of peripheral arterial disease, results in chronic pain, tissue loss, and often requires amputations. Therapeutic angiogenesis using autologous cells holds great promise for CLI treatment, while minimizing ethical and safety issues.

However, this approach is often limited by the cell numbers at harvest and prolonged *in vitro* expansion. Our group has recently developed a monocyte-derived cell type named Blood Derived Angiogenic Cells (BDAC) [1]. Using pulsed macromolecular crowding (MMC), we are able to generate clinically relevant numbers of 10 to 40 million functional cells from 100 ml of human peripheral blood in 5 days. BDAC were shown to be pro-angiogenic *in vitro* and *in vivo*. The therapeutic efficacy of BDAC was demonstrated through a pre-clinical model of murine hindlimb ischemia. A single injection of BDAC rescued most limb tissues from ischemia-induced necrosis. Magnetic resonance angiography (MRA) studies demonstrated that BDAC treatment accelerated reperfusion in the ischemic limbs. Histologically, BDAC-treated animals showed reduced fibrosis, attenuated adipose-replacement, and reduced neutrophil infiltration. Our study validated the therapeutic potential of BDAC and its pro-angiogenic capacity in a disease model. Future work will focus on other possible mechanisms of BDAC treatment, such as its interaction with myocytes and endogenous macrophages.

**Acknowledgments:** Singapore Bioimaging Consortium (SBIC), A\*STAR; Singapore-MIT-Alliance for Research & Technology (SMART) Innovation Centre.

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## Natural Nanostructured Materials for Tissue Engineering Applications

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Advanced biomaterials that can mimic the properties of native tissue to possess antibacterial properties, deliver drugs, and permit stem cells to adhere and differentiate are of paramount importance in the development of stem cell therapies for bone defects. Successful bone repair approaches may include an osteoconductive scaffold that permits excellent cell adhesion and proliferation, and cells with osteogenic potential. It is now well established that synthetic materials composed of nanomaterials promote bone formation. However, little is known about natural nanostructured material composites. The objective of this study was to prepare and characterize humanderived bone marrow mesenchymal stem cells (hMSCs) seeded on various natural nanostructured materials including (but not limited to) microporous, nano silver and nano gold bacterial cellulose (BC) scaffolds fabricated using a lasercutting instrument. Biocompatible BC was synthesized using the bacterium *Gluconacetobacter sucrofermentans*. To introduce highly reproducible micropores that will permit diffusion of nutrients into the scaffold, a lasercutting instrument was used. To improve the antimicrobial properties of the scaffold, silver nanoparticles (AgNPs) were added to the scaffolds. To assess the potential of the scaffold for potential nanoparticle-based drug and gene delivery applications, gold nanoparticles (AuNPs) were also used. The adhesion, viability, proliferation, and differentiation potential of hMSCs on the BC composites were analyzed using the colorimetric MTS assay, fluorescent microscopy and phase contrast microscopy. In summary, these microporous nanostructured BC composites and other natural nanomaterials formulated in our lab supported the proliferation and osteogenic differentiation of hMSCs, allowing for their future potential use in tissue engineering therapies.

## Elastic Properties of 3D-Bioprinted Stem Cell-loaded Hydrogels guide the Success of Osteogenesis *In Vitro*

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Differentiation of mesenchymal stem cells (MSC) inside 3D-bioprinted hydrogels renders multiple challenges, including 1) cell

viability after bioprinting, 2) cell spreading in supporting hydrogels, and 3) geometrical printability of supporting hydrogels regarding the final application. We investigated MSC-loaded agarose and collagen hydrogels mixed at varying ratios (A25-COL75, A50-COL50, and A75-COL25), which were 3D-bioprinted with a micro-valve based bioprinter, and cultured with osteogenic induction medium for 21 days *in vitro*. Increased compressive tangent modulus of  $18.1 \pm 3.5$ ,  $53.1 \pm 10.3$ , and  $89.1 \pm 13.9$  kPa at 20% strain were observed for hydrogels with higher agarose concentrations. The 3D-printing reproducibility of agarose-collagen constructs significantly depended on the concentration of agarose in the mixture. Improved contour accuracy was achieved for the stiffest hydrogels. The success of MSC osteogenesis in 3D-bioprinted hydrogels was evaluated regarding cell spreading (CS), presence of calcium accumulations detected by Alizarin Red staining, and osteogenic gene expression quantified by qPCR (3 independent donors). A75-COL25 did not allow cell spreading (<1% CS) and exhibited the lowest expression of osteogenic markers. Interestingly, MSC in A50-COL50 revealed moderate cell elongation (max 30  $\mu$ m and 6% CS) and osteogenic gene expression, but the most pronounced evidence of calcium accumulations. On the contrary, A25-COL75 guided cell spreading (max 120  $\mu$ m and 26% CS) and provided significantly enhanced expression of osteogenic markers compared to A50-COL50 and A75-COL25. Stiffer hydrogels demonstrated improved contour accuracy but inhibited osteogenic gene expression, whereas less stiff hydrogels that guided cell spreading exhibited difficult troubleshooting issues during 3D-bioprinting but encouraged osteogenesis.

## Evaluation of the Expansion and Expression of Cxcr4 Homing Molecule on Umbilical Cord Blood Hematopoietic Stem Cells in Biocompatible Microwells

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**Background:** Hematopoietic stem cell transplantation (HSCT) is a therapeutic approach for treatment of hematological malignancies and incompatibility of Bone marrow. Umbilical cord blood (UCB) has known as an alternative for hematopoietic stem/progenitor cells (HPSC) in allogeneic transplantation. The low volume of collected samples is the main hindrance in application of HPSC derived from umbilical cord blood. So, *ex vivo* expansion of HPSCs is the useful approach to overcome this restriction. The goal of using this system is to produce appropriate amount of hematopoietic stem cells, which have the ability of transplantation and long term haematopoiesis.

**Material & Methods:** In current study CD133+ cells were isolated from cord blood (CB). Isolated cells were seeded on microwells. Then expanded cells proliferation rate and ability in colony formation were assessed and finally were compared with 2 Dimensional (2D) culture systems.

**Results:** Our findings demonstrated that CD133+ cells derived from UCB which were cultivated on microwells had significantly higher rate of proliferation in compared with routine cell culture systems.

**Conclusion:** In Current study, it was shown that CD133+ cells' proliferation and CXCR4 expression on Hematopoietic stem cells which were seeded on PDMS microwells coated with collagen significantly increased. We hope that 3 dimensional (3D) microenvironment which mimics the 3D structure of bone marrow can solve the problem of using UCB as an alternative source of bone marrow.

## Investigation into the Role of Cd34 Expression in Corneal Stroma-Derived Stem Cells

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Keratocytes are specialised mesenchymal fibroblasts populating the corneal stroma; characterised by expression of the progenitor marker CD34. We have demonstrated that CD34+limbal

keratocytes become multipotent progenitor cells *in vitro*, known as corneal stroma-derived stem cells (CSSC). CSSC possibly play a regenerative role *in vivo*, and therefore have considerable potential as a stem cell therapy for ocular surface regeneration. However, expansion in conventional FBS-containing medium leads to loss of CD34 and associated progenitor activity. We investigated methods of optimising *in vitro* culture for maintenance of CD34, following on with assessment of the importance of CD34 expression in CSSC.

CSSC were extracted from human corneoscleral rims and phenotype assessed at early (P1) and late (P4) passage, when cultured in either: M199 with 20% FBS; DMEM-F12 with 20% KSR, bFGF and LIF (SCM); endothelial growth medium (EGM); or MethoCult™. Gene expression of isolated CD34+CSSC and effect of CD34 siRNA knockdown were then evaluated by RT-qPCR.

SCM was the only medium to maintain CD34 expression from P1 to P4 and exhibited significant upregulation of progenitor cell markers ABCG2, PAX6, THY1, SSEA4 and SOX2, compared to other media. CD34+CSSC had significantly increased expression of pluripotency markers compared to CD34- cells. Knockdown of CD34 had significant effects on the regulation of pluripotency genes.

Results demonstrate that maintenance of an optimal progenitor phenotype is medium-dependent, and CD34 expression is linked to CSSC stem cell properties. Once standardised and bankable, a CSSC therapy could lead to the next generation of ocular regeneration strategies.

### Evaluation of Human Fibroblast Viability and Migration of Polyelectrolyte Complexes of Chitosan, Alginate and Aloe vera

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Commercial chitosan (QCH) prepared by conventional thermochemical methods and chitosan obtained from biologically extracted chitin (BCH) were employed for preparation of hydrogels formulated with Aloe vera (*Aloe barbadensis* Miller) (AV) and Alginate (ALG). Polyelectrolyte complexes were prepared with ALG-QCH-AV (HQCH) and ALG-BCH-AV (HBCH) at pH 6 using Ca<sup>2+</sup> ions and without Ca<sup>2+</sup> ions. The ionic interaction of hydrogels was evidenced by band at 1595 cm<sup>-1</sup> determined with FTIR spectroscopy, due to formation of polyelectrolyte complex. Polyanionic surface charge prevailed in the hydrogels in a range of -20 and -24 mV allowing a stable system. In despite of the surface charges that might prevent cell adhesion, considering the anionic charge in the cell membrane, fibroblast viability was determined due to components in hydrogel as it was showed in the MTT assay with >90%. The wound assay was evaluated for all the systems including individual components and binary and ternary mixtures. Cell migration and proliferation were significantly higher with hydrogels prepared with Ca<sup>2+</sup> and BCH than biomaterials prepared with QCH.

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### 3D Printed Construct Supporting Multilineage Differentiation of Stem Cells

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3D bioprinting has exciting prospect for fabricating tissue-analogs by depositing cell-laden biomaterial at the intended location in layer-by-layer fashion, to recapitulate the precise architecture of native tissue. Advances in this field are relatively slow due to limited choices of bioink

for cell encapsulation, bioprinting and cytocompatible gelation mechanism. Unique mechanical properties, modifiability, chemistry of Bombyx mori silk fibroin protein make it a potential bioink for bioprinting. Problems related to frequent clogging of micronozzle while printing and lack of bioactive molecules in Bombyx mori silk fibroin protein could be solved by development of silk-gelatin blend, to impart biofunctionalization. Based on two different *in situ* cytocompatible gelation techniques (enzymatic crosslinking by mushroom tyrosinase or physical crosslinking via sonication), we could successfully develop bioinks having varied  $\beta$ -sheet crystal content, hence different gel stiffness. By careful optimization of rheology, self-supporting 3D constructs were fabricated by extruding human nasal turbinate tissue-derived mesenchymal stem cells encapsulated in silk-gelatin blend. Long term cell viability, gene and protein expression studies demonstrated that multilineage differentiation of stem cells can be achieved in tailor-made architecture. Furthermore, cell-laden structures by encapsulating single cells or aggregated chondrocytes and mesenchymal stem cells generate fundamental insights about difference in signalling pathways during chondrogenic differentiation while fabrication of anatomically relevant sized cartilaginous tissue constructs in patient-specific manner.

### Investigation of the *In Vivo* Vascularisation And Mineralisation Potentials of Endochondrally Primed Cellular Aggregates in a Rat Subcutaneous Delivery Model

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In this study, we test the hypothesis that a bone regeneration approach that pre-cultures cellular aggregates *in vitro* to mimic the endochondral ossification process, specifically the formation of the cartilage template and subsequent pre-vascularisation of that template, will improve cell viability, vessel infiltration and thus mineral formation once implanted subcutaneously *in vivo*. Human MSCs were chondrogenically primed for 21 days, after which they were co-cultured with MSCs and Human Umbilical Vein Endothelial cells (HUVECs) and cultured in endothelial growth medium for another 21 days. These aggregates were then embedded in BMP-2 containing alginate and implanted subcutaneously in nude rat model for 4 weeks. Bioluminescent Imaging (BLI), histology (Masson's trichrome and Alizarin Red) and immunohistochemistry (CD31, CD146, and  $\alpha$ -smooth actin) analyses were performed.

Prevascularised cartilaginous aggregates had mature vessels (indicated by  $\alpha$ -smooth muscle actin walls and erythrocytes) within the aggregates after 4 weeks *in vivo*, and also had viable human MSCs (detected by BLI imaging) 21 days after subcutaneous implantation. In contrast, aggregates that were not prevascularised had no vessels within the aggregates and human MSCs did not remain viable beyond 14 days. Interestingly, the prevascularised cartilaginous aggregates were also the only group to have mineralisation nodules within the cellular aggregates, whereas mineralisation occurred in the alginate surrounding the aggregates for all other groups. Taken together, these results indicate that a combined chondrogenic priming and prevascularisation approach for *in vitro* culture of MSC aggregates promotes improved survivability, enhanced vessel formation and increased mineralisation within the cellular aggregate when implanted subcutaneously.

### Oxygen Tension Regulates Human Mesenchymal Stem Cell (hMSC) Paracrine Functions

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Mesenchymal Stem Cells (MSCs) have captured attention and research endeavors of the scientific world because of their differentiation potential. However, there is accumulating evidence suggesting that the beneficial effects of MSCs are predominantly due to the multitude of bioactive mediators secreted by these cells. Since the paracrine potential of MSCs is closely related to their microenvironment, the present study investigated and characterized select aspects of the hMSC secretome and assessed its *in vitro* and *in vivo* bioactivity as a function of oxygen tension, and specifically near anoxia (0.1% O<sub>2</sub>) and hypoxia (5% O<sub>2</sub>), conditions which reflect the environment MSC are exposed during MSC-based therapies *in vivo*. In contrast to supernatant conditioned media (CM) obtained from hMSCs cultured at either 5 or 21% of O<sub>2</sub>, CM from hMSCs cultured under near anoxia exhibited significantly ( $p < 0.05$ ) enhanced chemotactic and pro-angiogenic properties, and a significant ( $p < 0.05$ ) decrease in the inflammatory mediators content. An analysis of the hMSC secretome revealed a specific profile under near anoxia, hMSCs increase their paracrine expression of the angiogenic mediators VEGFA, VEGFC, IL-8, RANTES and MCP1, but significantly decrease expression of several inflammatory/immunomodulatory mediators. These findings provide new evidence that elucidates aspects of great importance for the use of MSCs in regenerative medicine and could contribute to improving the efficacy of such therapies.

### Tissue Engineered Blood Vessels using Human Ips Cells

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The utility of human induced pluripotent stem cells (hiPSCs) to create tissue engineered vascular grafts (TEVGs) was evaluated in this study. hiPSC lines were first induced into a mesenchymal lineage via a neural crest intermediate using a serum-free, chemically defined differentiation scheme. Derived cells exhibited commonly known mesenchymal markers (CD90, CD105, CD73; negative markers - CD45), and were shown to differentiate into several mesenchymal lineages (osteogenic, chondrogenic, adipogenic). Functional vascular grafts were then engineered by culturing hiPSC-derived mesenchymal progenitor cells in a pulsatile bioreactor system over 8 weeks, to induce smooth muscle cell differentiation and collagenous matrix generation. Histological analyses confirmed layers of calponin-positive smooth muscle cells in a collagen rich matrix. Mechanical tests revealed that grafts had an average burst pressure of 700 mm Hg, which is approximately half that of native veins. Additionally, studies revealed that karyotypically normal MSC clones led to generation of grafts with predicted features of engineered vascular grafts, while derived clones having chromosomal abnormalities generated calcified vessel constructs, possibly due to cell apoptosis during culture. Overall, these results provide significant insight into the utility of hiPSC cells for vascular graft generation. They pave the way for creating personalized, patient-specific vascular grafts for surgical applications, as well as for creating experimental models of vascular development and disease.

### Estradiol Releasing Scaffolds for the Treatment of Pelvic Floor Disorders

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Stress incontinence (SUI) and prolapse (POP) are common debilitating conditions which have a major impact on the quality of life of as many as 1 in 5 women as they age. Reduced local collagen expression has been reported in patients and it is known that estradiol therapy can improve local collagen content. The most common surgical treatment is the implantation of non-degradable synthetic polypropylene mesh. This provides good mechanical support but shows poor host integration and in some patients leads to erosion through the patient's tissues.

Our aim is to produce which are closer mechanically to that of the native fascia of the patients and which can also release estradiol to stimulate new collagen production. Two polymers, polyurethane to give resilience and elasticity and polylactic acid to promote tissue integration, were co-electrospun so that fibres intermingled. In some of these PLA scaffolds estradiol was incorporated and its release measured over 5 months. To evaluate the impact of estradiol on new tissue production scaffolds were combined with adipose derived mesenchymal cells and the incorporation of estradiol significantly doubled total collagen production over 14 days. Estradiol releasing scaffolds had similar mechanical properties to native fascia, achieving strength and Young's modulus within the range reported for native tissues.

In conclusion estradiol can be incorporated into PLA to be released as fibres break down over several months. The incorporation of estradiol does not compromise the mechanical properties of the scaffolds and we suggest that its addition may improve the integration of implants post surgery.

### Low Adhesive Scaffold Collagen, Inducing Spheroid Formation, Promotes the Cell Crawling of Fibroblasts

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**Background:** Fibroblasts exist in the connective tissues and are generally separated by extracellular matrices (ECMs). One of functions in fibroblast is synthesis, secretion, and deposition of the ECM proteins such as collagen molecules. In addition, fibroblasts can migrate in connective tissues for remodeling and renewal. We succeeded in developing low adhesive scaffold type I collagen (LASCOL) (patent pending), and we found that fibroblasts cultured on LASCOL spontaneously form an aggregated sphere body (spheroid). In this study, we report the cell motility of fibroblast spheroid.

**Materials & Methods:** The culture dish was coated with LASCOL solution or atelocollagen solution. Subsequently, mouse NIH/3T3 cells were cultured on the dish. After 24hrs, to confirm spheroid formation, we observed cell morphology by using a phase-contrast microscope. To investigate the cell motility, we stained filamentous actin (F-actin) with DyLight 555-labeled phalloidin. Finally, cell crawling was monitored and analyzed by a time-lapse observation.

**Results:** By SEM observation, single cells cultured on atelocollagen adhered strongly on the scaffold, showing flat and smooth appearance. In contrast, we showed that a lot of protrusions existed on the surface of NIH/3T3 cells of spheroid cultured on LASCOL. F-actin was highly expressed in each cell of spheroid, as compared with a single flat cell. In conclusion, we demonstrated that the adherence of fibroblasts to the LASCOL is weak, and cells gather and adhere each other to form spheroid. [Funding] This work was supported by Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST (AS2414037P to K.M.).

### A Novel Mesenchymal Stem Cell Spheroids Based Therapy For Hind Limb Ischemia

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Stem cell based therapy is a promising new strategy to achieve functional improvement of cardiovascular disease. Mesenchymal stem cells (MSCs) have been widely studied for their differentiation potential as well as paracrine abilities. Recent studies have shown that MSC spheroids, due to their 3D structure, have longer retention and greater therapeutic effect than MSCs suspension. However, with the direct injection of MSC spheroids *in vivo*, spheroids are likely to dissociate, which hinders them from reaching their full potential. Herein, we hypothesized that nanoparticle enhanced MSC spheroids loaded on micropatterned scaffolds can help MSC spheroids maintain their structure meanwhile having better efficacy in treating cardiovascular.

Scaffolds were prepared by electrospinning of Polyurethane (PU). Microwell array patterns were introduced on electrospun fibres by applying micropatterned Polydimethylsiloxane (PDMS) as collector. Adhesive peptide functionalized nanoparticle was synthesized by conjugating IKVAV peptides to surface of PAMAM dendrimer. We tested our hypothesis in mouse hind limb ischemia model. Micro-patterned scaffold was loaded with nanoparticle enhanced MSC spheroids and transplanted to the ischemic leg of hind limb ischemic (HLI) mice ( $2 \times 10^5$  MSCs per mice). In Laser Doppler Perfusion Imaging study, the treated mice showed a 95% increase of perfusion in ischemic area than non-treated mice 7 days after surgery. Lectin staining indicated that treatment group has greater vascular density.

Our study demonstrates a novel and effective method of applying MSCs to hind limb ischemia. This result may provide insights into how stem cell therapy may be designed for regenerative applications.

### Spinner Basket Culture of Adipose-Derived Mesenchymal Stem Cells on Large-Scale 3D PCL/TCP Scaffolds Increases the Efficiency of Osteogenic Differentiation

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Culturing ADSC on large three dimensional (3D) scaffolds is challenging due to inefficient seeding, proliferation and differentiation of cells. Therefore, the effect of stirred-tank culture on seeding, proliferation and osteogenic differentiation of human ADSC in large-scale polycaprolactone/tricalcium phosphate (PCL/TCP) composite scaffolds was studied.

Cylindrical, porous PCL/TCP scaffolds ( $h = 25$  mm;  $d = 15$  mm) were manufactured using 3D-printing. Human ADSC were seeded onto scaffolds in a drop of medium (static seeding and culture) or the cell suspension was directly added into a 500 ml vessel with PCL/TCP scaffolds fixed in a porous basket and a stirrer enforcing medium flow through the scaffolds (dynamic seeding and culture-spinner basket). Part of the scaffolds seeded statically, was transferred into the spinner basket tank 24 h after seeding (static seeding-dynamic culture). XTT assay, live/dead staining, SEM observation, bone mineral staining and gene expression analysis were performed at 14 days of culture in osteogenic medium.

Dynamic culture conditions decreased scaffold seeding and cell number. Upon dynamic culture, the morphology of bone mineral changed from a scaffold surrounding layer to loosely attached spatial aggregates. Furthermore, dynamic culture resulted in a 2-fold up-regulation of Runx2, 3.5-fold up-regulation of osteopontin and 22.5-fold up-regulation of Osterix genes after 14 days of culture. The gene expression level of cells cultured in standard polystyrene plates or cultured in scaffolds statically was similar to undifferentiated cells. Therefore, dynamic conditions decreased scaffold seeding and proliferation while up-regulating osteogenic differentiation probably due to shear stress. Culture conditions should provide a compromise between ingrowth of cells into the scaffold and differentiation.

### VEGF-transfected Adipose-derived Stromal Cells Enhances Bone Regeneration and Neovascularization from Bone Marrow Stromal Cells

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Vascular endothelial growth factor (VEGF)-transfected adipose-derived stromal cells (ADSCsVEGF) were devised to promote bone regeneration and neovascularization of bone marrow stromal cells (BMSCs). ADSCs were transfected with pEGFP-C1 vector

containing human VEGF gene using electroporation. ADSCsVEGF was added to BMSCs and cocultured in variable proportion. ADSCsVEGF or ADSCsVEGF with BMSCs (BMSCs:ADSCs:VEGF ratio of 1:0.025 ~ 0.5) induced significantly greater number of tube formation in human umbilical vein endothelial cells than untransfected ADSCs. The coculture of BMSCs and ADSCsVEGF at the ratio of 1: 0.025 ~ 0.1 showed significantly greater *in vitro* osteogenic differentiation and mineralization than BMSCs alone. When critical size calvarial defects in rats were implanted with mixture of BMSCs and ADSCsVEGF along with hydroxyapatite/ $\beta$ -tricalcium phosphate granules, BMSCs and ADSCsVEGF at the ratio of 1:0.05 showed significantly greater bone area, volume and mineral density on the regenerated defect than BMSCs alone. The co-plantation of ADSCsVEGF with BMSCs significantly increased microvessel density and staining intensity of CD31 on the regenerated bone of the repaired defect than BMSCs alone. In conclusion, ADSCsVEGF added in small proportion to BMSCs effectively promotes bone regeneration and neovascularization.

### The Tissue Response to Biologic Scaffold Materials Used for Breast Reconstruction

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The clinical use of biologic scaffolds to provide support and coverage of the inferior pole in two-staged expander/implant breast reconstruction procedures has significantly increased over the last decade. While the results of these clinical studies appear promising, only a small amount of data exists on the kinetics of scaffold integration and host tissue response to clinically available biologic scaffold materials in this application. Furthermore, a robust, low cost, small animal model that accurately recapitulates the clinical breast reconstruction procedure has yet to be established. The aim of the present study was to develop a small animal model to be used as a means to evaluate the temporal host soft tissue response to clinically relevant biologic scaffold materials. Evaluation criteria included characterization of the degree of material contracture and quantification of aspects of the host cell response and tissue remodeling events using established histomorphologic metrics at various interfaces. The data presented herein show a decreased thickness of the collagenous tissue layer at biologic device/silicone interface compared to the abdominal wall/silicone interface across a 12 week experimental time course. Furthermore, all test materials were found to be readily incorporated into surrounding host tissue as shown by the presence of neovascularization and marked cellular infiltration, including macrophages.

### A Novel Human Epithelial *In Vitro* Wound Healing Assay for the Evaluation of Materials and Growth Factors Used in Corneal Tissue Engineering

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**Background:** There is a current need to develop relevant human models to determine the effects of materials and growth factors in corneal tissue engineering. The aim of this study was to generate an *in vitro* wound healing assay based on stratified human corneal-epithelial (HCLE) cells.

**Methods:** Immortalized stratified HCLE cells were cultured on a polystyrene surface. Afterwards, a mechanical injury with a trephine,

combined with epithelial debridement, was performed. Cells were grown in KSMF or DMEM/F12 culture media, both supplemented with EGF, with or without serum. The effect of the different culture media on the wound healing response was evaluated in the de-epithelialized area using phase-contrast microscopy. Barrier function and actin distribution were determined with the rose bengal assay and phalloidin fluorescence microscopy, respectively.

**Results:** Mechanical wounds using a trephine were reproducible. Addition of DMEM/F12 and serum resulted in complete re-epithelialization at 72 hours ( $83 \pm 16\%$  of the wounds). The de-epithelialized area was  $38 \pm 26\%$  at 24 h,  $8 \pm 15\%$  at 48 h and  $2 \pm 6\%$  at 72 h. The de-epithelialized area using other conditions was higher than  $80\%$  at 72 h ( $p < 0.05$ ). Of those wounds that completely healed,  $76 \pm 9\%$  showed stratification and barrier function in more than  $50\%$  of the re-epithelialized area. An actin re-arrangement in the leading edge during migration was observed.

**Conclusions:** Wound healing models based on cultured stratified human corneal epithelial cells could potentially be used to evaluate the effects of growth factors and materials in corneal epithelium for tissue engineering.

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### Meniscus-Derived Extracellular Matrix Hydrogels for Meniscus Regeneration

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Meniscus regeneration is a critical need for treating meniscus injuries and diseases, which are caused mainly by sports-related injuries and age-related degeneration. Tissue-derived material gains increasing interest as a biological scaffold for specific tissue regeneration because of its biomimetic compounds and high bioactivity. We processed porcine menisci into an injectable meniscus-derived extracellular matrix (ECM) hydrogel through decellularization using sodium dodecyl sulfate (SDS) and enzymatic digestion. The obtained ECM pre-gel solution was flowable at  $4^\circ\text{C}$  and formed a solidified hydrogel under a physiological condition. The hydrogel facilitated cell proliferation and cell infiltration for both bovine chondrocytes and 3T3 fibroblasts. Furthermore, the hydrogel was smoothly injected into a mouse subcutaneous model and showed good biocompatibility with minor immune response. To better preserve ECM compounds, we further used extra two detergents, Triton X-100 (TX) and peracetic acid (PAA) to decellularize the porcine meniscus. They effectively decellularized the meniscus, while the outcomes varied. The PAA-treated meniscal ECM exhibited a relatively well-preserved architecture, the lowest DNA residual, and the highest glycosaminoglycan retention compared to the SDS and TX-treated meniscal ECMs. All the three hydrogels exhibited similar gelation curves and fibrous structure. The PAA-treated ECM hydrogel had significantly higher peak compression strength than for the SDS and TX-treated ECM hydrogels. From above results, the attractive meniscus-derived ECM hydrogel, especially for the PAA treated, might find opportunities to be applied as a bioactive material for meniscus regeneration with minimally-invasive surgery.

### A 3D Human Lung Tumor Test System on an Acellular Scaffold: Simulation of a Biomarker-guided Therapy

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Lung cancer is often diagnosed late after the appearance of clinical symptoms, which implies that drugs for Non-Small Cell Lung Cancer treatment must be efficient to counteract tumors of advanced stages. Therefore, tumor models are required which reflect the clinical situation and allow a reliable drug testing.

In the present study, we introduced a 3D human lung tumor model developed on the basis of a decellularized porcine jejunum. In order to simulate the approach of personalized medicine, we used either a human cell line with an activating EGFR mutation (HCC827) or EGFR wild-type cell lines (H441, A549).

To block EGFR activation, we treated the tumor models with the EGFR inhibitor gefitinib: only in HCC827 cell proliferation was inhibited and apoptosis was induced, while in H441 and A549 cells, gefitinib treatment had no substantial effect. This correlates with the situation in the clinic as EGFR inhibitors have an effect only in patients with an EGFR mutation.

Often, these patients that are sensitive to tyrosine kinase inhibitors (TKIs) develop a resistance to EGFR-TKI. Therefore, we established a gefitinib resistant cell line, which proliferates also under gefitinib treatment.

To come closer to the *in vivo* conditions, we cultured our models dynamically in a bioreactor: a constant media flow was applied which enabled better nutrient supply and led to higher cell densities on the matrix mimicking tissue-like morphologies.

Thus, our models are promising tools to investigating the efficacy of new substances, the mechanisms of resistance, and the signaling pathways involved.

### Fucoidan-based Particles for Diabetes Mellitus Treatment

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Marine organisms are rich in a variety of materials with potential use in Tissue Engineering and Regenerative Medicine. One important example is fucoidan, a sulfated polysaccharide extracted from the cell wall of brown seaweeds. Fucoidan is composed by L-fucose, sulfate groups and glucuronic acid. It has important bioactive properties such as anti-oxidative, anticoagulant, anticancer and reducing the blood glucose (1). In this work, the biomedical potential of fucoidan-based materials as drug delivery system was assessed by processing modified fucoidan (MFu) into particles by photocrosslinking using superamphiphobic surfaces and visible light. Fucoidan was modified by methacrylation reaction using different concentrations of methacrylate anhydride, namely  $8\% \text{ v/v}$  (MFu1) and  $12\% \text{ v/v}$  (MFu2). Further, MFu particles with and without insulin ( $5\% \text{ w/v}$ ) were produced by pipetting a solution of  $5\% \text{ MFu}$  with triethanolamine and eosin-y onto a superamphiphobic surface and then photocrosslinking using visible light. The developed particles were characterized to assess their chemistry, morphology, swelling behavior, drug release, insulin content and encapsulation efficiency. Moreover, the viability assays of fibroblast L929 cells in contact with MFu particles showed good adhesion and proliferation up to 14 days. Furthermore, the therapeutic potential of these particles using human beta cells is currently under investigation. Results obtained so far suggest that modified fucoidan particles could be a good candidate for diabetes mellitus therapeutic approaches.

1. Silva T.H., *et al.*, Biomater, 2(4), 278, 2012.

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### Prevention of Collagen Scaffold Contraction using an Inhibitor of Rho-associated Protein Kinase Y-27632

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Collagen scaffolds are frequently used in the field of tissue regeneration since they mimic the extracellular matrix, thus providing an appropriate microenvironment for cells. A major issue associated with wound healing is contraction, a phenomenon that also occurs in the presence of biomaterials, such as collagen scaffolds. This can result in adhesions and limitation of movement of the repaired tissue. Contraction may be prevented by an inhibitor of rho-associated protein kinase (ROCK), Y-27632, as it may deregulate the actin fiber organization of cells that is responsible for contraction. In this study, the *in vitro* effect of Y-27632 was investigated using 3T3 fibroblasts and collagen scaffolds. Evaluation was by HE and immunostainings, cell viability assays, DNA quantification and contraction measurements. Immunostainings of 3T3 cells exposed to Y-27632 showed less intense staining of  $\alpha$ -actin, which may indicate lower levels of  $\alpha$ -actin or altered actin filament organization. When fibroblasts were seeded onto collagen scaffolds, they showed up to 50% contraction of the original length after 14 days of culture. However, when Y-27632 was added 1 day after seeding, only marginal scaffold contraction was observed after 14 days. HE staining, AlamarBlue cell viability assay and DNA quantification showed no reduced cell viability in scaffolds treated with the ROCK inhibitor. Taken together, these results suggest that Y-27632 can inhibit collagen scaffold contraction *in vitro* without affecting cell viability, and that it may be a promising tool to inhibit scaffold contraction *in vivo*.

### Creating a New Company to Commercialize Academic Research Relating to Peripheral Nerve Regeneration

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A polymer has been discovered that accelerated the growth of axons *in vitro*. Next, this polymer was spun into fibers, which were used to create a braided nerve regeneration conduit with superior physicochemical attributes. Then a collagen-based filler was introduced and the braided conduit was coated with a hyaluronic acid-derived coating, creating a uniquely effective device for peripheral nerve regeneration. Test results *in vivo* were so promising that it seemed appropriate to consider the creation of a new start-up.

Convincing investors that we had a winning technology was the difficult part. In this presentation, I will provide some of the technical details of our conduit technology and share with the audience the long process that most inventors endure before a well-funded start-up can be established. I will also discuss the perils of an "underfunded" enterprise that will run out of funds before reaching a reasonable milestone for subsequent financing.

Some of the key questions, any investor will ask, relate to market potential, technical risk, ability to create a commercial manufacturing process, calculating the cost of goods and profit margin, and the regulatory pathway for FDA market clearance. Anticipating these questions and having compelling answers greatly assisted us in our interactions with potential investors. As a final step, it is necessary to negotiate the financial arrangements between the inventors, their institution, and the investors.

While often difficult, the creation of a start-up can be a rewarding learning experience for scientists who want to realize the full potential of their creative ideas.

### Biomimetic Scaffolds for *In Vitro* Bone Marrow Tissue Engineering

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*In vitro* human bone marrow tissue analogues represent unique potential for studying bone marrow biology and realizing *ex vivo* hematopoiesis. Major cellular and molecular components constituting the bone marrow microenvironment have been identified, but recapitulating the full-range of bone marrow functions *in vitro* remains an elusive task. This is in part due to the lack of relevant

culture platforms that reflect bone marrow-like extracellular milieu. From a mechanical aspect, marrow is the softest tissue that is formed inside of the hardest tissue in the body, bone. We hypothesized that extreme contrast of biophysical properties in a microscale porous geometry would be critical in delineating stromal cell function and supporting hematopoiesis. To test this hypothesis, we have developed a hybridized porous hydrogel scaffold composed of glass beads and methylcellulose, reflecting both the soft and hard parts of the bone marrow in a controlled and analytical manner. The hybrid scaffold design enables the study of biophysical, biochemical, and structural cues that play a role in directing primary human bone marrow derived stromal cell behavior. We further investigated the biological significance of biomimetic scaffolds in supporting *ex vivo* expansion of human CD34 hematopoietic stem/progenitor cells. Our results indicate that integrating soft and hard materials in a single 3D platform is an effective strategy to create functional *in vitro* bone marrow tissue models.

### Decellularized Retina Matrices Controlling Retinal Progenitor Cell Response

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Tissue decellularization has enabled engineering of biologic scaffolds that maintain native extracellular matrix (ECM) composition. In this study, we developed decellularized retina (decell-retina) based substrates that mimic the retina niche, thereby stimulating human retinal progenitor cell (hRPC) attachment, proliferation and differentiation. Retinas isolated from bovine eyes were decellularized using 1% w/v sodium dodecyl sulfate (SDS) and pepsin digested, and the resulting decell-retina was biochemically analyzed for its composition and growth factor content. Decell-retina was cast dried to develop two-dimensional thin films and facilitate examination of its interaction with cultured hRPCs. Cell attachment, viability, morphology, proliferation and gene expression were studied *in vitro*. Biochemical analysis of decell-retina compared to native retina showed ~94% removal of cellular component, while ~55% glycosaminoglycans (GAGs), ~83% collagen, 87% hyaluronic acid and essential cytokines were retained after the decellularization process. The decell-retina films showed good cell attachment and ability to maintain hRPCs *in vitro*, with a cell number increase of 1.5 fold over a week. RT-PCR analysis revealed expression of rhodopsin, rod outer membrane, neural retina-specific leucine zipper neural and cone-rod homeobox genes on the decell-retina films, indicating photoreceptor development from the retinal progenitor cells. The developed matrices are being further explored to assess the ability of RPCs delivered on them to integrate into retina in an *in vitro* explant system. In conclusion, decell-retina films show promise as potential substrates for culture and/or transplantation of retinal progenitor cells to treat retinal degenerative disorders.

### Additive Manufacturing of Wet-spun Microbially Produced Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate] Scaffolds Tailored on a Critical Size Long Bone Defect

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Microbially produced polyhydroxyalkanoates (PHAs) are fully biodegradable polyesters that have attracted much attention as alternative polymeric materials that can be produced from renewable and biowaste resources. Among them, poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate] (PHBHHx) has been studied for bone Tissue Engineering (TE), showing better ductility, processing properties and osteoblast proliferation than the other PHAs. In this study, predefined three-dimensional PHBHHx scaffolds were produced layer-upon-layer by a computer-controlled wet-spinning technique. The scaffolds were designed with anatomical geometry

and dimensions modeled on a critical size defect in the radius of New Zealand White rabbits. Scaffolds with a longitudinal central channel to facilitate cell penetration in the inner parts of the constructs were also designed and produced. Optical Microscopy analysis showed a good reproducibility of the internal architecture and high pores interconnection, while the internal channel appeared with a roughly rectangular geometry. FT-IR spectroscopy and thermal analysis showed that the employed technique did not affect the material's chemical-physical properties, which have been demonstrated dependent on the biomass batch used for the polymer extraction. Scaffolds with the internal channel showed significant higher compressive modulus than not-channeled scaffolds. The compressive strength of the structure was correlated to the direction of the stress, in particular maximum stress was significantly higher when strain was perpendicular to the top, rather than the section, of both types of scaffolds. Furthermore, the developed supports showed compressive and tensile mechanical properties comparable to those of analogue scaffolds based on three arm star branched poly( $\epsilon$ -caprolactone), a synthetic polymer previously studied for bone TE.

#### Albumin Removal from Human Fibrinogen Preparations for Manufacturing Human Fibrin-based Biomaterials

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Fibrin is an excellent biomaterial choice for tissue engineering as it is biocompatible, enhances cell attachment and migration, promotes angiogenesis and plays a pivotal role in important physiological processes such as wound healing. Fibrin is often used in combination with other materials and/or chemically modified to enhance its physical properties. Commercially available two component fibrin sealants are commonly used as the starting product to manufacture human fibrin-based biomaterials. However, this option is expensive and allows little further modification of the biomaterial. Human fibrinogen solutions are a more cost-effective option as starting material as well as versatile in terms of further tuning of the biomaterial. Certain impurities (salts and albumin) accompany these products in order to stabilize fibrinogen. Albumin, which can have an inhibitory effect on fibrin activity during tissue regeneration, must be removed to optimise the tissue regeneration process. Within the context of biomaterials and tissue engineering we offer a simple yet novel solution based on classical biochemical techniques (optimised dialysis, SDS-PAGE and image analysis) to significantly reduce albumin in human fibrinogen solutions. This method can be used for various tissue engineering and biomedical applications as a previous step in the manufacturing of human fibrin-based biomaterials to optimise their regenerative application.

#### Electrospun Produced Small Diameter Vascular Grafts: Modification of Physico-chemical Properties and Evaluation of Biocompatibility *In Vitro* and *In Vivo*

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Scaffolds produced by electrospinning represents a convenient materials for production of small diameter vascular grafts (VG) but their low flexural strength, kink and dishevel resistance as well as low porosity and tendency to neointima formation must be improved.

3D matrices (3DM) or VG (i.d. 1,7 mm) were produced using NF-103 electrospun setup from polycaprolactone (PCL), nylon 6, polylactic-co-glycolic acid 50:50 and their mixtures with proteins in 1,1,1,3,3,3-hexafluoroisopropanol solution. 3DM were irradiated

with electron beam by 2 MeV ILU-6 electron accelerator in doses 25 ÷ 150 kGy and tested using testing machine/scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy, porosity was tested as described in ISO7198-98. Human primary endothelial cells (HUVEC) and gingival fibroblasts (HGF) were used in biocompatibility test *in vitro*. Vascular grafts were implanted in abdominal aorta of Wistar rats; intravital MRI, duplex ultrasound scanning (DUS), histochemical or survey light/fluorescent microscopy were used to evaluate grafts functioning.

Introduction of gelatin and irradiation treatment were shown to increase proportional limit and yield stress of PCL almost twice. Irradiation increase surface absorption of proteins in a dose dependent manner but decreases stability of 3DM and efficacy of protein release. Semi-permeable 5–10 micron inner layer allows to vary permeability of VG in a range 20 ÷ 0,05 ml. The data of *in vitro* experiments demonstrate that irradiation does not interfere with adherence, viability and efficacy of HGF and HUVEC proliferation on 3DM. Intravital functioning of VG using MRI and DUS, histochemical and light/fluorescent microscopy of explanted VG demonstrates the excellence of VG with semi-permeable inner layer.

#### Therapeutic Angiogenesis to Treat Heart Ischemia using Controlled Growth Factor Delivery

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**Purpose:** The spatiotemporal controlled delivery of growth factors (GFs) holds great potential to treat ischemic heart disease and repair the damaged muscle. VEGF triggers angiogenesis followed by PDGF to stabilize nascent blood vessels that have the potential to revascularize the ischemic tissue. We utilize fibrin gel and a heparin-based coacervate system to sequentially release these two GFs and evaluate their therapeutic effects in a rat myocardial infarction (MI) model.

**Methods:** VEGF was embedded into the fibrin gel, while PDGF was loaded into the coacervate then embedded into the gel. GF Release was determined by ELISA. A rat MI model was used with intramyocardial injection of saline, empty vehicle, free VEGF + PDGF, or sequentially delivered VEGF + PDGF (n=7 per group). Echocardiography was performed at multiple time points. Immunohistochemical and histological analyses were performed at 4 weeks. Angiogenesis was assessed by staining for VWF and  $\alpha$ -SMA, cardiomyocyte survival by staining for cardiac troponin I, inflammation by staining for CD68, and picosirius red staining for collagen deposition.

**Results:** Nearly 100% of VEGF was released by one week compared to 40% of PDGF whose release sustained to 75% by three weeks, thus achieving sequential release. *In vivo*, sequentially delivered VEGF + PDGF significantly improved cardiac function, angiogenesis, cardiomyocyte survival, and reduced fibrosis and inflammation compared to all other groups.

**Conclusions:** Results suggest the importance of spatiotemporal GF delivery to induce robust and mature neovasculature that contributes to the repair of ischemic heart muscle and improvement of cardiac function after MI. This approach warrants investigation in large animal models.

#### Human Umbilical Cord Vein Pericyte Differentiation for Vascular Tissue Engineering

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Cardiovascular diseases are the number one cause of death globally. Because of the limitation of the number of autologous arteries that can be used for the treatment of vascular diseases, vascular grafts are needed emergently. Synthetic materials have been proven to be suitable for the production of large-diameter blood vessels, but clinical research studies proved that they were not suitable for small-diameter vessels. We investigated whether pericytes could be

differentiated into three different cell types (fibroblast, smooth muscle cell and endothelial cell) for the purpose of small vessel-like three-dimensional structure. Human perivascular cells were isolated from umbilical cord vein and pericytes were purified by using MACs cell separation with CD146 microbeads. Pericytes were cultured in FGM-2, SMCGM-2 and EGM-2 medium for 14 days to investigate fibroblast, smooth muscle cells and endothelial cells differentiations. Cell differentiation was confirmed by immunofluorescence staining and flow cytometry analysis (fibroblast markers; Tenascin-C and Collagen type I, smooth muscle cell markers; Caldesmon and alpha smooth muscle actin, endothelial cell markers; VEGFR1, VEGFR2 and CD31). Immunofluorescence staining and flow cytometric evaluation demonstrated increased expression of fibroblast and smooth muscle cell markers but lack of expression of endothelial markers. The results indicated that pericytes could differentiate into fibroblast and smooth muscles cell but other supplement should be determined for endothelial differentiation. As a conclusion, differentiated pericytes offer an alternative cell source for constructing tissue engineered vascular graft. The authors wish to thank the The Scientific and Technological Research Council of Turkey (Project Number: 113S815) for their financial support.

### Human 3D Vascularized Organotypic Microfluidic Models for the Study of Breast Cancer Cell Extravasation

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Breast cancer is the most common and lethal cancer among women. Noteworthy, 90% of cancer-related deaths are due to metastases and 70% of advanced breast cancer patients develops skeletal metastases. Extravasation is a critical step in the metastatic cascade and consists of cancer cell adhesion to the endothelium and transmigration to invade secondary loci [1]. Despite effective *in vitro/vivo* models have been developed, they lack the high control over the microenvironment and the possibility to perform high resolution imaging, which characterize microfluidic models. The aim of this work is to develop organ-specific human 3D microfluidic assays analyzing breast cancer cell (BCC) extravasation through perfusable microvascular networks (MVNs). The model is a quad-culture in which BCCs extravasate through mural cell supported MVNs embedded within a bone mimicking (BMi) or a muscle-mimicking microenvironment. Extravasation rate and permeability were quantified through real time confocal imaging. Extravasation was significantly higher in bone ( $56.5 \pm 4.8\%$ ) compared to muscle ( $8.2 \pm 2.3\%$ ) or acellular matrices ( $14.7 \pm 3.6\%$ ). However, the highest vessel permeability was detected in the muscle-specific microenvironment ( $(8.37 \pm 1.53) \times 10^{-6}$  cm/s), which was significantly higher compared to bone ( $(4.12 \pm 0.75) \times 10^{-6}$  cm/s) [2]. We demonstrated that muscle-secreted adenosine was responsible for this phenomenon and we showed that adenosine increased permeability when introduced in the BMi microenvironment, despite surprisingly reducing extravasation. Our human organ-specific models demonstrated for the first time that tissue-specific molecules influence both endothelium and circulating BCCs, also regulating permeability and highlighting it as only one of the key factors driving extravasation.

1. Chambers *et al.* Nat Rev Cancer. 2002;2(8):563–572.
2. Jeon *et al.* PNAS. 2015;112(1):214–219.

### Engineering of Corporal Tissue Constructs using Non-human Primate/Human Corpus Cavernosum Smooth Muscle and Endothelial Cells for Clinical Applications

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Conditions such as congenital anomalies of the genitalia, penile cancer, traumatic penile injury, and some types of vasculogenic

erectile dysfunction often require extensive reconstructive procedures to correct anatomical and functional deficiencies of the penis. Various reconstructive procedures have been attempted to achieve functional and cosmetic properties, but these are often limited by a shortage of native penile tissue. In addition, these reconstructive procedures often involve multiple-stage surgeries, which may include the use of silicone penile prostheses or autograft implantation, but corporal tissue function is not restored. We previously demonstrated that reconstitution of autologous cells derived from corpus cavernosum were able to reconstruct functional corporal tissue after being seeded onto the collagen matrix in rabbit model. In this study we investigated the feasibility of applying this approach clinically. Acellular corpora collagen matrices were obtained from non-human primate (NHP) and human donor penile tissue using an established decellularization process. Autologous corpus cavernosal smooth muscle and endothelial cells were isolated from donor monkey and reseeded using a multistep static/dynamic procedure. This study demonstrates that NHP and human cavernosal smooth muscle and endothelial cells seeded onto 3D acellular matrices from donor corpora are able to form well-organized corporal tissue *in vitro*, and this technology may provide a therapeutic solution clinically.

### Magnetic Targeting of Stem Cell Therapies

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The development of regenerative medicine applications have to manage the requirement for large stem cell doses with the needs of cell manufacture, delivery and targeting. A number of groups have developed targeting strategies to guide and hold cells *in situ* whilst performing a repair. These approaches utilize superparamagnetic iron-oxide nanoparticles (SPIONs) to label stem cells and allow control outside the body using magnets. SPIONs can be tracked by MRI, however questions remain around SPION safety and effect both for the labelled cells and the recipient.

This study describes magnetic labelling of a range of therapeutically relevant cell types directed *in vitro* using magnetic fields. Particle retention, excretion and labelling kinetics have been measured with flow cytometry and super resolution microscopy. We demonstrate intracellular distribution of the particles and localization regions. Cell labelling was found to have minimal effects on cell identity, viability and therapeutic potential. Based on these results, we developed and tested a scalable method of cell labelling that could be integrated to a cell manufacturing process.

These results show that magnetic labelling represents a safe and effective potential tool to enhance stem cell therapies by improving targeting without compromising therapeutic potential. This labelling approach could be incorporated to scalable cell manufacturing processes to improve the potential outcome for all patients.

### The Biocompatibility of Piezoelectric PVDF-TrFE Scaffolds with Stem Cell Derived Cardiovascular Cells

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Electroactive piezoelectric scaffolds have an intrinsic property of producing local transient electric fields in response to minute mechanical deformation caused by cell contraction. As myocardium is an electroactive tissue, piezoelectric polymers may be an attractive material for cardiovascular tissue engineering applications. In this study, the biocompatibility and the effects of piezoelectric poly(vinylidene fluoride trifluoroethylene) (PVDF-TrFE) scaffolds on the function of mouse embryonic stem cell (mESC) derived cardiomyocytes (mESC-CM) and endothelial cells (mESC-EC) were examined.

Spontaneously contracting MESC-CM were seeded onto aligned PVDF-TrFE scaffolds and cultured for up to 6 days. MESC-CM adhered well, became highly aligned and demonstrated registered sarcomeres. MESC-CM cultured on PVDF-TrFE scaffolds exhibited cardiomyocyte phenotype by highly expressing cardiac specific markers including cardiac troponin T, myosin heavy chain and connexin43. MESC-CM also exhibited a positive inotropic response to  $\beta$ -adrenergic stimulation through a doubling in beating frequency upon isoproterenol administration. Calcium transients in response to caffeine exposure demonstrated normal calcium handling properties with functional sarcoplasmic reticulum. In addition, mESC-EC seeded onto PVDF-TrFE scaffolds remained highly viable and expressed endothelial cell markers including platelet endothelial cell adhesion molecule, Notch 1 and endothelial nitric oxide synthase. MESC-EC also demonstrated an uptake of low-density lipoprotein, a hallmark of mature endothelial cells, further indicating the maintenance of their phenotype on the scaffolds.

These results demonstrate the biocompatibility of PVDF-TrFE with stem cell derived cardiovascular cells while opening the door for further investigation into the benefits of electroactive scaffolds on functional maturation of cardiovascular cells.

#### Scaffolds Generated using Naturally-derived Growth Plate Extracellular Matrix facilitate Osteogenesis *In Vitro* and induce Host-mediated Bone Healing *In Vivo*

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Growth plate (GP) matrix contains an array of biomolecules and growth factors which are known to promote vascularization and endochondral ossification. We hypothesize that osteogenic and angiogenic bioactive signals will be retained within a GP ECM-derived scaffold, and the objective of this study was to establish the osteoinductive nature of this material *in vitro* and investigate the ability of acellular GP scaffolds to induce healing within a critically-sized defect.

GP tissue was harvested from porcine hind limbs, blended, decellularised and freeze-dried to produce porous 3D constructs. For *in vitro* studies BM-MSCs were seeded onto the constructs ( $0.5 \times 10^6$ /scaffold) and cultured in either chondrogenic or osteogenic medium for 28 days. Acellular constructs were then implanted into 7 mm cranial defects of skeletally mature Fischer rats, and harvested at 4 and 8 weeks to analyse the quality of the repair tissue.

GP scaffolds seeded with MSCs supported calcium deposition *in vitro* in both media conditions, however increased levels of sGAGs, collagen type II and type X deposition were observed in constructs cultured in chondrogenic medium vs. osteogenic medium. Upon acellular implantation, GP ECM scaffolds were shown to accelerate endogenous bone regeneration, with significantly enhanced levels of mineralisation obtained at both time points. De novo bone tissue formed within the scaffold treated defects, in addition to the presence of strong sGAG staining, compared to predominantly fibrous tissue bridging the defect in the untreated group. This illustrates the potential of these scaffolds to serve as an off-the-shelf, cell-free treatment option for bone regeneration.

#### Characterization of Rabbit Urine-Derived Stem Cells for Potential Application in Urethral Tissue Regeneration

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**Introduction:** Rabbits are commonly used as an animal model in urethral tissue repair. The goal of this study was to characterize rabbit urine-derived stem cells (rUSCs) and induce them to differentiate into urothelial and smooth muscle cells for potential use in lower urinary tract tissue regeneration.

**Methods:** Urine was collected from six adult male rabbits (weight 2.0~2.5 kg) through an F8 catheter. Cells were isolated from the urine, cultured with a mixed medium of KSMF and EFM, and extensively expanded *in vitro*. Growth curves, cell surface markers, karyotyping, and differentiation into urothelial and smooth muscle cell lineages for rUSCs were assessed *in vitro*.

**Results:** rUSCs had a rice grain-like shape and appearance. Mean population doubling and average doubling times of rUSCs were  $45.0 \pm 6.2$  hrs and  $25.7 \pm 8.4$  hrs, respectively. Flow cytometry assays of rUSCs at *p3* were positive for CD29, CD90, and CD105, but negative for CD31, CD34, and CD45. Karyotype analysis indicated that chromosomes of cultured rUSCs remained normal up to passage 12. When exposed to TGF- $\beta$ 1 and PDGF-BB, rUSCs differentiated into spindle-like cells, expressing smooth muscle-specific proteins, including  $\alpha$ -smooth muscle actin, desmin, and myosin. Urothelially-differentiated rUSCs expressed urothelial-specific proteins, i.e. uroplakin-Ia and -III, AE1/AE3, when exposed to epidermal growth factor.

**Conclusions:** Rabbit urine-derived stem cells can be easily isolated and cultured *in vitro*. These cells possess strong proliferative ability, and are capable of differentiating in urothelial and myogenic lineages. Thus, they are a potential alternative autologous cell source for lower urinary tract repair in a rabbit model.

#### Automation of Tissue Decellularization - Increasing Stability, Safety and Possibilities

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In the form of decellularized small intestine tissues or pericardium, tissue engineered graft materials have reached the clinical routine. However, most published production processes strongly vary in their time steps and involve the manual incubation with chemical reagents or mixtures, followed by many washing cycles. Therefor human error, reproducibility and integration in the workflow display major obstacles in the production processes. To overcome this lack, we developed a fully automated decellularization device. The device allows an easy and automatic adaption of multiple individual decellularization protocols for the parallel treatment of five separated grafts. Therefore, the exposition to up to four different reagents and a washing solution, their sterilization by filtration and UV-C-exposition is possible. Additionally an ultrasonic power source for increased decellularization efficiency is integrated in the system. Treatment characteristics like concentration, duration or repetitions of the single decellularization steps as well as the ultrasonic intensity can be individually defined. An additional unique feature of the device is the process and storage container system. Prior to the process, the unsterile biological material is placed in sealable storage bags. After sealing, the complete procedure, form decellularization over washing to sterilization and the storage is performed completely independent from the surrounding environment. Thus, no further handling of the sterile material is necessary and the risk of contamination is reduced to a minimum. In summary, the device allows the ultrasonic-aided and fully automated decellularization of multiple tissue engineered graft materials. Additionally the innovative storage concept simplifies the process and guarantees reproducible results.

#### Development of Novel Wet-spun Poly( $\epsilon$ -caprolactone) and Poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate] Biodegradable Stents

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Intravascular stents are hollow cylindrical meshes that prop open an injured blood vessel and are frequently used as a component of peripheral artery angioplasty. The first stents developed were metallic and permanent in nature, which could contribute to intimal hyperplasia, mismatch of the stent to the vessel size, restenosis and late

thrombosis. Thus, the concept of bioabsorbable stents has emerged as an alternative to permanent metal stents. The aim of this study was to develop polymeric biodegradable stents for the treatment of injured small caliber (<3 mm) blood vessels. The polymers chosen for stent production were a microbially produced poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate] (PHBHHx) and poly(ε-caprolactone) (PCL), present in currently FDA approved devices. Furthermore, a novel computer-aided wet-spinning apparatus for the production of three-dimensional microstructured polymeric constructs with a tubular geometry was designed and assembled. The produced stents exhibited a well-defined tubular micro-fibrous structure. By tuning the fabrication parameters, it was possible to produce stents with different morphological characteristics (length, porosity and wall thickness), underlining the versatility of the developed technique in customizing stent structural and dimensional features. By axial and radial mechanical compression tests, PHBHHx stents demonstrated great elasticity, in particular a full elastic recovery up to radial deformation of 70% of the diameter, thus showing potential compliance with the treated artery. PCL stents showed mechanical strength comparable with PCL stents produced by different techniques and the ability to expand through a coronary stent system balloon, maintaining the expanded state after deployment.

#### Magnetic Nanoparticle Guided Corneal Endothelial Cell Delivery

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**Purpose:** The corneal endothelium is responsible for corneal clarity; however, it poses a challenge for clinicians due to its location, lack of regenerative potential, and reducing cell population with age. This study investigates human corneal endothelial cells (HCEC) loaded with magnetic nanoparticles as an alternative non-surgical technique cell delivery system to corneal transplants.

**Methods:** HCEC were cultured in human endothelial serum free media containing 10 ng/ml FGF-2, and loaded with super paramagnetic iron oxide nanoparticles (SPIONP). Cell lineage and cell viability of SPIONP loaded HCECs was evaluated using FACS analysis and Cy-Quant assays, respectively. Intracellular iron content was evaluated via Elzone particle analysis and Prussian blue staining. Finally, SPIONP loaded HCECs were evaluated for inner cornea attachment.

**Results:** HCEC maintained their endothelial lineage, as was confirmed by their simultaneous expression of CD200 and glycoproteins. Furthermore, verification of SPIONP internalization by HCEC was demonstrated by Prussian blue staining and Elzone particle analysis. Lastly, HCEC maintained similar viability ratios as unloaded control cells, and demonstrated the *in vitro* ability to attach to the backs of corneas, the target region of choice.

**Conclusions:** Overall, studies showed that HCEC readily incorporated SPIONPs without changing the overall health of the cell. Furthermore, proof of concept studies performed here indicate that SPIONP-loaded HCECs can be incorporated onto the back of the cornea and potentially directed towards a specific target area by exposure to a magnetic field. Therefore, this cellular delivery system could be an effective alternative to corneal transplant.

#### Critical Role of MCSF in Salivary Gland Branching Morphogenesis

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Submandibular salivary gland (SMG) branching morphogenesis is regulated by the crosstalk between epithelium and mesenchyme cells. In modern strategies in regenerative medicine and tissue engineering, biomaterials provide a provisional three-dimensional (3-D) support similar to the extracellular matrix (ECM) to interact biomolecules with the cells to control their functions and guiding tissue growth. Some previous report confirmed the presence of tissue

residence macrophages (M2 macrophages) in different types glandular tissue during developmental stages. In the present study, since M2 macrophage is led by macrophage colony stimulating factor (MCSF) stimulation, we explore the effect of the MCSF on glandular tissue morphogenesis and the mechanism below the phenomena using the synthetic biomaterials. We use mouse SMG explant cultured on the stiffer alginate hydrogel sheet and found that exogenous MCSF accelerated the tissue growth. We also observed that the expression of Neurturin, VEGF and other important molecules responsible for tissue growth was also upregulated after MCSF supplementation. *In vitro* self-organization and *in vivo* studies revealed that anti-MCSF attenuated the neuronal growth and subsequent epithelial morphogenesis. It's suggests that MCSF secreted from the surrounding mesenchyme, triggering the initiation of morphogenesis through the secretion of a variety of molecules.

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**Disclosures:** There is no conflict of interest.

#### Benzoic Acid Enhances Alp, Osteopontin Expression and Osteoblast Differentiation Via Erk Signal Protein Stimulation During Osteogenesis

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Benzoic acid (BA) is a constituent of Whitfield's ointment which is used for the treatment of fungal skin diseases such as tinea, ringworm, and athlete's foot. The aim of the present study was to determine whether there is relation between ALP, osteopontin and Erk pathway during osteogenesis by BA. Mouse bone marrow stromal cells were cultured in osteogenic differentiation medium (ODM) for 6 days and treated with BA for 1 day. Then the cells were subjected to various experiments such as alizarin red S staining, alkaline phosphatase (ALP) activity and calcium content analysis, real-time PCR, Western blot and fluorescence microscopy. Osteogenic differentiation was enhanced when cells were treated with BA, as determined by alizarin red S staining and ALP activity. To investigate whether benzoic acid enhanced ALP, osteopontin and signaling protein during osteoblastic differentiation we assessed at BA stimulated ALP, osteopontin and Erk expression using Western blot. After BA stimulation, cells were increased p-Erk expression. The results of the present study suggest that the Erk is involved in the APL and osteopontin enhancement BA during osteogenesis. These results suggest that BA increases ALP and osteopontin expression via Erk signal protein expression during osteogenesis. Better understanding of the BA signal mechanism can have potentially effects on therapeutic strategies from bone and to musculoskeletal related diseases.

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#### 3D Printing of Complex Biological Structures using Soft Hydrogels

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3D printing can create complex biomimetic structures that are difficult to fabricate using traditional approaches. However, the soft hydrogels used in tissue engineering, are challenging to print because they collapse when printed in air. This limits the achievable resolution and requires the use of support materials that can interfere with the printed structure. We hypothesized that a Bingham plastic bath composed of a sacrificial, thermo-reversible hydrogel could be used as a support within which we could print complex hydrogel scaffolds. Termed freeform reversible embedding of suspended hydrogels (FRESH), results show that we can (i) print hydrogels within a compatible support material, (ii) remove the



support material without disrupting the printed hydrogel, and (iii) do this under sterile conditions while maintaining cell viability, cytocompatible temperature, pH and osmolality. The support bath was produced by creating a gelatin slurry, with particle size less than 100 µm, and transferring it to a dish within which 3D printing was performed. Hydrogel inks including alginate, collagen and fibrinogen were loaded into a modified MakerBot Replicator 3D Printer with a syringe pump extruder, deposited layer-by-layer, and released by melting the slurry at 37°C. Complex structures printed using FRESH include a right coronary arterial tree with sub-millimeter wall thickness and a perfusable lumen. More complex internal structure was demonstrated by printing an embryonic chick heart with high fidelity and a well preserved trabecular structure. Future work is focused on using FRESH to print scaffolds for engineering cardiovascular tissues using a range of biological hydrogels.

### The Development of Plasma Modified Electrospun Poly(L-Lactide-Co-D, L-Lactide) Matrices for the Treatment of Corneal Scarring

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Corneal disease and scarring is a serious clinical problem affecting more than 10 million people worldwide. Corneal transplantation or keratoplasty is the main method of repairing visual loss as a result of corneal scarring but is limited by the lack of available donor cornea. This combined with the postoperative risks of persistent epithelial defects, corneal ulceration, unhealed wounds and additional scarring creates a clinical need for more effective treatment protocols that can promote healthy regeneration of the cornea and ultimately restore sight.

This study examines the potential of tissue-engineered biomaterials to treat corneal blindness. Electrospun (ES) matrices using a copolymer of poly(L-lactide-co-D, L-lactide) [PLDLLA] were manufactured and processed using a cold plasma dielectric barrier (DBD) treatment protocol to enhance the physical and chemical surface properties of the ES matrices. Characterisation of the matrices using X-ray photon spectroscopy analysis demonstrated a rise in surface oxygen content on DBD treated samples, indicating a potential increase in surface wettability. Fourier transform infrared spectroscopy and scanning electron microscopy both confirmed that no significant changes were made to the bulk composition or morphology of the ES matrices after plasma treatment. A 28-day biological study showed a higher rate of proliferation and monolayer formation of the HCE-T cell line, on plasma treated ES matrices versus that of the control.

This study concluded that plasma treated ES PLDLLA matrices have the potential to enhance corneal epithelial monolayer development for potential use as a tissue engineered product.

### Marine Collagen/Apatite Scaffolds Envisaging Tissue Engineering Applications

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Despite the vast investigation and the large amount of products already available in the market to treat the different bone defects there is still a growing need to develop more advanced and complex therapeutic strategies. In this context, a mixture of Marine Hydroxyapatite-Fluorapatite:Collagen (HA-FP:ASC) seems to be a promising solution to overcome these bone defects, specifically, dental defects. HA-FP particles (20–63 µm) were obtained through pyrolysis (950°C, 12h) of shark teeth (*Isurus oxyrinchus*, *P. glauca*), and Type I collagen was isolated from *Prionace glauca* skin as previously described

(1). After the steps of purification, collagen was solubilized in 0.5 M acetic acid and HA-FP added producing three different formulations: were produced, 30:70, 50:50 and 70:30 of HA-FP:ASC, respectively. EDC/NHS and HMDI binding agents were used to stabilize the produced scaffolds. Mechanical properties were evaluated by compression tests. SEM analysis allowed observing the mineral deposition, after immersion in simulated body fluid and also permitted to evaluate how homogenous was the distribution of HA-FP in the different scaffold formulations, also confirmed by µ-CT assay. It was readily visible by Cytotoxicity and life/dead CLSM assays that cells were able to adhere and proliferate in the produced scaffolds.

Scaffolds crosslinked with EDC/NHS showed lower cytotoxicity, being the ones chosen for further cellular evaluation.

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### Characterization of *in Vitro* Cultured Murine and Human Endometrial Stromal Cells in the Presence of Urinary and Recombinant FSH

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**Objective:** It is important for the generation of artificial uterus to culture endometrial cells efficiently *in vitro*. FSH receptors (FSHR) are expressed in extragonadal tissues such as endometrium. FSH is widely used for the stimulation of ovarian follicles in assisted reproductive cycles. To date direct effects of FSH on the endometrium is rarely elucidated. The effects of urinary and recombinant FSH on the proliferation of murine and human endometrial stromal cells *in vitro* were evaluated in this study.

**Methods:** Endometrial stromal cells (ESCs) were collected from the uteri of 6 week-old, c57/BL6 female mice by mincing and sieve filtration of acquired endometrial stromal tissues. Human endometrial stromal cell line (T-HESC, ATCC) was also used for the study. ESCs were plated as concentration of 1.5 × 10<sup>5</sup> cells/plate, and treated with 7.5, 15, 30, 150 mIU/mL of urinary and recombinant FSH for up to 72 hrs. Expression of cell cycle genes and the proliferation of treated cells were assessed using qRT-PCR and BrdU assay from 24, 48, 72 hrs groups.

**Results:** Regardless of cell type, urinary FSH tended to inhibit the cell proliferation up to 48 hrs. In contrast, recombinant FSH showed divergent effects on the ESC proliferation. No dose dependent effects were observed. Cell cycle-related gene expression was not significantly altered after FSH treatment.

**Conclusion:** Short-term treatment of FSH may affect the *in vitro* proliferation of murine and human ESCs. Further combined treatment of FSH and LH needs to be tested for the processing of artificial uterus (HI14C2289010014 and HI14C2259010014).

### Gold Nanoparticle-Decellularized Matrix Hybrids for Cardiac Tissue Engineering

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Cardiovascular diseases, such as myocardial infarction, remain the most common cause of morbidity and mortality in developed countries. The aim of cardiac tissue engineering is to develop functional 3-dimensional (3D) tissue patches *in vitro*, later to be delivered to the damaged area in the myocardium to promote regeneration. Decellularized matrices are valuable scaffolds for engineering functional cardiac patches. However, the lack of quick and efficient electrical coupling between adjacent cells, and a possible adverse immune response after patch transplantation, may jeopardize the success of their clinical use. To address these issues, we have deposited gold nanoparticles on fibrous decellularized omental matrices and investigated their morphology, conductivity and degradation. Since parts of the omentum can be easily, quickly and safely harvested from the patient, after efficient cell removal and quick processing, a personalized porous non-immunogenic 3D scaffold can be obtained. Next, the decellularized scaffold is decorated with conductive motifs using an e-beam evaporator. We have shown that cardiac cells engineered within these hybrid scaffolds exhibited elongated and aligned morphology, massive striation and organized connexin 43 electrical coupling proteins. Finally, we have shown that the hybrid patches demonstrated superior function as compared to pristine patches, including stronger contraction force, lower excitation threshold and faster calcium transients.

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#### Characterizing the Impact of 3D Bioprinting Parameters on Extruded Construct Properties

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In recent years, advances in tissue engineering have brought about the development of 3D bioprinting. Despite efforts to improve the cytocompatibility and structural complexity of the extruded biomaterial, the effects of hydrogel bioprinting parameters on construct properties have yet to be comprehensively characterized. Using a pneumatically-driven Biobots 3D bioprinter, micro-computed tomography, finite element analysis, mechanical testing and various microscopy techniques, we systematically assessed the fidelity, stress distribution, rigidity, resolution and cytocompatibility of extruded hydrogel constructs. Gelatin methacrylate (GelMA) was selected as a hydrogel for characterization and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was selected as a crosslinker for photopolymerization under visible light (405 nm). As expected, we found that biomaterial composition plays a major role in structural rigidity: the Young's modulus of constructs increased approximately six-fold, from  $64 \pm 8$  kPa to  $360 \pm 29$  kPa, as GelMA concentration was raised from 10 w/v% to 20 w/v% with 0.25% LAP. Modulation of extrusion pressure also revealed the existence of optimal extruding pressures, which increased from 80 psi to 130 psi as GelMA concentration increased from 10% to 20%. Moreover, an increase in printing resolution was observed with both increasing GelMA concentration and increasing printing speed. The viability of human vascular endothelial cells encapsulated in 15% GelMA was found to be approximately 50% two hours after extrusion, in agreement with reports describing the relative fragility of these cells. These results reveal complex interactions between various bioprinting parameters and their effects on printing resolution. Such a systematic characterization will be instrumental in developing a versatile and modular biofabrication platform.

#### Osteoblast Conditioned Medium Stimulates Osteogenic Differentiation of iPS Cell-derived Mesenchymal Stem Cells

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Recent studies showed that induced pluripotent stem cells (iPSCs) could be maintained in an undifferentiated state and subsequently be differentiated into mesenchymal stem cells (iPS-MSCs). The goal of the present study was to investigate the effect of osteoblast conditioned medium (CM) on osteogenic differentiation of mouse iPS-MSCs. Osteoblasts were harvested from one-day-old ICR mice cranium by adherent culture and enzyme digestion methods. Conditioned media from cultured osteoblasts were collected and added on mouse iPS-MSCs, in which iPS-MSCs were induced to osteoblasts. Defined conditioned medium distinctly promoted iPS-MSCs to become poly-edged morphologically after induction for 14 days and there was a significant increase in alkaline phosphatase (ALP) activity and Runx2 expression, as well as enhanced expressions of *runx2*, *alp*, *col* and *ocn* mRNA in the presence of CM. Mouse osteoblast conditioned medium have an active stimulatory role in steering iPS-MSCs towards osteoblasts, which may serve as a potential source of osteogenic seed cells for bone tissue engineering.

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#### Water Vapor Annealing of Silk for Vascular Grafts

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Clinically effective small diameter synthetic vascular grafts are lacking. Conventional graft polymers such as ePTFE and Dacron are non-compliant and lack biocompatibility. Bypass of blocked coronary arteries is therefore reliant on autologous grafts, which are unavailable in many patient cases due to prior removal or disease. We propose a holistic approach to the development of a durable, biocompatible arterial conduit, which is mechanically robust, favorably interacts with vascular cells and is hemocompatible. Silk fibroin is a versatile natural polymer with remarkable mechanical properties, highly tunable degradation profile which is extremely well tolerated *in vivo*. This work reports the engineering of electrospun silk conduits with highly controllable mechanical properties, influenced by electrospinning solvent (Hexafluoro-2-propanol - HFIP and H<sub>2</sub>O) and crosslinking method (H<sub>2</sub>O vapor annealing at 4°C, 24°C, 37°C and 55°C). All silk scaffolds supported human coronary artery endothelial cell attachment and proliferation to at least 4-days, superior to electrospun PCL controls. Preliminary mechanical testing showed that water-spun silk was more elastic than when using HFIP, irrespective of cross-linking conditions. Higher annealing temperatures yielded stronger materials. Pilot *in vivo* studies in a rat aortic replacement model show that electrospun silk grafts sutured well, did not leak and were well tolerated for up to 21 days. Further evaluation of the tissues will determine the immune response, degradation rate, rate of re-endothelialisation and neointimal hyperplasia. Ultimately, we propose that elastic water-spun silk conduits are promising synthetic vascular grafts that could provide an improved alternative to existing commercial conduits.

#### Exosomes Obtained from Mesenchymal Stem Cells as a Novel Therapeutic Tool for Aortic Aneurysm

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**Background:** We reported that intravenous injection of bone marrow-derived mesenchymal stem cells (BM-MSCs) could reduce the incidence rate of aortic aneurysm (AA) by way of the anti-inflammatory and tissue repair properties of MSCs. On the other hand, it is known "Exosomes (obtained from MSCs)" improve GvHD symptoms owing to its immunosuppression and anti-inflammation. Thus, we verified the effects of BM-MSCs-derived exosomes for treatment of AA *in vitro*.

**Methods:** The exosomes were collected from the serum-free conditioned medium of BM-MSCs by ultra centrifugation. They were characterized by transmission electron microscopy and flow cytometry. TNF- $\alpha$ -activated macrophages or aortic smooth muscle cells (SMCs) were incubated with or without 10  $\mu$ g/mL exosomes for 12 hrs and was performed quantitative RT-PCR to investigate expression of AA related gene.

**Results:** The exosomes were detected with the specific surface marker for CD9 and CD81. In macrophages, the gene expression of IL-1 $\beta$  (3.5 vs 0.3,  $p < 0.05$ ), TNF- $\alpha$  (0.6 vs 0.2,  $p < 0.005$ ), MMP-2 (1.1 vs 1.9,  $p < 0.05$ ) and MMP-9 (1.3 vs 2.1,  $p < 0.05$ ) was significantly decreased and the gene expression of Cystatin C (1.2 vs 0.8,  $p < 0.05$ ), MMP-2 (1.3 vs 0.8,  $p < 0.05$ ) and TIMP-2 (1.3 vs 0.8,  $p < 0.005$ ) in SMCs was significantly increased in the exosome group compared to the control group.

**Conclusion:** These results suggest that exosomes might be a novel therapeutic tool for AA.

#### A Hydrogel Bioink Toolkit for Bioprinting Tissue Constructs while Mimicking Native Tissue Biochemical and Mechanical Properties

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Advancement of bioprinting technology is limited by the availability of materials that both facilitate bioprinting logistics as well as support cell viability and function by providing tissue-specific cues. Herein we describe a modular hyaluronic acid (HA) and gelatin-based hydrogel toolbox comprised of a 2-crosslinker, 2-stage polymerization technique, and the capability to provide tissue specific biochemically and mechanically accurate signals to cells within biofabricated tissue constructs. First, we prepared and characterized several tissue-derived decellularized extracellular matrix-based solutions, which contain complex combinations of growth factors, collagens, glycosaminoglycans, and elastin. These solutions can be incorporated into bioinks to provide the important biochemical cues of different tissue types. Second, we employed combinations of PEG-based crosslinkers with varying molecular weights, geometries (linear, 4-arm, and 8-arm), and functional groups to yield hydrogel bioinks that supported extrusion bioprinting and the capability to achieve final construct shear stiffness values ranging from approximately 100 Pa to 20 kPa. Lastly, we integrated these hydrogel bioinks with a 3-D bioprinting platform, and validated their use by bioprinting primary liver spheroids in a liver-specific bioink to create *in vitro* liver constructs with high cell viability and measurable functional albumin and urea output. This hydrogel bioink system has the potential to be a versatile tool for biofabrication of a wide range of tissue construct types.

#### Optimisation of Hydrogel Injection for Regeneration of Myocardial Infarction

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Local delivery of stem cells is a promising treatment for patients with chronic heart disease (Strauer, JACC, 2011). However, it has been suggested to be limited by poor cell retention (Bartunek, Clin Pharmacol Ther, 2009). Embedding stem cells in hydrogels can increase retention of intramyocardially delivered cells (Wang, Acta Biomater, 2009). Subsequently, the objective of this study is to determine optimal injection parameters for retaining the largest volume of intramyocardially delivered hydrogel.

*In vitro*, stained Poloxamer hydrogel (100  $\mu$ L) was endocardially injected into body temperature equilibrated porcine ventricle. Injections were made with bevel and pencil tip needles of gauges (G) 17 and 26, each inserted at 50 and 1000 mm/min, and helical and L-shape needles of outer diameters 0.51 and 0.5 mm respectively. Samples were uniaxially compressed 60 seconds post injection for up to 1,000 cycles before examination using a 3D modelling technique.

Dispersion/retention was found to be significantly higher for helical, L-shape, 26G pencil tip and 26G bevel tip needles, compared to 17G needles. Boluses for 26G bevel, helical and L-shape needles lay remote of the endocardial surface, while boluses for 26G pencil tip needles lay just under it. Potentially, 26G pencil tip boluses are therefore more exposed to myocardial contraction and washout. Helical or L-shaped needles do not enhance gel retention over a standard straight needle. In conclusion, we therefore recommend injection with 26G bevel tip or 0.5 mm L-shape needles to maximise hydrogel retention. Further studies incorporating embedded stem cells and alternative hydrogels will lead to further optimisation.

#### Biofabrication of Cell-laden Tissue Engineering Constructs: Temporary Addition of Methylcellulose to Low Concentrated Alginate Strongly Improves Printing Properties

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Biofabrication is a growing research area in regenerative medicine which aims to provide tissue engineering constructs with tailored architecture and organized cell placement. Simultaneous processing of matrix material and sensitive biological components requires mild process conditions which can perfectly be fulfilled by the 3D plotting technology. We developed a novel alginate-based plotting material which combines suitable features for both cell embedding and accurate deposition by 3D plotting using the Bio-Scaffolder from GeSiM (Großberkmannsdorf, Germany). The addition of methylcellulose to low concentrated alginate resulted in a strong enhancement of viscosity allowing the generation of real 3D structures with defined geometry and dimensions in the range of centimeters. SEM analysis revealed that the alginate strands exhibit a porous inner structure caused by dissolving of methylcellulose after gelation of the alginate with Ca<sup>2+</sup> ions which might be advantageous in regard of nutrient and oxygen supply. Live/dead staining 24 h after plotting revealed that many cells suspended in the alginate-based hydrogel survived the plotting process. Viable cells have been also detected after cultivation of the cell-laden matrices for 21 days indicating the suitability of the hydrogel as cell carrier. Differentiation potential of the emedded hMSC has been proven for the adipogenic lineage.

The newly developed composite material enabled the fabrication of cell-laden matrices with defined geometry and clinically relevant dimensions applying a simple, inexpensive and versatile method. Through the incorporation of further components such as extracellular matrix molecules the material should be suitable to generate tissue substitutes for a number of different tissue types.

#### Development of Functional Tissue-engineered Artificial Cardiac Construct generated by Human Induced Pluripotent Stem Cells

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**Background:** Tissue-engineered artificial cardiac constructs (ACCs) derived from human induced pluripotent stem cells (hiPSCs) are promising for myocardial regenerative medicine. However, the appropriate ratio of cardiomyocytes (CMs) and non-CMs, which would substantially impact on functionality of the ACC, is poorly understood. We herein developed the well-functioning ACCs derived from hiPSCs.

**Methods:** Cardiomyogenic differentiation was induced in hiPSCs. The cell preparation was separated based on expression of the iPSC-CM specific marker, CD172, using a magnetic-activated cell sorting system to produce cell preparations with different ratios (25, 50, 70, and 90%) of iPSC-CMs, which were then prepared as scaffold-free constructs using thermoresponsive culture dishes.

**Results:** The ACC showed synchronized spontaneous beating when CMs constituted 50% or more of the total cells in the ACC. The electrical conduction velocity, assessed by a multi-electrode array, was positively related to the CM ratio in the ACCs. However, the ACC including 90% CMs failed to form a stable structure. Collagen type I and III were more abundantly expressed in the ACC including 25% and 50% CMs, whereas laminin alpha 2 and 4, were more abundantly expressed in the ACC including 70% CMs, as assessed by quantitative PCR. In addition, peak ratio, upstroke velocity, of  $Ca^{2+}$  transient were positively related to the CM ratio of ACCs, but ACCs including 90% CMs did not exceed ACCs including 70% CM.

**Conclusions:** The ACC composed of 70% CMs displayed greater stability and functionality compared to those of other ratios, indicating a possibility of optimal ACCs as a bio-cardiac support device.

### Increased Synergism of Human Mesenchymal Stem Cells and Delivery of BMP-2 Exert Potent Antitumorigenic Effects

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Traditional minimally invasive therapies in tumor ablation by one time delivery represent alternatives to surgery. The replication defective adenovirus containing human BMP-2 gene transfected human bone marrow mesenchymal stem cells (advBMP-2/hMSCs) are capable of continual secretion of BMP-2 within 3 months by single injection.

There was decreased proliferation via enhanced apoptosis, increased cell migration in Hep3B coculture advBMP-2/hMSCs group. The regional injected advBMP-2/hMSCs in a xenogenic subcutaneous human HCC Hep3 models induced tumor apoptosis and prolonged animals' survival.

It is a suggested nonsurgical regional cancer therapy that the synergic effects with transfected hMSCs intrinsic antineoplastic properties and their continual autocrine BMP-2 after one delivery may enhance the tumor apoptosis, compromise the tumor cachexia and postpone animals' death.

The engineered hMSC therapy may be a new alternative in minimally invasive tumor ablation.

### Pericyte Plasticity - Investigation of the Angiogenic and Multilineage Potential of Pericytes

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Pericyte recruitment is essential for the stability of newly formed vessels during angiogenesis. In addition, it was recently suggested that pericytes represent common ancestor cells providing an *in vivo* source of mesenchymal stem cells. Here, we investigated pericytes from different human tissues with regard to their angiogenic and multilineage potential *in vitro*.

Retinal and placental pericytes have been investigated as source of mature pericytes. MACS and FACS have been used to enrich

CD34neg/CD146pos cells from adipose tissue and bone marrow as potential sources of primary pericytes for tissue engineering applications. The multilineage potential of pericytes was assessed by testing their capability to differentiate towards osteogenic, adipogenic and chondrogenic lineage *in vitro*. To study the function of pericytes in angiogenesis, pericytes and endothelial cells were co-seeded on Matrigel and the formation of tube-like structures examined after 24 h.

Our results show that not all pericyte types had multilineage differentiation potential, both retinal and placental pericytes failed to differentiate into adipogenic or chondrogenic lineage. Expectably, adipose tissue-derived cells displayed poor chondrogenic differentiation upon stimulation with TGF- $\beta$ . Matrigel assays revealed that pericytes from all tissues integrated into tube-like structures with a significant enrichment of pericytes at branching points. The pericyte-endothelial cell ratio determined the efficiency of network formation. Here, an increasing number of pericytes resulted in inhibition of tube-like structure formation.

We show that bone marrow and adipose tissue-derived pericytes have the combined function to regenerate tissue of different lineages and to support neovascularization. This might be of particular interest for vascularized tissue engineering.

### Hydrogel Coatings on Decellularized Tissue Engineered Vascular Grafts (TEVGS)

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Vascular disease surgical treatments such as decellularized tissue engineered vascular grafts (TEVGs) are clinically hindered by high thrombogenicity. Decellularization destroys the cell glycocalyx, which is composed of "anchors" proteins (proteoglycans) that hold a continuous glycosaminoglycan (GAG) layer (2–10  $\mu$ m) of which the largest is hyaluronic acid (HA). Given that HA is a naturally occurring GAG shielding blood elements from the underlying vasculature, we speculated that surface modification of acellular grafts with HA would improve decellularized grafts hemocompatibility.

Using the amine groups present on decellularized TEVGs as "amine hooks" we crosslinked a 5  $\mu$ m thick continuous HA hydrogel layer. The HA coating was architecturally characterized via SEM, TEM and histology, demonstrating that the horizontally grown HA hydrogel layer recreates a luminal wall "hiding" exposed collagen. To test the efficacy of the coating *in vivo*, we HA coated decellularized syngeneic rat aortas and implanted into Fischer rats for 4 weeks. Since the rodent aortic grafting model is not highly predictive of graft function in either large animals or in humans, we undertook a pilot study in a dog recipient. Acellular canine TEVG were implanted as bilateral end-to-side carotid bypass grafts, with the intervening segment of carotid artery occluded using surgical clips: one side uncoated, and the other with HA-coated. The animal studies demonstrated preservation of the HA coating after 4 weeks implantation time, and reduced graft thrombosis compared to the control grafts. In conclusion early evidence suggests that HA coating architecturally and functionally recreates an antithrombogenic luminal wall on decellularized grafts.

### Utilizing Arteries from Adult Human Donors for Blood Vessel Tissue Engineering

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**Introduction:** Tissue engineered blood vessels promise to surpass vascular prostheses, auto- and allo- grafts in accessibility, size match, resistance against infection and thrombosis, mechanical compliance and endurance, non-immunogenicity, and physiological integration. We consider human acellular vascular matrix to be a strong candidate for clinical translation in midterm due to its properties either inherent or acquired by different means (mesenchymal stem cells (MSC), in particular).

**Material and Methods:** Iliac arteries (n=15) common, external and internal; small mesenteric arteries (n=3) were taken from deceased heart beating donors during organ procurement. Before decellularization biomaterial had been transported in preservation solution at +4°C within 24 hours, stored at -80°C. Histological, DNA fluorescence and extraction, primary cytotoxicity tests were done.

**Results:** The individual protocols of decellularization were developed for different arteries applying detergent-enzymatic method and simultaneous inside- and outside lumen perfusion, varying: the time of each step respectively to the inner diameter of vessels and wall thickness; and concentrations of detergents. Histological and fluorescence examination confirmed total cell elimination with the drop of DNA at least by 97%. Moreover the 3 layer structure of vessels, elastic membranes and lamellas, collagen fibers preserved their integrity. Primary cytotoxicity tests revealed that rabbit MSC are able to attach and successfully proliferate on the scaffold.

**Conclusion:** We successfully developed process of decellularization of human arteries of various diameters with preservation of vessel's microarchitecture and cell-compatibility, which is important for graft size matching and maintaining long-term graft patency. This increases the potential of grafts for vascular reconstructive surgery usage.

### 3D Bioprinting Malignant Tumor Micro-tissues for Cancer Research

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More than 1.66 million new cases of malignant tumor occurred and it caused about 25% of all deaths in the United States in 2014. The objective of this study is to develop a biomimetic three dimensional micro tumor model for cancer research. Tumor micro-tissues were directly 3D bioprinted into each well of a micro well plate using our custom developed 3D bioprinter. A bioprinted micro-tissue composed of human tumor cells, stromal cells, micro vessels formed by endothelial cell and stromal matrix. The size of the tumor micro tissues could be precisely controlled by the software. Viability staining assay shows that the bioprinted tumor tissue were viable over a 28-day period of time during *in vitro* culture. H&E staining revealed the inherent histological heterogeneity of the malignant tumor, which is similar to that of the counterpart native tumor tissue. The hypoxia core of the tumor micro-tissue, which is similar to the *in vivo* condition in the native tumor, was also successfully engineered. After implantation on the chorioallantoic membrane of chick embryos, the tumor micro-tissues show growth malignancy and the *in vivo* angiogenic capability. Additionally, we have observed that resistance to chemotherapeutic reagents within our 3D tumor micro-tissues is significantly higher than those in 2D cultures. In conclusion, the presented 3D bioprinted tumor micro-tissues are a versatile platform for anti-tumor therapy development deciphering the dubious nature of the cancer.

### 3D Plotted Extracellular Matrix (ECM) Scaffolds using a Direct Rapid Prototyping for Tissue Engineering Applications

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Naturally derived biomaterials are rarely used in advanced RP technology despite their superior biocompatibility. The main problem of natural material plotting is the high sensitivity of materials concentration and viscosity on the plotting nozzle. The aim of the current study was to develop a 3D plotting system capable of dispensing ECM-c (ECM powder blended collagen) and manufacture various shapes of ECM-c scaffolds to apply for irregular defects. We had adapted a powder-based plotting approach to print the stable 3D construct using only cartilage derived ECM materials. This study successfully developed the plotting method for high viscous ECM-c material and showed the 3D plotted scaffolds with high interconnected pores as well as complex shape. Furthermore, cell culture results proved that plotted ECM-c scaffolds were able to provide a suitable environment for cell attachment, proliferation and chondrogenesis. This study shows the 3D printing feasibility of ECM natural material has demonstrated as a first time. We believe our results will offer a meaningful step toward the 3D scaffold printing based on natural ECM materials for future organ printing.

### Quiescence Preconditioning of Human Multipotent Stromal Cells Enhances their Long Term Survival under Ischemic Conditions

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Owing to their multifaced properties, multipotent stromal cells (MSC) represent a promising cell population for cell therapy and tissue engineering. However, a major impediment to the development of MSC-based therapies is their poor survival and engraftment at the site of injury, probably because of the ischemic environmental conditions, i.e. a lack of both nutrients and oxygen supplies, that MSC faced when transplanted.

In the present study, we hypothesized that lowering the energetics demands of MSCs by driving them into a quiescent state would enhance their survival under ischemic conditions. In response to a 48-hour serum deprivation (SD), human MSC entered into a quiescent state with a reduced anabolic activity. When cultured under ischemic conditions (0.1% pO<sub>2</sub>, 0% FBS and 0g/l glucose), preconditioned cells (SD-hMSCs) remained viable cells for up to 14 days (60 ± 22% of survival vs. 2 ± 2% for unpreconditioned hMSCs). The viability of SD-hMSCs was correlated with the maintenance of their ATP contents throughout the ischemic episode. We provided evidence that SD-hMSC drew their energy from glycolytic metabolism using pyruvate as a metabolic fuel. Besides, disruption of autophagy in SD-hMSC led to a drastic loss of cell viability, emphasizing its key role in cell survival in such harsh conditions. Lastly, when reperfused in standard culture medium (10% FBS, 21% O<sub>2</sub>, 1 g/L glucose), SD-hMSCs had maintained their functionality as proved by same proliferative ability and differentiation potentials as fresh hMSCs. These findings propose quiescence preconditioning of MSC as a simple, promising and untapped strategy to sustain their viability post-implantation.

### A 3D Thermo-responsive Scaffold System for the Continued Culture and Passaging of Primary Human Corneal Stromal Cells

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*In vivo*, corneal stromal cells known as keratocytes exhibit a quiescent, dendritic cell phenotype, responsible for maintaining the extracellular matrix. During 2D *in vitro* culture, keratocytes differentiate into an undesirable fibroblastic repair phenotype which in the eye would lead to scarring and blindness. Our previous work has shown that 3D environments can promote the reversion of activated corneal stromal cells to a quiescent keratocyte phenotype. However, difficulties in using enzymatic digestion to extract cells from 3D culture has led to the development of thermo-responsive 3D scaffolds for longer-term culture and passaging. Primary human corneal stromal stem cells (hCSC) were extracted from the limbus of the cornea, and then seeded on thermo-responsive electrospun fibres or on 2D culture flasks. Six thermal and enzymatic passages on scaffolds and flasks respectively were performed. The effect of extended passaging and 3D-culture on hCSC was assessed by RT-qPCR and immunocytochemistry. hCSC were not affected by the thermo-responsive polymer as cells were viable and proliferated in a similar manner to those cultured on the control. Culture on 3D scaffolds promoted the quiescent keratocyte phenotype with increased expression of the keratocyte markers CD34 and ALDH and decreased expression of the myofibroblast marker ACTA2 compared to 2D-culture flasks. In summary, thermo-responsive 3D scaffolds supported the culture and detachment of hCSC without enzymes, and promoted a quiescent keratocyte phenotype over multiple passages. This culture system has the potential to provide high numbers of a desirable cell phenotype for regeneration of the ocular surface in cases of disease or trauma.

#### Composite Materials for Pelvic Floor Repair Which Cope with Dynamic Distension and Support Cell Integration

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Polypropylene mesh (PPL) commonly used in prolapse repair is associated with severe clinical complications. *In vitro* mechanical testing shows that PPL copes poorly with mechanical distension and is also poor at supporting cell proliferation.

Our aim was to produce materials with more appropriate mechanical properties for use in the pelvic floor which is subject to rapid changes in pressure as women cough or sneeze and which would better support cell integration. We need to develop materials better able to cope with this challenge.

Scaffolds of polyurethanes (PU) and of poly-L-lactic acid (PLA) and composites of the two by electrospinning and their mechanical properties assessed and compared to PPL. The ability of scaffolds to support adipose derived stem cells (ADSC) was assessed. Additionally uniaxial tensiometry of scaffolds was performed before and after 7 days of cyclical distension. Cell penetration (DAPI), viability (Alamar blue assay) and total collagen production (Sirius red assay) were measured for ADSC cultured on scaffolds.

PPL was stronger than polyurethanes and PLA as expected. However PPL plastically deformed after 7 days of sustained cyclical distension while polyurethanes maintained their elasticity. Scaffolds of PU containing PLA were weaker and stiffer than PU or PPL but were significantly better than PU scaffolds alone at supporting ADSC.

In conclusion prolonged mechanical distension *in vitro* causes PPL to fail. Materials with more appropriate mechanical properties for use in the pelvic floor can be produced using PU and a combination of PLA with PU greatly improves interaction of cells with this material.

#### Segmental Bone Repair using a Combination of Dense and Porous Hydroxyapatite Seeded with Osteo-differentiated Mesenchymal Stem Cells Enriched with Fibrin

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A novel tissue engineered bone composed of dense hydroxyapatite (HA) at the periphery and porous HA filled with osteo-differentiated marrow stromal cells at the core was fabricated. The design mimics the structure of a tibial bone where the spongy bone in the center filled with marrow elements is surrounded by the bone cortex at the periphery. This study evaluated the efficacy of this tissue engineered bone in repairing critical-sized segmental bone gap at a load bearing site. Sheep was used as the animal model. Bone marrow was harvested from the sheep iliac crests. Plasma was obtained from sheep whole blood. HA scaffold was fabricated via gel casting and sintering technique. Approximately 30–50 million osteoprogenitor cells were mixed with the plasma and seeded onto the porous HA. The cell-seeded construct was then maintained in osteogenic differentiation medium for a week before transplanting into a 5 cm segmental defect of a sheep tibia. Radiographs were taken periodically. After 6 months of implantation, animals were euthanized, the experimental and contralateral limbs amputated for further analysis. CT scan was performed. Analysis was done using CTAn software to quantify the bone structural index. The repaired tibia was further subjected to torsion and compression tests using a Universal Testing Machine. Finally, histological analysis was performed. The novel tissue engineered bone successfully bridged the defect within 3 months and histologically resemble that of native bone. Dense HA was still visible while porous HA has been fully replaced by new bone.

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#### Toward Understanding the Pathogenesis of Keratoconus: Insight from a Tissue-Engineered Stromal Model

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Keratoconus (KC) is a corneal pathology that causes progressive thinning and weakening of the corneal stroma. The pathogenesis of KC is unclear, however, gene and cellular changes have been reported. The goal of this study was to investigate the pathogenesis of KC using tissue-engineered (TE) stromal models.

Corneal stromal cells were isolated from human KC (n=2) and healthy eye bank (n=2) corneas. Cells, cultured in presence of ascorbic acid and fetal calf serum, were analysed on day 7 (cells) and day 30 (TE models). At both time points, a 24 h conditioned-medium was collected to analyse the secreted proteases (Proteome profiler human proteases array kit, R&D Systems) and RNA was extracted in order to perform transcriptome analysis by DNA chip (Agilent SurePrint G3 Human Gene expression microarrays).

Analysis of the proteases revealed that day 7 KC cells secreted a higher amount of MMP8 (3.4), MMP9 (4.5), MMP12 (3.0), MMP13 (2.8), ADAM8 (3.2), ADAMTS1 (2.7), ADAMTS13 (3.5), Cathepsin V (30.4), Kalikrein-6 (6.0), Kalikrein-7 (3.6), Kalikrein-10 (3.4), Neprilysin (2.6), Proprotein convertase 9 (8.0) and Proteinase 3 (4.6) as compared to healthy cells (fold-changes). Transcriptome analysis also showed an upregulation for ADAMTS1 (2.3), MMP8 (1.8), MMP12 (1.9), MMP13 (2.8) and Neprilysin (3.3) genes (fold-changes), at day 7 in KC cells.

This study demonstrated the possibility of to produce an *in vitro* model of KC that allows investigation of this complex pathology using omics approaches. Characterization of this model will be of great interest to develop new therapies for this eye disease.

### The Effect of Poly(4-Hydroxy Butyrate) upon Host Innate Immune Response and Antimicrobial Activity

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The bioactive metabolite, Butyrate, is a naturally occurring histone deacetylase (HDAC) inhibitor found in many tissues including the gastrointestinal tract. Hydroxylated forms of butyrate, such as Poly(4-hydroxy butyrate) (P4HB), is a byproduct of bacterial metabolism, and is associated with seemingly paradoxical anti-inflammatory and anti-microbial effects (1). P4HB is commercially available and used as an FDA approved 'biosynthetic' material for regenerative medicine and tissue engineering applications (2,3). The present study evaluated the immunomodulatory and antimicrobial effects of bioscaffolds composed of P4HB.

P4HB scaffolds were evaluated in a rat bilateral partial thickness abdominal wall defect model (4). Histomorphologic and immunolabeling quantification showed modulation of macrophage activation toward an M2 phenotype with antimicrobial peptide (AMP) expression following implantation. The mechanism by which P4HB influences macrophage phenotype involves transcriptional regulation of NF- $\kappa$ B production and histone deacetylase inhibition. Bioscaffolds composed of P4HB have the potential to provide the constructive regulatory and tissue "rebuilding" effects commonly seen with alternatively activated macrophages and the advantage of a reproducibly manufactured "synthetic" bioscaffold.

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### Success of *In-Vivo* Vascular Tissue Engineering Depends on Implantation Site and Species

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**Objectives:** Poly( $\epsilon$ -caprolactone) (PCL) was previously used as interposition abdominal aortic graft showing better results than ePTFE with good patency, endothelialisation, cellular infiltration, vascularisation, extracellular matrix (ECM) formation and mechanical properties. We investigated whether implanting the grafts in the carotid position of rats and pigs would reveal similar good results as in the rat abdominal position.

**Methodology:** PCL (1 mm ID) grafts were implanted in the common carotid artery in 15 anesthetized rats (A). Follow-up at

3,6,12 weeks. Similarly end-to-end pig carotid replacement was done with 4 mm ID PCL grafts in 11 Swiss Landrace pigs followed for 1-month (B). At each time point, *in-vivo* compliance, angiography and *ex-vivo* histological examination with morphometry were performed. Results of A&B were compared to rat abdominal replacement(C).

**Results:** Patency rates were 60% (A), 78% (B) compared to 100% (C). Graft compliance was reduced by 69% (A), 40% (B) and 62% (C). Complete endothelialization was achieved at 3 weeks in the rat (A&C) and near confluence (86%) in the pig at 1 month (B). Intimal thickness was low for A&C and not relevant for B. Early cellular infiltration (3–4 weeks) was 82% (A), 90% (C) and 44% (B).

**Significance:** The degradable PCL grafts showed fast endothelialization, good cellular infiltration, reduced compliance and low IH in the pig and rat carotid artery replacement models. However, compared to the abdominal position there was a higher occlusion rate in both species due probably to the small graft diameter and the different flow pattern. Therefore tissue-engineered vascular-grafts have to be tested at several time-points, implantation sites using various species before considering a clinical use as a coronary bypass graft.

### Human, Peripheral-blood Neutrophil Phenotype Regulation by Electrospun Vascular Tissue Regeneration Template Design

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Neutrophils' adaptability to microenvironmental cues and ability to synthesize and release an array of factors (cytokines and metalloproteinases) with multifaceted physiological effects including angiogenesis and regeneration have been demonstrated; however, no studies have been conducted regarding regeneration template (i.e. bioresorbable vascular grafts) modulation of human neutrophils. Hypothesis: Electrospun template architecture and composition can be engineered to temporally modulate interacting neutrophil phenotype (inflammatory, N1 vs. angiogenic/regenerative, N2) to promote, guide, and enhance *in situ* regeneration. This study examined neutrophil response to electrospun templates with defined architectures (large and small fiber diameter/pore (LD and SD)) and composition (polydioxanone (PDO) and collagen at 100:0, 90:10, and 0:100 PDO:collagen ratios). Fresh human, peripheral-blood neutrophils were seeded on the templates for 3 and 24 hours. Neutrophil cytokine release analysis (14 cytokines) demonstrated that collagen templates significantly reduced overall cytokine release compared to PDO alone. Specifically, significantly higher levels of MIP-1 $\alpha$  and MIP-1 $\beta$  were detected from LD PDO templates at 3 hours compared to 24 hours, whereas IL-10 and IL-8 were minimally detectable at 3 hours with a significant increase (84 $\times$ ) at 24 hours. Inhibitor-free MMP-9 was released on the LD PDO templates at significantly higher concentrations compared to other templates at 3 hours (38,100 pg/ml or 2.5 $\times$  increase over SD PDO templates that was sustained at 24 hours). These results for the first time demonstrate that human neutrophil phenotype and functionality can be temporally regulated by template design and more importantly can be engineered to direct/enhance *in situ*, neutrophil-guided template transmural angiogenesis and regeneration.

### Decellularization of Porcine Esophagus using a Perfusion Bioreactor System and Predictive Mathematical Modelling

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The current surgical treatment for many esophageal conditions involves resection and replacement with autologous stomach or intestine, a treatment associated with considerable morbidity and

mortality. A tissue engineered esophagus would eliminate the need to harvest patients' own organs, minimizing trauma to the patient and thereby potentially improving the surgical outcome. Appropriate decellularization of donor organs can yield non-immunogenic scaffolds that could be used for transplantations. Here, we aimed to translate our previous successful small animal model into large animals whilst also reducing decellularization time in order to minimize damage to the scaffold, risk of contamination and processing costs. Using a perfusion system and mathematical predictive modelling, we successfully obtained a decellularized porcine esophagus in less than 22 hours. The scaffold showed a well-preserved architecture while the majority of cell nuclei and 89% of the DNA was eliminated. The decellularization resulted in minimal disruption to i) extracellular matrix proteins such as collagens, elastin and laminin, ii) basement membrane and iii) biomechanical properties. Electron microscopy further revealed that the cuboidal epithelial cells were replaced by a smoothed surface with a complex ultra-architecture. Notably the abundant bacteria on the internal side were removed whilst the fine network on the external side of the organ was retained. Further, we found that the scaffold was cytocompatible as demonstrated by cell adhesion, survival and proliferation. In conclusion, our new protocol produced, in minimal time, a scaffold that fulfilled several important criteria for subsequent transplantation.

### An Innovative 3D Perfusion-Based Bioreactor System for the In-Vitro Maintenance and Expansion of Primary Breast Cancer Tissue

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Development of cancer treatment has traditionally been based on studies in experimental animal models or taking advantage of human established tumor cell lines for *in-vitro* and *in-vivo* assays. Here we present an innovative method to culture breast cancer tissues in porous 3D scaffolds by using a perfusion-based bioreactor system.

Freshly excised estrogen-receptor (ER) positive breast cancer (BrCa) specimens were fragmented and cultured in 3D "sandwich-like format" between porous scaffolds under perfusion flow. Culture medium was supplemented with 10% autologous human serum. Tumor cell survival and expansion into the scaffold in perfusion culture as well as the ability to recapitulate features of the original BrCa specimens was histologically assessed. In a second step anti-ER treatment (Fulvestrant<sup>®</sup>) was added to the cultures.

With this innovative method we were able to preserve viability and expand BrCa tissue with concomitant stromal and immune cells. Expanding cancer cells were viable after 21 days and recapitulating the initial histology with formation of glands. Administration of anti-ER treatment was associated with decreased expansion of cancer tissue into the scaffold after 21 days.

The 3D perfusion culture of BrCa tissue preserves malignant, interstitial and immunocompetent cells, thus allowing direct testing of basic cancer immunobiology research hypotheses in complex, human tumor microenvironments. Additionally, it allows direct evaluation of the effects of various treatments on malignant and non-malignant cells, including immune-therapy. This *in-vitro* model could be extended beyond the context of BrCa, as a platform allowing animal-free testing of approaches to treat human malignancies in the direction of personalized medicine.

### Cardiac ECM-Fibrin Hybrid Biomaterials Alter the Gene Expression of Human Pediatric Cardiac Progenitor Cells in both a Stiffness and Composition Dependent Manner

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Approximately 1% of infants are born with a congenital cardiovascular disorder, which include various cardiomyopathies, congenital heart defects (CHDs), or arrhythmias that can lead to heart failure at a young age. Currently, the majority of available therapies are geared toward slowing down the progression of heart failure and not toward restoring the proper contractile function of the myocardium. Solubilized cardiac extracellular matrix (ECM) is being developed as an injectable therapeutic that offers promise for promoting cardiac repair. However, the ECM alone forms a hydrogel that is very soft compared to the native myocardium. As both the stiffness and composition of the ECM are important in regulating cell behavior and can have complex synergistic effects, we sought to develop an ECM-based scaffold with tunable biochemical and mechanical properties. We used solubilized cardiac ECM from two developmental stages (neonatal, adult) combined with fibrin hydrogels that were cross-linked with transglutaminase. We show that ECM was retained within the gels and Young's modulus could be tuned to span the range of the developing and mature heart. C-kit+ cardiovascular progenitor cells from pediatric patients with CHDs were seeded into the hybrid gels. Both the elastic modulus and composition of the scaffolds impacted the expression of endothelial and smooth muscle cell genes. Furthermore, we demonstrate that the hybrid gels are injectable, and thus have potential for minimally invasive therapies. ECM-fibrin hybrid scaffolds offer new opportunities for exploiting the effects of both composition and mechanical properties in directing cell behavior for tissue engineering in pediatric patients.

### Effect of Vinpocetine on Smooth Muscle Cell and Endothelial Cell for Cardiovascular Applications

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Anti-proliferative drugs have been applied to improve the function of stent, so called drug-eluting stent (DES). Many researchers reported that the reduction in neointimal hyperplasia and restenosis in stent could be accomplished by stent-based delivery of anti-proliferative drugs, such as sirolimus, paclitaxel and so on. However, the application of these drugs still has some major problems. For example, these drugs cause endothelial cell (EC) toxicity to attenuate re-endothelialization, resulting in delaying wound healing. In this study, we hypothesized that the treatment of vinpocetine inhibits the proliferation of smooth muscle cells (SMC) and minimizes EC toxicity, as well as inflammatory responses. To compare the effects of vinpocetine and sirolimus on stent treatment, the drug-coated cobalt-chrome substrates were prepared by an electro-spraying method. The physicochemical properties of substrates were characterized, and *in vitro* cell study was performed by cell counting kit-8 assay, enzyme-linked immune sorbent assay and macrophage infiltration on the substrates. As a result, it was demonstrated that vinpocetine effectively inhibits SMC proliferation and enhance the cell survival of ECs, compared to sirolimus. Also, vinpocetine significantly decreased the expression level of pro-inflammatory cytokines, as well as macrophage infiltration. Therefore, vinpocetine can be a useful candidate to improve in-stent restenosis, alternative to sirolimus in DES.

### Differentiation of Human Tonsil-derived Mesenchymal Stem Cells into Schwann-like Cells

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Since Schwann cells (SCs) provide structural support and guidance for axonal regeneration by producing neurotrophic factors and adherent molecules, SCs are indispensable mediators of the repair process of the injured nervous tissue. Although autologous SCs transplantation has shown promising clinical results, this procedure is limited by donor site morbidity and difficulty to generate sufficient



number of cells. We have evaluated that the differentiation of tonsil-derived mesenchymal stem cells (T-MSCs) into SC-like cells (T-MSC-SCs) can be an alternative cell source for the generation of SCs. To neurosphere formation, T-MSCs were cultured in DMEM with 20 ng/ml bFGF, 20 ng/ml EGF, 2% B27 supplement and then to differentiate into SCs, mechanically dissociated cells were grown in DME/F12 with 10% FBS, 5 ng/ml PDGF, 10 ng/ml bFGF, and 200 ng/ml heregulin. At neurosphere stage, all of Schwann cell markers including CAD19, GFAP, MBP, NGFR, S100B and Krox20 were up-regulated. However, in fully differentiated stage, only GFAP, NGFR, S100B and Krox20 were up-regulated. The expression of CAD19 and MBP were down-regulated in differentiated SCs. The formation of myelin sheath on axons was observed by co-culture with mouse dorsal root ganglion neurons. Neurotrophic factor secretion of T-MSC-SCs was also evaluated by conditioned media test measuring neurite outgrowth of NSC34 mouse motor neuron cells. These results suggest that human T-MSCs can be a powerful stem cell candidate for peripheral nerve regeneration.

### Comparative Biology of Decellularized Lung Matrices

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Recently, regenerative medicine technologies have made great strides with the development of decellularized scaffolds for the construction of artificial lungs. In this work, we seek to 1) characterize the impact of decellularization on relevant models of lung regeneration, and 2) determine if there exists species dependent cell-matrix interactions in these systems. Therefore, we produced acellular lung scaffolds from rodent, porcine, primate and human lungs by perfusing a combination of Triton X-100/Sodium Deoxycholate via the pulmonary artery on a per gram wet tissue basis. We assessed decellularization and matrix retention by measuring residual DNA, mechanical properties, key matrix proteins (collagen, elastin, sGAGs), and the preservation of basement membrane in the resulting acellular tissue. To assess intrinsic matrix biologic cues, human endothelial cells (veravacs, 1 x 10<sup>6</sup> cells/slice) were seeded onto decellularized slices for 3 days and immunoblotted for markers of cell health and inflammation (VCAM, phosphorylated NF- $\kappa$ B). All tissues decellularized similarly (~95% reduction in DNA), retained native levels of collagen content, and possessed an intact basement membrane post decellularization. Surprisingly, human and primate acellular tissues were stiffer, contained more elastin, and retained less sGAGs than porcine or rodent lung scaffolds. Additionally, veravacs seeded onto human and primate tissue demonstrated reduced "inflammatory behavior" as evidenced by a reduction in VCAM and NF- $\kappa$ B, and also better engraftment than those seeded onto rodent or porcine tissue. This work provides a comprehensive analysis of decellularized lung tissue in relevant model systems, and also provides new information regarding species-dependent biologic cues intrinsic to lung matrix.

### Culture Expansion of Undifferentiated Human Endometrial MSC using a Small Molecule Inhibitor; in Preparation for Cell-based Therapy for Pelvic Organ Prolapse

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**Introduction:** Pelvic organ prolapse is a debilitating condition affecting women and we are proposing cell-based therapy as a new approach. Human endometrial MSC, eMSC, are a novel source of MSC which are purified by bead-sorting using the SUSD2 antibody. Like other MSC, eMSC undergo spontaneous differentiation into fibroblasts during culture expansion decreasing their purity and efficacy for use in cell-based therapies. The aim of this study was to

determine if A83-01, a TGF $\beta$  receptor inhibitor, prevents spontaneous differentiation of eMSC in culture.

**Methods:** SUSD2<sup>+</sup> eMSCs were obtained from dissociated endometrial tissues and cultured in serum free medium (SFM) with bFGF/EGF in 5%O<sub>2</sub>/5%CO<sub>2</sub>. At passage 6 (P6), eMSC were incubated with or without A83-01 for 7 days, then analysed for MSC properties.

**Results:** A83-01 promoted SUSD2<sup>+</sup> cell proliferation with maximal effect at 1  $\mu$ M, increasing the %SUSD2<sup>+</sup> cells in P6 cultures. A83-01-treated cells had higher cloning efficiency than untreated cells, differentiated into mesodermal lineages and expressed MSC phenotypic markers. By cell cycle analysis and Annexin V flowcytometry, we demonstrated that A83-01 promoted SUSD2<sup>+</sup> cell proliferation and blocked apoptosis. Fewer A83-01-treated cells were autofluorescent or stained with  $\beta$ -galactosidase indicating A83-01 promotes SUSD2<sup>+</sup> cell proliferation and blocks senescence in late passage cultures. Decreased Smad2/3 phosphorylation in A83-01-treated cells indicates that A83-01 binding to TGF- $\beta$ R blocks downstream signalling leading to apoptosis.

**Conclusion:** Small molecules such as A83-01 that promote eMSC proliferation and block apoptosis may provide an approach for the expansion of undifferentiated MSC for use as a cell based-therapy for pelvic organ prolapse.

### Tissue Engineered Urinary Conduits

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Regenerative medicine and tissue engineering techniques hold great promises in treating patients who undergo cystectomy and urinary diversion surgery by replacing diseased tissues and creating or regenerating healthy urinary conduits. Current surgical technique to create urinary conduits employs gastrointestinal tract as a tissue source; however, it can lead to health problems, such as chronic infection and stone formation. Stem cells together with biomaterials are shown to enhance and accelerate partial tissue defect healing; however, their clinical translations in patients who need urinary diversion surgery are limited due to either insufficient mechanical strength or inadequate biological responses, such as lack of vascularization, and anti-fibrosis properties. Therefore, we developed collagen-based tissue-engineered tubular urinary diversion conduits with controlled mechanical properties by using a novel fabrication process that employs a porous hydrophilic material to deposit and condense collagen fibers under vacuum. Collagen tubular conduits with various thicknesses up to 300  $\mu$ m and radial burst pressure strengths more than 1.0 MPa were fabricated. We also extended this technology to fabricate tissue engineered bladder-like hollow structures. Furthermore, we compared the biomechanical performances of these tubular scaffolds with the decellularized trachea-, and electrospun PLGA nanofibers-based tubular conduits. The fabricated elastomeric collagen-based scaffolds supported proliferations of Lgr5 progenitor cells, bladder-derived urothelial and smooth muscle cells, and maintained their phenotypes and biological functions. We believe that these tissue-engineered scaffolds can benefit patients who undergo treatments for replacing hollow-structured tissues and organs.

### Development of a Polycaprolactone (PCL) Scaffold as a Platform for Human Corneal Stroma Regeneration

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Around 100.000 corneal transplants are performed worldwide every year, and over half of them are due to corneal stromal opacity. Corneal transplantation is the only effective way for visual rehabilitation of these patients. However, graft failure or global shortage of

donor corneas are important drawbacks of this procedure. Alternatively, several substitutes to corneal tissue have been proposed for corneal stromal replacement. However, although some of these prostheses can restore vision, their prognosis is eclipsed by a significant risk for infection, extrusion and dehiscence, due to the lack of integration of the non-biological material within the recipient corneal tissue. Our goal is to fabricate a biologically customized three-dimensional polycaprolactone (PCL) scaffold to facilitate corneal stromal regeneration. We hypothesize that human keratocytes will be able to migrate into the scaffold and produce native extracellular matrix. PCL scaffolds were fabricated by melt electrospinning writing with a 0–90° lay-down pattern and 100 microns of fiber distance. Scaffolds were plasma treated and coated with amniotic membrane extract to help maintaining stromal keratocytes phenotype. Human corneal stromal explants were placed on the surface of the scaffold and maintained in culture medium for 3 months. Keratocytes attachment and migration into the scaffold was confirmed by immunohistochemistry, and extracellular matrix production was evaluated by immunofluorescence confocal microscopy. At 3 months, keratocytes had migrated and completely repopulated the scaffold. Additionally, keratocytes produced extracellular matrix components (collagen and proteoglycans) that resembled natural human corneal stroma. In conclusion, these preliminary results disclose a novel and exceptional alternative to corneal transplantation.

#### Bio-printing Personalised Tissue Engineered Cell Constructs for Nasal Reconstruction

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Carcinoma of the nose is a debilitating condition with 2000 new cases diagnosed each year in the US and 400 in the UK. One of the severe social and functional side effects of this condition is nasal deformity following a life-saving re-section of the tumour. The complex three-tissue architecture of the nose does not lend itself to simple surgical reconstruction. Instead, reconstructive surgery must recreate the internal nasal lining, the cartilaginous inner and bony upper sections as well as restore the external skin covering. Current surgical approaches involve autologous cartilage grafts, alloplastic materials and titanium meshes which result in poor functional and aesthetic results..

Our approach involves a personalised bio-printed composite for nasal reconstruction. The composite consists of a bio-degradable thermoplastic polymer to provide structural support, and a cell-laden thermoresponsive and UV cross-linkable hydrogel. This composite strategy aims to emulate the mechanical properties and architecture of nasal cartilage.

Specifically, we have developed a printing method that allows us to modify both the surface architecture and porosity of the scaffolds. The effect of scaffold surface porosity on cell infiltration has been examined *in vitro*. Moreover, we have co-printed a cell-laden hydrogel and a thermoplastic into a composite scaffold with optimal architectures for the regeneration of neotissue. Bio-printed cells have been demonstrated to remain viable and functional, secreting tissue specific matrix. The hydrogel composition has been found to play an important role in cell viability and function *in vitro*.

#### Deciphering a Novel *in Vivo* Hierarchy of Vessel Resident Endothelial Progenitors using the Human Term Placenta

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**Aims:** Endothelial progenitor cells (EPCs) have been used for the treatment of ischemic diseases in previous clinical trials. Major limitations of these trials were the lack of proper definition of EPCs

and the incapacity to deliver large numbers of cells. We aimed to define EPC populations *in vivo* using a highly vascularised organ, the human term placenta.

**Methods and Results:** Human placenta was enzymatically digested to obtain a single cell suspension. Upon digestion significant heterogeneity could be observed within placental endothelial cells using common markers such as CD34 and CD31 after exclusion of hematopoietic cells. As expected most high proliferative endothelial colonies could be found in the CD45-CD34+ population. Further examination identified the high-proliferative potential endothelial colony forming EPCs to be almost exclusively in the CD31low population which could be further passaged and expanded. We also showed that these progenitors were vascular in origin as labelled with VE-Cadherin *in vivo*. The CD45-CD34+CD31 neg, low and high populations were of fetal origin and in culture displayed no hematopoietic but mesenchymal capacity with osteogenic and adipogenic potential only in the CD31neg population. Finally, single cell seeding, quantitative analysis revealed a clear hierarchy between the CD31low and CD31hi cells, the latter giving rise only to small colonies with limited proliferative potential.

**Conclusion:** This establishes a novel *in vivo* hierarchy amongst endothelial cells narrowing the identity of EPCs *in vivo*. This work implies the possibility of efficient isolation of EPCs, providing the purity and the large quantity of cells needed for future cell therapies.

#### Perfluorocarbon Nanodroplets as Oxygen Carriers for Improved Stem Cell Angiogenic Therapies

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**Objective:** Adipose stem cell (ASC) therapies are used to promote angiogenesis needed for restoration of injured ischemic tissue. However, clinical translation of the therapy is challenging due to low ASC viability upon implantation in the severely hypoxic injury site. While moderate hypoxia promotes the secretion of ASC angiogenic factors, extreme hypoxia results in ASC death. Therefore, a system capable of controlled oxygen delivery to minimize cell death while promoting angiogenesis would greatly improve current ASC therapies.

**Methodology:** We have developed a therapeutic system consisting of an implantable hydrogel embedded with ASCs along with oxygen-rich perfluorocarbon nanodroplets (PFCnD). The PFCnDs aim to deliver controlled oxygen levels to the implanted ASCs with the goal of increasing their viability while maintaining the angiogenic effects of moderate hypoxia. By incorporating an optical absorber in the PFCnDs, we can controllably trigger oxygen release from the PFCnDs through laser-induced optical droplet vaporization. Lastly, ultrasound and photoacoustic imaging techniques can monitor the particle location, activation, and thus oxygen release.

**Results:** ASCs cultured under hypoxic conditions (1% O<sub>2</sub> for 48 hours) in the presence of oxygen-loaded PFCnDs exhibited increased viability over ASCs incubated without PFCnDs. Oxygen release from PFCnDs occurs via two methods: (1) passive diffusion and (2) triggered release. Studies of the laser-activated release mechanism indicate that the amount of oxygen released from the PFCnDs depends on the concentration of particles as well as the laser energy irradiating them, demonstrating control over oxygen delivery.

#### Evaluation of a Multilayered Biomimetic Cardiac Patch in a Rat Model

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Approximately 10,000 children undergo surgery each year in the US to repair a congenital heart defect, and most require implantation of an artificial patch, baffle, or conduit. Current materials for patched heart repair induce an inflammatory response, hinder contraction, disrupt

electrical conduction, and serve as a nexus for calcification. Patients with heart patches have an increased risk of heart failure, arrhythmias, infection and aneurysm. In this study, we investigated a biomimetic and biodegradable engineered patch made from polycaprolactone, chitosan and decellularized porcine heart matrix for right ventricle free-wall replacement in a rat model 1, and compared to engineered patches with collagen replacing heart matrix, and commercially available bovine pericardium patches. Inflammation, cell invasion and heart function were analyzed using MRI and immunohistology after 2, 4, and 8 weeks post surgery, with 5 rats per material per time point.

MRI results revealed no outflow tract obstruction. No significant areas of clotting or fibrosis were observed with any patch. After 8 weeks, hearts with both engineered patches had significantly higher LVEF compared to pericardium controls. Histology revealed that both engineered patches promoted aligned cardiac myofibroblast formation throughout the patched area, whereas the pericardial patches showed no cell integration. A continuous epithelial layer formed only on the surface of the heart matrix patches. Furthermore, engineered patches promoted neo-vascularization throughout the patched region. These results demonstrate that engineered patches promote normalized heart function, cell invasion and regeneration along with the native tissue.

#### **Adaptation of a Mendelmax 1.5+Printer at El Bosque University for Extruding a Collagen Hydrogel in Acetic Acid Solution**

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This project focuses on solving an actual problem that is increasing; the need for organs and tissues. In Colombia, in the year 2012 there were 1322 cases presented and by 2013, 2414 cases. One of the innovative solutions is the implementation of 3D bioprinting that is applied in tissue engineering and regenerative medicine. Printing scaffolds with personalized designs to allow an efficient the recovery.

Project consisted of adapting the 3D printer 1.5+ MendelMax from El Bosque University for extruding a collagen hydrogel in acetic acid solution; for this, an extruder carriage was fabricated using the 3D printer and it was adapted to perform tests with collagen, extracted from bovine tail. Furthermore, we developed a manual detailing the use of the printer and a protocol to achieve a consistent collagen structure.

In this project biodegradable materials were implemented with University resources, to achieve an innovative design in Colombia. It is important to consider that the selected printer is located in the Engineering Faculty of the University and uses free software; this allows modifications for different materials. The use of this printer is strengthened by one of the courses that the University offers and in the different activities of research group of Biomaterials.

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#### **Production of a Scaffold from Bovine Muscle Tissue**

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Lesions involving greater portions of muscle cannot be naturally regenerated. The mainly used treatment of such lesions consists of muscle mass transplantation from the own patient. This approach assures aesthetics and functionality, but is highly associated with increased levels of morbidity. Thus, bioengineered tissue emerges as a promising alternative for such situation, since it is associated with

low morbidity. A commonly used strategy consists in growing cells in scaffolds composed of synthetic biodegradable polymers providing the proper three-dimensional structure for adhesion, proliferation, differentiation and secretion of Extracellular Matrix (EM). However, such type of scaffold lack signals hindering the formation of functional tissue, also being associated with delay in remodeling process and prolonged immune response. Another problem is the structural uniformity of scaffolds composed of only one type of synthetic polymer, which cannot reproduce the complexity of tissues. Alternatively, decellularized tissue has been used as scaffolds, such as the use of EM, which can be explained by its role in the maintenance of tissue architecture, besides participating in the regulation of proliferation, migration and differentiation processes. Such approach for skeletal muscle reconstitution is also the target of numerous studies, which use muscles of small animals, yielding little material. Thus, this work aims to produce a scaffold by decellularization of bovine skeletal muscle. For decellularization, different detergent was applied, after what the material presented translucent appearance consistent with decellularization process, and maintained initial 3D structure, with DNA content below 1 ng/mg, showing a degree of decellularization much higher than recommended in the literature.

#### **Impact of Electrospun Conduit Composition on Remodeling of Constructs Initially Grown within Rat Peritoneal Cavities after Aortic Grafting**

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Tissue engineered vascular grafts require strategies to allow graft remodeling, but avoid thrombosis and loss of graft mechanics. In this study, we determined the amount of collagen needed to both modulate the inflammatory response and maintain graft structural stability. This was tested in the peritoneal cavity and then subsequently after grafting into the abdominal aorta of the same rat. Electrospun conduits were produced from 100%, 90%, and 75% blends of poly( $\epsilon$ -caprolactone)(PCL)/collagen (w/w), enclosed in PTFE porous pouches, and then implanted in the peritoneal cavity for 4 weeks. Mechanical testing via a ring test procedure showed an increase in tensile moduli from  $\sim 1.3$  MPa to  $\sim 2.3$  MPa for 100% and 75% samples ( $p < 0.007$ ). The percent elongation and ultimate tensile strength also suggests construct remodeling. Lipid oxidation results using HPLC/mass spectrometry showed lower levels of lipid oxidation for PCL/collagen blend samples, including decreased accumulation for all-HETE species (i.e., arachidonic acid oxidation products) with 75% PCL ( $p = 0.034$ ). PCR results included higher  $\alpha$ -smooth muscle actin expression in cells recruited to 90% PCL blend conduits ( $p = 0.041$  vs. 75%). Opposite trend were observed for macrophage markers. Aortic grafting studies to investigate remodeling and endothelial cell recruitment are ongoing. In preliminary data, 100% PCL and 75% PCL/collagen samples without initial peritoneal cavity implantation remained patent over 6 weeks, as visualized with H&E. A 100% PCL peritoneal construct has also been grafted. Overall, these results demonstrate that incorporating collagen reduces inflammation, and this affect might be collagen ratio dependent. Further, these grafts remain patent after aortic grafting.

#### **Tumor Necrosis Factor-Alpha Inhibits Neurite Outgrowth from Major Pelvic Ganglion by Inducing Apoptosis *Ex Vivo***

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**Objectives:** Erectile dysfunction (ED) is a major sequela of radical prostatectomy (RP). Cavernous nerve (CN) injury at the time of RP induces ED. However, neuroinflammation often impairs CN

regeneration. Previously, we demonstrated that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is increased following bilateral CN injury (BCNI). This study examines the effect of exogenous TNF- $\alpha$  (eTNF- $\alpha$ ) on neurite outgrowth from major pelvic ganglion (MPG) *ex vivo*.

**Methods:** Whole MPGs were harvested from male Sprague-Dawley rats (age 12–14 weeks) and cultured in Matrigel with eTNF- $\alpha$  in concentrations of 0, 10, 20, 30 ng/mL (n = 3/group). Media and eTNF- $\alpha$  were changed every 24 hours. Neurite lengths were measured at 72 hours after culture. MPGs cultured without eTNF- $\alpha$  and with eTNF- $\alpha$  20 ng/mL were processed to evaluate gene expressions of TNF- $\alpha$ , Caspase-1, Caspase-3, and Caspase-9 by qPCR (n = 6/group).

**Results:** Neurite outgrowth from MPG was inhibited by eTNF- $\alpha$  in a dose-dependent fashion (neurite length: 586  $\pm$  20.5, 455  $\pm$  11.5, 408  $\pm$  28.7, 361  $\pm$  2.65  $\mu$ m; eTNF- $\alpha$  0, 10, 20, 30 ng/mL, respectively; p < 0.01). MPGs cultured with eTNF- $\alpha$  displayed increased gene expressions of TNF- $\alpha$  (p0.05).

**Significance:** This study demonstrated that eTNF- $\alpha$  impaired neurite outgrowth from MPG in a dose-dependent manner by upregulating gene expressions of apoptosis markers. Furthermore, eTNF- $\alpha$  upregulated gene expression of TNF- $\alpha$ , indicating positive feedback. These results suggest that TNF- $\alpha$  antagonists may promote CN regeneration by inhibiting neuronal apoptosis and thus prevent post-RP ED.

### Engineered 3D Microtissues Promote Maturation of Human Pluripotent Stem Cell-Derived Cardiomyocytes

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Human pluripotent stem cells (hPSCs) represent a potentially unlimited source of cardiomyocytes for the study of cardiovascular development and disease, as well as for use in regenerative cardiac therapies. However, current differentiation protocols of hPSCs generally yield cardiomyocytes exhibiting embryonic-like phenotypes, with properties distinct from mature adult cardiomyocytes. In order to realize their therapeutic potential, effective methods to mature hPSC-derived cardiomyocytes must be developed. In this study, we model cardiac microenvironments and modulate cardiomyocyte maturation by generating engineered 3D cardiospheres and controlling their formation and culture conditions. Cardiomyocyte differentiation from hPSCs was initiated by controlling Wnt signaling in 2D monolayer cultures. Cells were dissociated at various time points throughout the differentiation and seeded into microwells for 24 hrs to aggregate and form cardiospheres. Cardiospheres were transferred to rotary suspension culture and maintained for 7–21 days. The formation of 3D cardiospheres promoted up to 8-fold enrichment of the cardiomyocyte population and by day 7 of cardiosphere culture nearly 100% of the cardiospheres exhibited spontaneous beating, synchronous intracellular calcium transients, and expression of cardiac markers. Subsequent culture of the 3D cardiospheres promoted defined sarcomeric structures within cardiomyocytes reflecting a more mature phenotype found in 3D constructs compared to 2D controls. Together this data suggests that 3D cardiosphere culture provides a method for maturation and enrichment of cardiomyocyte cultures independent of the initial differentiation purity. Ongoing studies aim to understand the complex physical and biochemical elements of 3D cardiac microenvironments and how we can utilize engineered 3D approaches to further direct maturation of cardiomyocytes.

### Dynamic Seeding of Valve Interstitial Cells in Decellularized Porcine Pericardium

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Despite being widely used in the clinical practice, glutaraldehyde fixed porcine pericardium based aortic valve bioprosthesis are prone to severe calcification after long-term implantation, requiring re-intervention. In this work, we devise a novel procedure to optimize the seeding of

patches of porcine-derived fixative-free decellularized pericardium with native human aortic valve interstitial cells (aVIC). Pericardium patches (6 mm diameter) were repopulated by direct perfusion of cell suspension (5E+5 cells/patch) through the pericardium sheets, using a bioreactor system (U-Cup, Celtec). Dynamic cell seeding efficiency was tested applying three flow rates (0.03 ml/min, 3 ml/min, 6 ml/min) for three different times (8 hours, 24 hours, 48 hours). Cell adhesion onto the scaffold was evaluated through qualitative 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, histological analysis (Hematoxylin & Eosin) and nuclear fluorescence staining (DAPI). After seeding both MTT assay and DAPI staining confirmed the presence of cells on both sides of the patch. Finally, the more efficient seeding was obtained applying a flow rate of 3 ml/min for 48 hours. In conclusion, our data show, for the first time, the feasibility to dynamically seed decellularized porcine pericardium and to use it as a support for human aVICs three-dimensional seeding and culture.

### Regeneration of Endometrium Tissue using Cell Sheet Engineering

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Pregnancy occurs when a fertilized egg has been implanted in the endometrium of the uterus. Endometrial disorder can cause uterine infertility. Endometrial tissue comprises of two layers, the functional layer and the basal layer. The functional layer is shed during menstruation, and is then regenerated by the basal layer and, it has been suggested, stem cells within.

Therefore, endometrial regeneration is difficult with a basal layer defect. We propose the use of a new type of regenerative medicine for endometrial disorder.

We will show that three-dimensional endometrial tissue was assembled by layering two types of cell sheet. Moreover, healthy endometrium can be regenerated by implanting the layered-cell-sheet. These results indicate that the endometrial disorder was improved and the endometrium tissue was re-established by endometrial cell-sheet-transplantation.

We expect that endometrial cell sheet transplantation will be able to find application in the human endometrial disorder. As a result, the method can become a novel therapy for uterine infertility in women.

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**Disclosures:** Teruo Okano is a director of the board of CellSeed Inc. Tatsuya Shimizu and Masayuki Yamato are scientific consultants for CellSeed Inc. These 3 authors are the stakeholders of the company and are inventors of cell-sheet-related patents. Tokyo Women's Medical University is receiving research fund from CellSeed Inc.

### Study on Genetic Stability in Human Urothelial Cells *in Vitro*

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**Introduction:** Quality control studies are crucial to secure biosafety in regenerative medicine.

Analysis of gene expression changes and genetic stability are two approaches to better understand the effects of *in vitro* propagation. In this study we aimed to investigate genomic stability of human urothelial cells in long-term culture.

**Methods:** The Affymetrix, human genome U133 2 plus set (HG-U133) was used to compare gene expression profiles in cells at early and late passage (p2 vs. p8). Ingenuity Pathway Knowledge database and biological function software tools (IPA<sup>®</sup>, QIAGEN Redwood City, www.qiagen.com/ingenuity) were used to interpret the observed gene expression profiles and to identify upstream regulators.

Chromosomal structural instability was investigated with high resolution Comparative Genomic Hybridization (CGH) with special focus on oncogenic regions.

**Results:** Bioinformatics analysis of the differentially expressed genes identified several upstream transcriptional regulators and its target molecules. These regulators play an important role in controlling cell-fate outcomes that are frequently associated with changes in the cell cycle. Long-term cultured cells had a normal expression pattern of oncogenes/tumor suppressor genes and also of gene associated with bladder cancer. Furthermore, CGH analysis indicated no gain or loss of chromosomal regions down to 20 kbp and no structural changes in bladder cancer related gene regions.

**Significance:** Urothelial cells *in vitro* are stable at a genomic level. Patterns of gene expression activity demonstrated a decrease in proliferative capacity and no increase in oncogenic activity after long-term culture. We suggest arrayCGH and gene expression analysis of selected genes as a routine in clinical regenerative medicine.

### Determination of Optimum Matrix Stiffness for Maintenance of Human Colon Cancer Stem Cells

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Metastasis, resistance to chemo- and radiotherapy, and eventual relapse has been attributed to a tumor subpopulation known as cancer stem cells (CSCs). CSCs are regulated in their tumor microenvironment by various factors. Synthetic hydrogels can be used to investigate the effects of individual environmental factors on CSCs by providing inert 3D matrices. In this study, the role of matrix stiffness on tumorsphere formation by human colon cancer cells in an inert microenvironment was determined. HCT116 human colon cancer cells were encapsulated in poly ethylene glycol diacrylate (PEGDA) hydrogels and the effect of gel stiffness on tumorsphere formation was investigated by fluorescent microscopy, qRT PCR and DNA content assay. Hydrogel stiffness affected the tumor formation; At day 9, as the gel stiffness was increased from 5 kPa to 10, 15, 20, 25 and 30 kPa, the average tumorsphere size changed from  $52.36 \pm 12.39 \mu\text{m}$  to  $68.91 \pm 13.49 \mu\text{m}$ ,  $73.06 \pm 11.22 \mu\text{m}$ ,  $80.69 \pm 15.91 \mu\text{m}$ ,  $100.47 \pm 18.16 \mu\text{m}$ , and  $81.82 \pm 12.37 \mu\text{m}$  respectively. HCT116 cells had the largest tumorspheres, highest density of tumorspheres and highest expression of CSC markers in the gel with the stiffness of 25 Kpa. The inert polyethylene glycol hydrogel with the optimum modulus of 25 kpa can be used as an inert 3D matrix to study the role of individual environmental factors on the maintenance of human colon CSCs.

### Mapping Scientific Dynamics in Regenerative Medicine Research

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Regenerative medicine takes an increasingly important place in medical research. However, it is a heterogeneous research domain bringing together different scientific fields. The aim of this study is to characterize the history of this concept, the scope of this new research field, and its current trend using a scientometric approach.

Literature search was conducted both in PubMed & Scopus databases. Then, we led a descriptive statistics and a network analysis on the corpus (11203 documents). Thus, we have been able to draw a landscape of the «regenerative medicine» field, track its history, examine its trends, rank the actors (author, institution, country, journal) and determine the patterns of scientific collaboration in order

to gain an understanding of the diffusion of ideas and the institutionalization of this new field.

If the term “regenerative medicine” is connoted for the first time in 1992, it began to spread in scientific publication since 2000, with a marked acceleration in the last 5 years. An analysis of the underlying concepts shows a very heterogeneous and unstable research domain with in/out scientific fields along the years. This is accompanied by a lack of autonomy revealed by the absence of leading journal, and calls into question an accomplished legitimation of this new field. Indeed, to sustain its institutionalization, numerous universities had undertaken a communication strategy by entitling their department regenerative medicine. We will discuss our results in the light of the various research public policies in the world and from a science studies point of view.

### Effect of the Decellularization of Pericardial Tissue on the Macrophage Response

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Biomaterials derived from decellularized tissues have shown promoting properties on the regeneration of tissues such as abdominal wall and dermis. The tissue remodeling induced by biologic material remaining after decellularization has been associated to the polarization of the response of immune cells. In this work, we report the decellularization of bovine pericardium (DBP) by non-ionic detergent 4-octylphenol polyethoxylate (Tx) and Tx combined with reversible alkaline swelling (STx), as well as the residual composition of laminin, fibronectin, DNA and sulfated glycosaminoglycans (sGAG). Also, we report the effect of the leachates (RPMI media, 37°C, 72 h) from DBP and non-decellularized tissue (nDBP) on the stimulation of RAW264.7 macrophage cell line. The results indicated a higher residual amount of DNA, sGAG and fibronectin in Tx-DBP than STx-DBP, however we detected a lower residual amount of laminin in STx-DBP than Tx-DBP. Macrophages proliferated on all three biologic meshes as well as in the presence of nDBP- or DBP-leachates. The gene expression (4 h of culture) and secretion (12 and 36 h of culture) of cytokines IL6, IL10 and TNF $\alpha$  were significantly promoted by Tx- and STx-DBP-leachates, in comparison with non-stimulated macrophages and macrophages stimulated with nDBP-leachates. Our results suggest that Tx and STx decellularization methods favored a high leaching of components from biologic material, promoting a faster stimulation of macrophages in comparison with non-decellularized tissue.

### Transplantation Adipose-derived Stromal Cell Sheets for Prevention of Stricture after Esophageal Endoscopic Submucosal Dissection in Pig Models

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**Background:** Endoscopic submucosal dissection (ESD) is an accepted treatment for early esophageal cancer. However, large mucosal defects cause esophageal strictures after esophageal ESD. Thus, mucosal regeneration will be expected for preventing esophageal strictures.

We have developed novel regenerative medicine to prevent such complication using fabricated epithelial cell sheets. However, the fabrication of epithelial cell sheets needs patients' own oral mucosa as the autologous cell source. Therefore, we examined adipose-derived Stromal Cells (ASCs) for another cell source to treat the

ulcers after ESD. In the present study, we evaluated the feasibility of transplantation of autologous ASC sheets for preventing esophageal strictures after ESD in a pig model.

**Methodology:** ASCs were isolated from subcutaneous fat of pigs and expanded under a culture condition. The surface antigens of expanded ASCs were analyzed by flow cytometry. Furthermore, the capacities of differentiation and proliferation of ASCs were evaluated. ASCs were seeded on temperature-responsive dishes and cultured for 5 days. Then, ASCs sheets were easily harvested upon temperature reduction. To confirm the presence of transplanted ASCs, cell sheets were labeled with a fluorescent dye, PKH26GL, immediately before the transplantation to the esophageal ulcer sites after ESD.

**Results:** Primarily cultured ASCs expressed the known surface markers specific to ASC (CD29, CD44, CD90, and CD105). Endoscopic transplantation of ASCs sheets were performed with a device as reported previously (1). Fluorescence microscopy of frozen sections of transplanted ulcer sites revealed that PKH26GL-positive cells were adhered on the surface of the submucosal layer.

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### Engineered Cardiac Microtissue Model Reveals No Detrimental Effect of Fibrosis on Beating Frequency

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Cardiac fibrosis is characterized by deterioration of cell and matrix alignment due to extracellular matrix accumulation that may hamper ventricular function. However, it remains unclear how fibrosis affects the microenvironment of the cardiomyocytes and thereby influences tissue contractility on the cellular level.

Here we describe an *in vitro* cardiac microtissue model composed of a mixture of neonatal mouse cardiomyocytes and cardiac fibroblasts which are seeded in uniaxial and biaxial constraints to induce (an)isotropy. Uniaxial constraints were used to mimic 'healthy' aligned organization, while biaxial constraints resulted in 'diseased' disorganized matrix. Furthermore, this model allows studying the effect of matrix accumulation by manipulating the collagen concentration.

Disorganization of the matrix had no detrimental effect on the beating frequency and the force generated by the cardiomyocytes, although disarray had a negative effect on the distribution and homogeneity of the contraction. Furthermore, the average dynamic contraction was decreased after increasing the collagen content, while the beating frequency of the microtissues was unaffected.

In this study, cardiac microtissues were used to unravel the effect of matrix disorganization and accumulation on cardiac contractility. Our results indicate that changes in the ECM have no direct influence on the beating frequency, although the dynamic contraction force exerted by the microtissues is affected. Furthermore, the model system presented is suitable for investigating pathophysiological events associated with cardiac fibrosis and may thereby facilitate in the optimization of new therapies.

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### Implantable Microenvironments for Studying Human Tumor Metastasis

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The metastatic spread of cancer is responsible for more than 90% of tumor-associated death while remaining a poorly understood as-

pect in carcinogenesis. Bone marrow, a sponge-like gelatinous tissue found inside of bone cavities, has been observed as a major metastatic site for prominent tumor types. Yet, studying this important interaction is difficult by current methods; there are no good model systems to explore the natural, dynamic course of metastases to the bone marrow. In this presentation, we will introduce a bioengineering strategy to study bone marrow metastases of human tumor cells by applying tissue-engineering principles. We will first discuss the development of humanized marrow implant that is composed of a biomimetic scaffold pre-seeded with human bone marrow stromal cells. This implant can retain and support host and transplanted human bone marrow cells. Multiple genetically-engineered human microenvironments were also achieved in a single host mouse. We will also present a model system to study the spread of human metastasis in a mouse, beginning from an orthotopic tumor, to circulating tumor cells, and finally a metastatic nodule that was captured using our bone marrow implant. This model system was also leveraged as a platform for testing anti-metastatic drug compounds. The presented approach can be readily applied to other types of human cancer types with bone marrow homing prevalence.

### Photo-Cross-Linked Amniotic Membranes for Preservation of Limbal Epithelial Progenitor Cells: Ultraviolet Irradiation Dosage Effect

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To avoid potential toxicity induced by chemical cross-linkers, the amniotic membrane (AM) was physically cross-linked by ultraviolet (UV) irradiation to stabilize the tissue collagen matrix. In this study, we investigated the irradiation dosage effect on the biophysical characteristics of tissue matrix and its ability to preserve limbal epithelial progenitor cells. After exposure to different dose levels of UV irradiation (0.9–18 J/cm<sup>2</sup>), the AM was investigated by determinations of its cross-linking degree, nanostructure, and biocompatibility. To evaluate the cell stemness, the limbal epithelial cells (LECs) were cultivated on these photo-cross-linked AM substrates for 5 days. The number of cross-links per unit mass of AM significantly increased with increasing irradiation dose. Transmission electron microscopic observations showed that UV irradiation-mediated change in the matrix nanostructure of AM could be attributed to the aggregation of tissue collagen fibrils. The results provide a basis for understanding the relationship between collagen nanofiber size and the number of cross-links generated during AM photo-cross-linking. *In vitro* and *in vivo* biocompatibility studies demonstrated that all the physically cross-linked AM materials studied here are not detrimental to corneal cells and tissues. The ABCG2 gene and protein expressions were significantly up-regulated with increasing UV irradiation dosage, suggesting the important roles of cross-linking density and matrix nanostructure in the maintenance of undifferentiated precursor cell phenotype. It is concluded that biophysical characteristics controlled by UV-induced cross-linkage may affect the ability of photo-cross-linked AM to preserve limbal epithelial progenitor cells and its potential application for corneal epithelial tissue engineering.

### Peptide-CNT Hydrogels for Tissue Engineering and Cancer tumor Studies

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Self-assembling peptides are promising nanomaterials for tissue engineering and 3D cell culture due to their advantages compared to synthetic or protein-based biomaterials. They are formed from amino acids; can be found throughout the body; at the same time, they can be synthesized with precise control of its chemical composition and form hydrogels with nano-sized fibers/pores upon injection into the body. On the other hand carbon nanotubes as multifunctional

nanomaterials can enhance the efficacy of biomaterials if they are incorporated to the hybrid systems successfully.

Here EFK8 peptide hydrogels and EFK8-SWNT hybrid hydrogels have been prepared and used to culture NIH-3T3 fibroblast and A549 cancer cells. It was shown that presence of SWNTs in the peptide hydrogel improves attachment, growth, spreading and migration of NIH-3T3 cells. Indentation tests showed that this effect is not related to a change in the mechanical property as the hydrogel stiffness did not change by adding SWNTs.

Then it was shown that EFK8 hydrogels induce A549 cancer cell spheroid formation, but stiffening the hydrogel by increasing the peptide concentration leads to cells with a more stretched morphology and migratory phenotype. On the other hand, cells adopt a stretched morphology with high mobility when seeded on hybrid EFK8-SWNT hydrogels (formed at normal EFK8 concentration). Overall the effect of SWNT can be attributed to its role in serving as locations for cell anchorage facilitating cell adhesion and migration. This effect can be used to study another effect of the microenvironment, namely cell-binding motifs, on tumor progression and metastasis.

### Biocompatible Nanocellulose Hydrogels for 3D Bioprinting of Tissue Constructs

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The objective of this research has been to prepare bioink based on bacterial nanocellulose. Bacterial nanocellulose (BNC) hydrogel is an emerging biomaterial in tissue engineering applications due to its biocompatibility and tissue integration. This natural polymer, produced by the aerobic bacterium *Gluconacetobacter xylinus*, is composed of highly crystalline and hydrated (99% water) nanosized cellulose fibrils with high mechanical strength. Due to its outstanding properties BNC has been used already commercially as an FDA approved wound care product. In a recent study BNC was used clinically as a graft for dural replacement in 62 patients. BNC is synthesized extracellularly as nanosized fibrils when the bacteria utilize glucose as a source and form an exopolysaccharide. The cellulose chains are held together due to the hydrogen bonds of the hydroxyl groups. Due to its high crystallinity the mechanically strong 3D nanosized network of BNC is difficult to be converted and be used as a bioink for bioprinting applications. We have developed an efficient homogenization process which combines chemical, enzymatic and mechanical treatment to convert 3D network into bioink with shear thinning properties which provide outstanding results in printability and shape fidelity. Furthermore the bioprinting of BNC with alginate and fibroblasts produced 3D crosslinkable constructs. The cell viability after 7 days proved to be high (79%) indicating great future for BNC as a bioink in tissue engineering applications.

### Reference

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### A Modular Glycosaminoglycan-Based Hydrogel Platform to Establish 3D Co-Culture Models

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Angiogenesis, the outgrowth of new capillaries from pre-existing vessels, is crucial in development, disease and regeneration. Studying angiogenesis *in vitro* remains challenging because the capillary

morphogenesis of endothelial cells is controlled by various exogenous cues. Therefore, a modular platform of *in situ*-forming starPEG-glycosaminoglycan hydrogels have been developed allowing for the independent control of their mechanical and biomolecular matrix parameters. Upon optimization of matrix stiffness, adhesion ligand density and growth factor administration primary human vein endothelial cells (HUVECs) were cultured together with human bone marrow mesenchymal stem cells (MSCs) to create mature capillary networks that were stable for more than 4 weeks *in vitro* (Chwalek K *et al.*, Sci. Rep. 10.1038/srep 04414, 2014). Furthermore, we utilized the hydrogel platform to establish a complex 3D microenvironment of human cancer angiogenesis by culturing breast or prostate epithelial carcinoma cells (MCF-7, MDA-MB-231, LNCaP, PC3) together with HUVECs and MSCs (Bray LJ *et al.* Biomaterials 53, 609, 2015). The study was executed in three stages: comparative evaluation of cancer tumor growth within the starPEG-glycosaminoglycan hydrogels and within Matrigel<sup>1</sup>™, evaluation of the bioengineered tumor angiogenesis microenvironment, and evaluation of the responsiveness of the culture model to chemotherapeutics and angiogenesis inhibitors. The multiple cell types cultured within the cell instructive hydrogels were less sensitive to chemotherapy when compared with 2D cultures, and displayed comparative tumor regression to that displayed *in vivo*. We expect that this biomimetic 3D model will provide a platform for the in-depth analysis of cancer development and the discovery of novel therapeutic targets.

### Development of Engineered Valved Venal Grafts with Increased Antimicrobial Resistance

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Chronic venous disease is a common cause of morbidity. Conventionally dysfunctional veins are replaced by synthetic grafts, which demonstrate early infection<sup>1</sup> in about 10% of cases and reduced patency rates. Electrospinning has shown great potential in drug delivery<sup>2</sup>. The aim of this study was to develop decellularised valved venal conduits, encaged by a biodegradable polymer cage with encapsulated antibiotics, for venal reconstructions.

Porcine saphenous veins were decellularised using SDS and Triton X-100. Samples were assessed histologically (H&E and DAPI) and immunohistochemically (Collagen IV). The electrospinning apparatus was utilized to prepare 150 mg/ml polycaprolactone and 50 mg/ml polyethylene glycol copolymer loaded with Vancomycin and Gentamicin at a concentration of 5 mg/ml each. The antimicrobial efficacy of the antibiotics on *S. aureus*, *S. epidermidis*, *E.coli* and *Pseudomonas* was assessed *in vitro*. The cumulative drug release of both antibiotics from the polymeric cage was measured by UV-vis spectroscopy.

Histological and immunohistochemical staining confirmed the complete removal of cells and the preservation of the extracellular matrix. Antimicrobial assessment showed that there was efficient amount of antibiotics released from the polymeric scaffolds, in order to inhibit growth of bacteria during the first day, while decreasing the incidence of bacteria colony formation within the first week. These results were in agreement with the drug release experiments which demonstrated an initial burst release of antibiotics, followed by a later gradual release.

This work demonstrated the feasibility of using a decellularised venal graft encaged in a biodegradable antibiotic-releasing polymer scaffold for preventing infection at the implantation site.

### Meniscal Regeneration using a Bovine Dermal Collagen Matrix

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**Purpose:** To investigate the regenerative capacity of a bovine dermal collagen matrix, SurgiMend, placed into a meniscal defect in swine over 6 months.

**Materials & Methods:** Three, 4 mm cylindrical defects were made in the left medial meniscus of nine swine: in the anterior horn, posterior horn, and body of the meniscus. 5 mm cylindrical implants of scaffold were press fit into the defect and secured with a single 3-0 Ethibond suture. Control sites were untreated without any implant. Other control sites were treated with a cylinder of meniscus removed when making the defects. Tissues were harvested at 3, 8, and 24 weeks and studied grossly, immunohistochemically, and histologically using polarized light and the following stains: H&E, Trichrome, and Safranin-O.

**Results:** No healing or angiogenesis was evident in the meniscal tissue control implant and the empty defect was forced closed. At three weeks there was evidence of angiogenesis and cells populating the interstices of the SurgiMend matrix. By 8 weeks, the collagen scaffold implants had vessels throughout and were fully integrated with both the peripheral and central regions of the native meniscus, and fibrochondrocyte-like cells were noted throughout. Safranin-O showed typical GAG staining from peripheral to central locations. Polarized light demonstrated collagen fibril formation typical of native meniscus. In many of the 24-week animals the implant appeared to be fully integrated into the meniscus.

**Conclusion:** SurgiMend was capable of regenerating meniscal lesions by promoting vascularization, fibrocartilage development, and healing over 6 months in a swine model of meniscal injury.

### Three-Dimensional Morphological Assessment of Hypertrophy in hiPSC-derived Cardiomyocytes

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Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes are a promising cell source for cardiac tissue regeneration. Little is known about how these immature cardiomyocytes respond to their extracellular environment at the cellular level in three dimensions, information that is crucial to predicting cellular behavior in engineered tissues. Here we develop an analysis of plated single hiPSC-derived cardiomyocytes to assess 2D area, 3D volume, shape, and location of the nuclei within the cell. HiPSC-derived cardiomyocytes plated on glass coverslips were treated with phenylephrine (PE) daily for 3 days to induce hypertrophy. Fixed cells labeled with  $\alpha$ -actinin were imaged with confocal microscopy to obtain z-stacks for 3D cell volume analysis in CellProfiler and Matlab. Area increased 73% with PE stimulation ( $1125 \pm 238$  versus  $650 \pm 75 \mu\text{m}^2$  (control),  $P < 0.05$ ), while volume increased 45% with PE ( $4130 \pm 970$  ( $n = 33$ ) versus  $2852 \pm 271 \mu\text{m}^3$  ( $n = 32$ ),  $P = 0.11$ ). A similar trend was observed in neonatal rat ventricular myocytes where area increased 54% with PE stimulation ( $2064 \pm 203$  versus  $1433 \pm 199 \mu\text{m}^2$  (control),  $P < 0.05$ ), and volume increased 21% ( $11306 \pm 203$  ( $n = 46$ ) versus  $9333 \pm 1059 \mu\text{m}^3$  ( $n = 46$ ),  $P = 0.11$ ). Further morphological analysis of hiPSC-cardiomyocytes is ongoing. These results reveal that conclusions about cardiomyocyte morphology in 3D cannot simply be extrapolated from 2D measurements and thus our imaging and analysis method is a valuable tool for further understanding and characterizing cell populations. Notably, this technique will be compatible with cells in 3D gels and engineered tissues for regenerative medicine applications.

### Dual-Reporter Induced Pluripotent Stem Cells for Vascular Cell Sheet Engineering

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Current treatments for vascular occlusive disease (angioplasty, stenting, and bypass surgery) have limitations, including restenosis,

multiple surgeries, and lack of suitable autologous grafts. Synthetic grafts and tissue-engineered blood vessels (TEBVs) have overcome some limitations and have sought to avoid issues with thrombosis/occlusion; however, immunogenicity remains a problem. The discovery of reprogramming somatic cells to induced pluripotent stem cells (iPSCs) provides an alternative cell source, where non-immunogenic cells of multiple lineages can be derived from patient-specific iPSCs. In this study, we differentiate a novel iPSC line with a dual-reporter (green fluorescent protein (GFP) for smooth muscle actin (SMA) and red fluorescent protein (DsRed) for neural/glial antigen 2 (NG2)) into endothelial cells (ECs) (using VEGF) and vascular smooth muscle cells (vSMCs) (using PDGF-BB and TGF- $\beta$ ). ECs were isolated using co-expression of CD31/CD144, and vSMCs, which are characterized by co-expression of SMA/NG2, were isolated using our dual-reporter system. iPSC-derived ECs and vSMCs express characteristic markers of ECs (CD31, CD144, Flk-1) and vSMCs (SMA, SM22 $\alpha$ , MHC, Calponin, NG2). Ongoing studies of traction force microscopy measure contractile force of iPSC-derived vSMCs (SMAGFP/NG2DsRed), SMCs (SMAGFP), and primary vascular and bronchial SMCs to characterize the functional phenotype of different SMCs. We are also using thermo-responsive substrates to produce cell sheets generated from differentiated iPSCs that we evaluate for mechanical strength for comparison to native vessel mechanics and for correlation with SMC phenotype. With this study, we will gain a better understanding of SMC phenotype from different anatomical locations and how those phenotypes affect functional properties of TEBVs.

### Osteoporosis Affects Stem Cell Migration that is Ameliorated by CXCR4

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**Background:** Osteoporosis and bone fractures cause immobility, chronic pain and high patient care costs. Mesenchymal stem cells (MSCs) from postmenopausal women have a slower growth rate and osteogenic differentiation ability causing lower bone density and reduced fracture healing capacity compared to premenopausal women. Cellular movement and relocation are necessary for many physiologic properties. Local MSCs from injured tissues and circulating MSCs aid in fracture healing. Cytokines and chemokines such as SDF-1 and its receptor CXCR4 play important roles in maintaining mobilization, trafficking and homing of stem cells from bone marrow to the site of injury. We investigated the effect of CXCR4 over-expression on the migration of MSCs from ovariectomised, normal and young rats.

**Methods:** MSCs were harvested from femora of young, normal and OVX rats, genetically modified to over-express CXCR4 and put in a Boyden chamber to establish their migration towards SDF-1. This was compared to the non-transfected stem cells.

**Results and discussion:** MSCs from OVX rats migrate significantly ( $p < 0.05$ ) less towards SDF1 ( $9 \pm 5\%$ ) compared to MSCs from normal ( $15 \pm 3\%$ ) and juvenile rats ( $25 \pm 4\%$ ). Cell transfected with CXCR4 migrated significantly more towards SDF-1 compared to non-transfected cells irrespective of whether these cells were from OVX ( $26.5 \pm 4\%$ ), young ( $47 \pm 17\%$ ) or normal ( $21 \pm 4\%$ ) rats. MSCs migration is impaired by age and osteoporosis and this may be associated with significant reduction in bone formation in osteoporotic patients. The migration of stem cells can be ameliorated by up regulating the CXCR4 levels which could possibly enhance fracture healing in osteoporotic patients.

### Age-dependent Regulation of hASC Osteogenesis Quantified via Electrical Cell-substrate Impedance Spectroscopy

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Human adipose derived stem cells (hASC) are an attractive cell source for bone tissue engineering applications. However, a critical issue to be addressed before widespread hASC clinical translation is the dramatic variability in osteogenic potential between hASC from different donors. The goal of this study was to use electrical cell-substrate impedance spectroscopy (ECIS) to apply a low, non-invasive alternating current to hASC in order to monitor complex impedance patterns during hASC proliferation and osteogenesis. We hypothesized that ECIS impedance patterns could be utilized to elucidate donor age-related differences in hASC proliferation and osteogenic differentiation in real time, thus providing a means to better understand and predict variability between hASC cell lines.

ECIS osteogenesis curves were generated from superlots comprised of hASC from young (age: 24–36), middle-aged (age: 48–55), and elderly (age: 60–81) donors. hASC were seeded on electrode arrays and impedance measurements were taken during proliferation and osteogenic differentiation. hASC superlots were analyzed via live/dead staining for viability, Alizarin red and calcium liquorcolor assays for mineralization, and Alamar Blue for proliferation. Four distinct phases were identified in impedance curves: proliferation, confluent stabilization, mineralization, and differentiated stabilization. Mineralization was characterized by a distinct impedance drop, initiating at approximately day 12 in young, day 6 in middle-aged, and day 2 in elderly hASC. This is the first study to use ECIS to elucidate and screen hASC proliferation and osteogenic variability. These findings could be critical for development of patient-specific tissue engineering and regenerative medicine therapies.

#### Development and Bio-mechanical Evaluation of a Bio-hybrid Scaffold for Heart Valve Tissue Engineering

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**Objective:** Currently available heart-valves (HV) do not allow growth and remodelling after implantation in children. Tissue engineering (TE) approach mimics the properties of native valve using decellularized tissues, synthetic polymers and hybrid scaffolds for HV-TE [1]. The objective was to modify bovine pericardium (BP) with Polycaprolactone-Chitosan-nanofibers (PCL-CH-NFs) and cells to obtain sufficient mechanico-elastic properties with regenerative potential for HV-TE.

**Methodology:** BP was decellularized using 1% deoxycholic acid and analyzed for acellularity and extracellular matrix (ECM) preservation (histology & DNA estimation). PCL-CH-NFs were coated by electrospinning onto BP without cross linking agents to fabricate Bio-Hybrid scaffold. Surface and functional characterization (SEM & FT-IR), mechanical properties (uni & biaxial), hemocompatibility and biocompatibility (MTT assay & direct contact analysis) were analysed for Bio-Hybrid scaffold. Human fibroblasts, endothelial and mesenchymal stem cells were used for cell adhesion studies.

**Results:** Histological staining (H&E and DAPI) and DNA estimation showed acellularity of BP with ECM retention. The electrospun PCL-CH nanofibers (134.68 ± 49.4 nm) showed polymer integration to BP with unique peaks observed in FT-IR. This biocompatible, hemocompatible Bio-Hybrid scaffold demonstrated biomechanical properties comparable to native valve such as contact angle (21.6 ± 4.2°) and mechanical properties (tensile strength-23.63 ± 1.222 MPa, Young's modulus-45.987 ± 1.22 MPa and burst strength-24.34 ± 0.08 MPa).

**Conclusion:** We developed a Bio-Hybrid scaffold suitable for HV-TE with possible regenerative capacity for pediatric use. Further analysis has to be done to evaluate the fatigue and regenerative potential of the scaffold.

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#### *In vitro* Endothelial Cell Response to Ionic Dissolution Products Released From a Lithium-doped 45S5 Bioactive Glass

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As it has been established that lithium (Li) may perform functions in angiogenesis, the controlled and localized release of Li ions from bioactive glasses (BGs) is expected to provide a promising therapeutic alternative for regenerative medicine of vascularized tissues. The aim of this study was to assess the *in vitro* angiogenic effects of the ionic dissolution products (IDPs) from BGs in the SiO<sub>2</sub>-CaO-Na<sub>2</sub>O-P<sub>2</sub>O<sub>5</sub> (45S5) system and of those from 45S5 BG doped with 5 wt% Li<sub>2</sub>O (45S5.5Li). IDPs were obtained by incubating 45S5 BG or 45S5.5Li BG particles (< 5 μm) in M199 growing medium for 72 h on an orbital shaker at 37°C. The soluble ions (Li, Si, P, Ca, and Na) were determined by ICP-MS. The results show, for the first time, that the IDPs from 45S5.5Li BG stimulated human umbilical vein endothelial cells (HUVECs) proliferation and migration via an activation of β-catenin signaling. It was also shown that IDPs from 45S5.5Li BG could enhance *in vitro* HUVECs tubule formation and secretion of IGF1 and TGFβ. The effects observed are attributed to the presence of 0.2 mM Li in the IDPs. These findings are relevant to tissue engineering and regenerative medicine because the IDPs from 45S5.5Li BG may act as inexpensive inorganic angiogenic agents providing a convenient alternative to the application of conventional angiogenic growth factors.

#### Graphene Oxide Membranes for Ocular Tissue Engineering

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**Purpose:** We analyze the viability of graphene oxide (GO) membranes as a carrier of ocular cells and their potential use as a treatment of ocular lesions.

**Methods:** Eyes were obtained from a local eye bank. Limbal cells were obtained from corneoscleral-ring tissue. Explants, 2–3 mm in diameter, of the limbal region were cultured onto GO membranes, as well as on a plastic culture dish as control group. Retinal pigment epithelial (RPE) cells were obtained by digesting RPE tissue with Trypsin/EDTA. Once confluent, RPE cells were detached and sub-cultured on GO membrane.

GO membrane were obtained by casting into a silicone mold a GO solution and air-dried at room temperature.

Human limbal and RPE cells growing on the GO membranes were fixed and analyzed by phase contrast microscopy, scanning electron microscopy and immunocytochemistry against Cytokeratin High Molecular Weight (CK) and p63 for limbal cells and against CK18 and ZO-1 for RPE cells.

**Results:** Limbal and RPE cells were able to attach and grow onto GO membranes. All the cells maintained their morphology and cellular markers, both in the membrane and on the culture plate.

Limbal cells exhibited a positive stain to CK and p63 and RPE cells for CK18 and ZO1.

**Conclusions:** GO membranes have shown to be a good scaffold for culturing limbal, and human RPE cells; therefore, future

applications of graphene oxide membranes may be developed for reconstruction of the eye.

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### First Successful One-Year Follow Up of Decellularized Tissue Engineered Heart Valves in Sheep

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Traditional valvular prostheses lack growth potential, representing a major problem for pediatric patients. They currently need staged interventions to accommodate for growth of the valve accompanied by increased risks of morbidity and mortality. Decellularized tissue engineered heart valves (DTEHVs) hold great promise to overcome this limitation by providing a valve for life.

Based on a first generation DTEHV design, we showed complete host cell repopulation already after 5 hours and an increase in extra cellular matrix content with time, indicative for regenerative and growth potential [1]. These valves maintained functional up to 8 weeks, where after competence was gradually lost over time, likely due to cell-mediated leaflet retraction. Based on computational simulations, a second generation of DTEHVs with an improved geometry was developed. These valves were designed to increase strain in radial direction to counteract for cell mediated leaflet retraction by means of an enhanced coaptation area and belly curvature implemented via a patented bioreactor insert [2].

These second generation DTEHVs (n = 11) were transvenously implanted into sheep as pulmonary valve replacement and functionality is assessed monthly via MRI and intracardiac echocardiography. These valves seem to provide competent mechanical behavior without developing clinically relevant regurgitation up to at least 6 months. Being still an ongoing study, we have currently obtained the first successful one-year follow up.

The European Union's Seventh Framework Program is acknowledged for funding this study.

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### Effect of Alginate Incorporated Fmoc-FF Hydrogels on Chondrocyte Behaviour

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In the last decade, peptide based hydrogels has gained an important interest in the tissue engineering studies due to having great advantages such as non-toxicity, ability of spontaneous forming without any additional crosslinker, injectability and tunable nature with required amino acid sequences. Fmoc-diphenylalanine (Fmoc-FF) is one of the earliest and widely used example of these small molecule gelators that have been utilized in biomedical area. However, mechanical integrity of Fmoc-peptides is insufficient for long term use and the low stability problem in almost neutral pH seriously hinders their utilization [1]. Therefore, in this study Fmoc-FF dipeptides were mechanically enhanced by incorporation of alginate, a biocompatible and absorbable polysaccharide. The hybrid hydrogel is consisted of Fmoc-FF and alginate and obtained via molecular self-assembly of Fmoc-FF dipeptide

in alginate solution and following ionic crosslinking of alginate moieties with varying concentrations of calcium chloride. Hydrogel characterization was evaluated in terms of morphology, viscoelastic moduli and diffusional phenomena and the structures were tested as 3D scaffolds for bovine chondrocytes. The *in vitro* chondrocyte culture in scaffolds lasted 14 days and sulphated glycosaminoglycan (sGAG), collagen type II synthesis were monitored with immunohistochemical staining. According to the results, it was found that alginate incorporation speeds up establishing a stable and favourable environment for the chondrocytes to secrete sGAG and collagen type II.

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### Trans-wall Oxygen Gradient invokes Adventitial Neovascularization in Human Saphenous Veins *Ex Vivo*: a Bioengineering Approach

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Although several cellular and molecular causes for vein graft disease have been identified in cell culture and *in vivo* models, the mechanical and metabolic cues induced by the interruption of blood supply in the *vasa vasorum* have remained unexplored.

We devised an *ex vivo* culturing platform allowing the generation of an oxygen gradient between the luminal and the adventitial sides of the human saphenous vein (hSV). hSV segments were mounted within the system, and two conditions were compared: *i*) CABG condition (intraluminal oxygen level equal to 21%; extra-adventitial oxygen level equal to 5%) and *ii*) standard condition (both regions with oxygen level equal to 21%). In all experiments, culture medium was recirculated at 5 ml/min, resulting in a luminal pressure of 5 mmHg. At the end of the oxygen conditioning period, hSV samples were recovered and the central portion was processed for the histological analyses. Another part was used for RNA studies.

Compared with the standard condition, the CABG condition displayed a significant increase in the density of adventitial small and large caliber *vasa vasorum*, as well as a higher proliferation of cells within and around these vessels. Analysis of hypoxia-related mRNA and miRNA is currently ongoing. Our results suggest that vein adventitial hypoxia invokes neovascularization effects and growth of *vasa vasorum*, two factors known to predispose the arterialized vein conduits to restenosis.

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### Establishment of the Culture Condition to Maintain the Primary Human Colorectal Cancer Microenvironment by using a 3D Perfusion-Based Bioreactor

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Two-dimensional (2D) *in vitro* culture systems and *in vivo* animal models are the primary tools used to test cancer cell responses to drugs. However, drug sensitivity data obtained via 2D culture systems can be misrepresentative, while, in the opposite end, patient derived xenografts (PDX) are time consuming, expensive and the non-transformed components of tumor microenvironment are lost.

The use of three-dimensional (3D) systems based on human tissue could be an innovative and efficient tool able to bridge the gap between 2D cultures and animal model for the development of new chemotherapeutic strategies.

Fresh surgically colorectal cancer (CRC) specimens were cut into fragments, inserted into a collagen scaffold sandwich and then cultured for 10 days using a perfusion-based bioreactor system. We assessed tissue *in vitro* survival, proliferation and the maintenance of the microenvironment cell components by histo-morphological analysis, immunofluorescence and gene expression for Ki67, Ep-CAM, vimentin, CD90, CD45, CD4, and CD8.

After 10 days of culture the tissue partially maintained its original architecture with typical neoplastic disorganization. Phenotypic analysis confirmed that expanded tissues included epithelial and stromal cells. Tumor cell proliferation, as provide by Ki67 staining, was assessed. Furthermore, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were detected within cultured tumor fragments.

Our results indicate that culture of primary tumor fragments within perfused bioreactors allows the preservation of the diverse CRC cellular components, thus representing a relevant tool for the evaluation of anti-tumor treatments.

### Development of an Innovative Three-dimensional Culture Model of Human Breast Cancer to Study Multi-drug Resistance

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Multidrug resistance (MDR) is one of major reasons for the poor outcomes of chemotherapy. However, limited progresses have been achieved to overcome this phenomenon effectively due to our poor understanding of its underlying mechanism. This might mainly be caused by the shortage of suitable culture models that could highly mimic tumor MDR *in vitro*. In the present study, we developed a more pathologically relevant, three-dimensional (3D) culture system of human breast cancer that included adriamycin-resistant breast cancer cell line MCF-7/ADM in collagen hydrogel. Distinct growth profile of MCF-7/ADM cells was observed when they were embedded within collagen matrix, compared to that of the monolayer cultures. Presence of collagen matrix not only promoted MCF-7/ADM cell proliferation but also induced aggregate formation. These aggregates displayed good viability during cultivation, suggesting a suitable microenvironment provided by the collagen matrix for cell growth. Significantly increased expression of MDR-related genes was also observed in the 3D cultures by RT-PCR, which was consistent with their improved expression profiles of drug resistant proteins assayed by western-blotting or immunofluorescence staining. Most importantly, compared to the 2D cultures, a significantly enhanced functionality of MCF/ADM cells was found in the 3D cultures, evidenced by their increased IC<sub>50</sub> values of chemotherapy drugs. Taken together, this collagen-embedded culture system of human breast cancer cells effectively represent an improved model of tumor MDR, providing a robust tool to explore the mechanism of MDR and its reverse in drug resistant tumors.

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### In Vitro Physiologic Erythropoiesis in a 3D Bone Marrow Biomimicry

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Erythropoiesis is a complex process which takes place in the bone marrow (BM) of adults and is regulated by several microenvironmental factors, including BM architecture, cellular interactions, growth factors, oxygen, etc.. Traditional 2D cultures rely on addition of high concentrations of exogenous cytokines and neglect BM architecture which favours formation of niches, where paracrine and autocrine cell communication occurs.

We have previously created a 3D BM biomimicry using collagen-coated polyurethane scaffolds for culture of human cord blood mononuclear cells. We now investigate the role of oxygen and the

microenvironmental niches that support erythroid differentiation. Using this 3D system, serum-free cultures were supplemented with 10 ng/mL SCF, from day 0–28, and near-physiologic concentration of EPO at 100 mU/mL, from day 7–28. Three conditions were studied: normoxia (20% oxygen), hypoxia (5% oxygen) and 2-step oxygenation (hypoxia from day 0–7 and normoxia thereafter). *In situ* proliferation and erythroid maturation was monitored by flow cytometry weekly using cell surface markers for CD45, CD71 and CD235a. Proliferation (10-fold at day 21) and expression of erythroid-specific markers (40% CD45<sup>+</sup>CD71<sup>+</sup>CD235a<sup>+</sup> at day 21) indicated expansion and commitment towards the erythroid lineage. Hypoxia did not change erythroid expression profile. Adjacent CD68<sup>+</sup> and CD71<sup>+</sup> cells were detected by confocal microscopy at day 19 suggestive of *in situ* erythroid islands. The presence of niche-like structures was also observed by SEM at days 17 and 25. These findings suggest the creation of physiologic erythroid niches within the *ex vivo* BM biomimicry.

### Enhancement of Adult Retinal Pigment Epithelial Stem Cell Survival and Integration Using an *in vitro* Testing Model for Cell Delivery

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Retinal pigment epithelium (RPE) cells are polarized, highly specialized neural cells that have several functions essential for vision. Progressive deterioration of the RPE in elderly individuals promotes a cascade of pathologic events, and results in visual impairment and ultimately blinding diseases such as age-related macular degeneration. Sub-retinal transplantation of healthy stem-cell-derived RPE cells suspended in saline solution has been explored as a strategy to recover the damaged retina. However, the benefit on visual acuity can be limited by poor post-injection cell viability, and subsequent distribution and integration of RPE cells. To overcome these limitations, a more biocompatible delivery vehicle that supports *in vivo*-like morphology and function are introduced. FDA-approved natural polymers, including alginate, methylcellulose and hyaluronic acid (HA) were tested. Using human retinal pigment epithelium stem cells (RPESCs) obtained from human cadaver globes and expanded and differentiated into polarized RPE cells, we compared vehicles' effect on overall proliferation and functional surface expression after 1–96 hours in free suspension. Cell attachment, distribution and morphology were monitored by microscopy over the course of 8 weeks. The ability to form an effective blood-retina barrier was tested by measuring the transepithelial electrical resistance, polarization of membrane domain and distribution of tight junction proteins. Our *in vitro* results demonstrate that 0.2% HA solution promotes better initial cell distribution, proliferation, cobblestone formation, and RPE surface marker expression after 96 hours of storage, suggesting that HA is capable of overcoming injection limitations and could be further pursued as an alternative delivery vehicle for RPE transplantation *in vivo*.

### Chitosan based Hydrogels for Vascular Tissue Engineering Applications

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Chitosan is a linear polysaccharide obtained industrially from deacetylation of chitin and particularly interesting for vascular tissue engineering applications. We prepared chitosan physical hydrogels (2 concentrations, 5% acetylation degree and 2 gelation routes) to check *in vitro* (i) proliferation of EPCs (Endothelial Progenitor Cells, from umbilical cord-blood) on chitosan disks, (ii) their behavior under shear

stress, (iii) ability of EPCs to line chitosan tubes, and *in vivo* (iv) implantation in a large animal model as end-to-end carotid interposition.

**In vitro:** EPCs were able to grow on chitosan and confluence was obtained at day 10. When exposed to laminar shear stress (1.2 Pa) in parallel-plate flow chambers, EPCs aligned in the flow direction at 24 H and 30 H when compared with static conditions and EPCs phenotype was maintained. Concerning tubular constructs, chitosan tubes were filled with EPCs suspension and placed on a perfusion device for 7 days in the incubator. Analysis of live/dead staining of cellularized grafts confirmed the presence of EPCs at the graft luminal surface.

**In vivo:** After success of patches (2 cm<sup>2</sup>) implanted on arteriotomy for 2 hours, uncellularized tubes of chitosan (6 mm ID) were implanted in sheep. Carotid Doppler ultrasound exam was assessed at the end of surgery. Tissues including chitosan were harvested for scanning electron microscopy and histology. Results are ongoing.

The present study aimed the elaboration of a cellularizable chitosan based hydrogel tube for small diameter vascular tissue engineering, which could be implanted in a large animal model.

### High-throughput Screening Identifies Novel Polyacrylates Competent for Human Aortic Valve Interstitial Cells Growth and Physiologic Differentiation in a 3D Environment

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Aortic valve stenosis is a widely diffused pathology resulting from pro-calcific differentiation of valve interstitial cells (aVICs), leading to native valve substitution. The limitations of currently employed devices call for the identification of biomaterials promoting the physiological growth of aVICs, for the manufacture of novel tissue engineered heart valve prostheses. For this purpose, we used a high-throughput polymer array screening method. Primary human aVICs (n = 3) were seeded (3E + 5 cell/array) and cultured for 72 hours onto arrays fabricated by spotting onto standard glass slides a polyacrylate (PAK) library (n = 381). Cell adhesion and spreading were assessed through immunofluorescence (Vimentin, Collagen-I,  $\alpha$ SMA, Phalloidin, DAPI), followed by computer-assisted fluorescence quantification (ImageJ). Results indicated 7 PAKs promoting aVICs adhesion (cell number per spot ranging from 18 ± 3 to 60 ± 6, mean ± SE; n > 14 replicates/polymer), although with different cytoskeleton organization. These PAKs were spin coated onto 22 mm diameter glass coverslips and they were used as substrates for aVICs culture up to 14 days. Based on qRT-PCR (n = 5) results on the expression of the most common calcification related genes (BMP2, ALP, OPN, RUNX2), one PAK was sorted for coating onto a 3D polycaprolactone scaffold (Mimetix<sup>®</sup>). Bioreactor based (U-Cup, Cellec) culture of aVICs into the novel scaffold was performed up to 14 days, showing an increase in adherent cells after seeding (ratio *coated/uncoated* = 2.5). In conclusion, our data show, for the first time, the applicability of high-throughput screening method to identify polyacrylates for manufacturing novel scaffolds for bio-valve implants.

### Therapeutic Effects of FK506 on Prevent Intervertebral Disc Degeneration through Suppression of IL-2

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**Background:** It has been reported that immune reactions could play a key role in intervertebral disc degeneration (DDD). Although many recent studies have focused on IL-1, associated with degeneration, few studies have yet attempted to investigate IL-2.

**Methods:** Ten human intervertebral discs were obtained during surgery. The intervertebral discs of twelve New Zealand white rabbits were punctured with a 16-gauge spinal needle at L3-4 and L4-5. MRI was checked to evaluate disc degeneration. FK506 (inhibitor of

IL-2) were then injected into six rabbits' discs randomly, and saline injected into other six as a control.

**Results:** H-E staining in the samples of patients exhibited obvious signs of degeneration, including destruction of lamellar pattern, decreased cellularity, deviated extracellular matrix, and fibrocartilaginous change. The significantly higher levels in disc samples of mRNA and protein expressions of IL-2 were found in the DDD patients compared to the controls. In the rabbit model, MRI revealed the signal from the discs that had been injected with FK506 had begun to recover by the 4-week time point. The trend continued to the 8-week time point. There were no visible signs of recovery of the signal intensity in the control group at 8 weeks. Based on histological analysis, the FK506-injected intervertebral discs exhibited elevated extracellular matrix secretion and little ossification compared with control discs.

**Conclusion:** In our study, we found that IL-2 was expressed in tissues of the human degenerative intervertebral discs. And intradiscal injection of FK506 could prevent intervertebral disc degeneration *in vivo*.

### Interrogating Adhesivity in Alginate Hydrogels for Mesenchymal Stem Cell Spheroids

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Mesenchymal stem cell (MSC)-based therapies are promising for applications in tissue repair but are limited by poor cell survival upon transplantation. While MSC spheroids exhibit improved survival and angiogenic potential *in vitro*, these benefits are rapidly lost when spheroids are delivered *in vivo* as cells migrate from the aggregate. We hypothesized that deploying MSC spheroids in RGD-modified alginate gels would reduce apoptosis while maintaining their robust bone-forming potential. The function of MSC spheroids in Arg-Gly-Asp (RGD)-modified alginate hydrogels was compared to non-modified alginate and corresponding dissociated cell groups. Live/dead imaging of spheroids in RGD-modified hydrogels confirmed significantly greater cell survival than spheroids entrapped in non-modified alginate gels. After 5 days in culture, spheroids in RGD-modified gels exhibited similar caspase activity, an indicator of apoptosis, but more than a 2-fold increase in vascular endothelial growth factor (VEGF) secretion compared to spheroids in unmodified gels. Regardless of material, MSC spheroids had reduced caspase activity and greater VEGF secretion compared to an equal number of dissociated cells. MSCs (40x10<sup>6</sup> cells/mL) were entrapped in gels and implanted subcutaneously to determine bone-forming capacity. Upon explantation at 8 weeks, radiographs demonstrated similar mineralization in both spheroid and dissociated groups. Immunohistochemistry revealed more diffuse osteocalcin staining in gels containing spheroids compared to dissociated controls. This study demonstrates the promise of cell-instructive biomaterials for spheroid delivery for bone tissue engineering applications.

### A Reconstructed Urethra by Tissue Engineering: *In Situ* Grafting in Rabbits for Proof of Concept

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**Introduction:** Urethral pathologies often need reconstruction but procedure complications and the limited availability of urologic tissues raised our interest for the development of a tissue-engineered urethral substitute. We propose a substitute produced by the self-assembly method, free of exogenous materials. The aim was to create a rabbit model for autologous urethral replacement *in vivo*.

**Materials and Methods:** Enzymatic cell extraction was accomplished from bladder and skin biopsies. Harvested dermal and vesical fibroblasts produced and assembled their own extracellular matrix and sheets were rolled on a mandrel. Ring tensile tests were performed to evaluate load failure and strain as well as estimated burst pressure. To demonstrate the proof of concept, reconstructed urethras (1.5 cm long, 6.2 mm diameter) were grafted in 4 rabbits. Complete

circumferential replacement surgeries were performed. Characterization was performed by histology and immunofluorescence.

**Results and discussion:** After 3 weeks of maturation, constructs presented suture retention strength  $32.7 \pm 8.1$  gram-forces. The estimated burst pressure averaged  $514 \pm 99$  mmHg, whereas native porcine urethras reached a maximum of  $418 \pm 66$  mmHg. Exploratory surgeries confirmed the feasibility of the technique used for the reconstruction and provided information about future improvement. The size and mechanical resistance of tissues were appropriate for suturing to the native urethra. Four weeks after the surgery, vascularisation was abundant and urothelial cells completely covered the urethral lumen.

**Conclusion:** This study showed our capacity to produce an autologous rabbit urethra that can resist to physio-mechanical constraints and is ready for urothelial cell seeding before long-term *in-vivo* study in rabbits.

### Stimulation of Beta-Cell Function by Extracellular Matrix Proteins

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Intrahepatic islet-transplantation is a promising therapy for treatment of type 1 diabetes. During islet isolation, collagenase is used to extract islets from the pancreas, leading to loss of important cell-matrix interactions. The islet extracellular matrix (ECM) comprises a specific combination of collagen, laminin and fibronectin. It has been shown that these proteins can either boost function or migration of beta-cells. The aim of this study was to investigate the effect of specific islet ECM molecules, fibronectin, collagen type IV, laminin-111, laminin-332 and mixture of laminin-111, 211, 121, 311, 411 and 511, on beta-cell function, proliferation and survival. We studied the behavior and function of INS-1E isletoma-cells on microcontact printed surfaces, patterned with 100  $\mu$ m spots, comprising individual or combinations of ECM proteins. We showed that a INS-1E-cells were able to adhere and proliferate on fibronectin, collagen IV and laminin 111 in an equal manner, and that glucose stimulated insulin secretion was enhanced on fibronectin and collagen IV. Formation of the 3D aggregates of INS-1E-cells on spots during 7 days of culture partially improved endocrine function compared to cells grown in a monolayer. Additionally, we showed that a combination of collagen type IV and laminin-322 improved beta-cell function in human donor islets cultured on ECM coated TCPS surfaces. We conclude that biofunctionalization of inert biomaterials with a specific subset of islet ECM molecules can support and improve islet function. We suggest that creation of a biomimetic islet niche using biofunctionalized substrates could potentially lead to a better transplant outcome in the future.

### A Shape-controlled Microgel Cell Delivery Platform for Delivery of Primed Stem Cells for *In Vivo* Therapeutic Neovascularization

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Tissue regenerative processes are driven by reparative mesenchymal stem cells under the influence of biochemical cues dictated by the microenvironment. Prolonged ischemia and lack of blood supply drive cellular apoptosis and tissue death. However, delivery of primed stem cells via a tunable microenvironment triggers an 'angiocrine' response, driving therapeutic angiogenesis. In this study, it is hypothesized that delivery of primed human mesenchymal stem cells

(hMSCs) on a shape-controlled microgel platform at a low-cell dose promotes angiogenesis in a murine model of hind-limb ischemia (HLI). Optimized 2 mg/ml collagen microgels were fabricated by dispensing type-I collagen with 4S-Star-PEG and hMSCs at  $0.8 \times 10^6$  cell density on to a hydrophobic surface. Balb/c nude mice underwent unilateral HLI and were divided into five groups ( $n = 12$ /group); PBS; microgels alone; microgels with 50,000 hMSCs; 50,000 and 1,000,000 cells alone. Laser-Doppler perfusion and pathological markers of disease severity were assessed between the animal groups. Histological and molecular evaluation of inflammation and angiogenesis were assessed using immunohistochemistry, multiplex ELISA and gene expression arrays. Statistical analysis was performed using one-way ANOVA with  $p < 0.05$ . Perfusion analysis revealed higher perfusion in hMSC embedded microgels  $60\% \pm 20\%$  compared to controls. Immunohistochemistry revealed increased angiogenesis and reduction in inflammation at day 21. hMSC embedded microgels also showed significant changes in gene and protein expression compared to the controls. Microgel delivery of primed hMSCs at a low-cell dose promotes functional angiogenesis in a severe murine model of HLI.

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### Single Source of Human ADMSC to Produce Functional Tissue Engineered Vascular Graft with Deposited Elastin to Favour Blood Vessel Compliance

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Tubular scaffold fabricated using biodegradable synthetic polymers could be strong to resist the hemodynamic forces of flow; therefore, suitable for tissue engineered vascular graft (TEVG) construction. Fibrin based niche may be deposited on hydrophobic polymer scaffold to promote growth and differentiation of adipose derived mesenchymal stem cells (ADMSC) to endothelial cells (EC) in the lumen in response to shear stress. This study was aimed to demonstrate extra cellular matrix (ECM) protein deposition by fibroblasts differentiated from ADMSC, seeded on the outer surface. Fibrin-based composite matrix with hyaluronic acid and gelatin was deposited on biodegradable polymer scaffold, electrospun over solvent-cast smooth lumen. Human ADMSC (IEC approved) from P2/P3 was seeded on the scaffold and cultured under static condition with step-wise increase of shear stress to 15 dynes  $\text{cm}^{-2}$ . On 8th day, nitric oxide release was analyzed; phenotype of EC and fibroblasts were characterized using cell-specific markers. The deposition of ECM proteins, collagen and elastin, was analyzed using commercial assay kits. Nitric oxide released into medium confirmed EC phenotype in the lumen. The lumen was blood compatible after contact with anticoagulated blood *in vitro* as per ISO10993 part4 tests. No significant platelet adhesion, activation or up regulation of von Willebrand factor on EC was detected. Real-time PCR analysis indicated presence of EC and fibroblasts. Collagen-elastin deposition were found to be significant. The study established that using autologous ADMSC, functional TEVG may be constructed with deposited elastin in the electrospun fibrous compartment for improved pliability and vascular activity in response to flow after implantation.

### Impact of Different Biological Scaffolds derived from Extra Cellular Matrix on Behaviour of Stem Cells

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Decellularised extracellular matrices (dECM) have been widely accepted as an ideal substrate for repair and remodulation of numerous tissues. Recent studies have claimed tissue specificity as well as complex tissue formation in preclinical settings when the biological scaffolds were derived from site-specific homologous tissues. The present study focused on the confirmation of therapeutic utility and suitable mode of delivering apical papilla (SCAP) derived mesenchymal stem cells via 3 different hydrogels derived from dECM. The biological scaffolds (8 mg/ml) were prepared from dentine (human), Spinal cord (porcine) as well as bone (bovine) and characterized for their molecular structure using SEM. The biochemical composition of scaffolds confirmed the DNA content was < 50 ng and the amount of collagen present in dentine, spinal cord and bone was  $83.15 \pm 5.5$ ,  $67.41 \pm 23.16$ ,  $84.23 \pm 10.20$  respectively. The rheologic modulus of ECM scaffold from dentine ( $4.30 \pm 0.99$  Pa) and spinal cord ( $19.53 \pm 0.59$  Pa) was lower than scaffold derived from bone ECM ( $286.4 \pm 2.12$  Pa). All three scaffolds showed sigmoidal shape of gelation kinetics with similar rate of gelation but different lag time. Assessment of cell viability after 2 days of SCAP encapsulation in scaffolds demonstrated cell viability of  $94.17 \pm 3.1\%$  on bone ECM,  $68.75 \pm 81\%$  on spinal cord ECM and  $64.0 \pm 12\%$  on dentine ECM. The cell proliferation was determined using presto blue assay and there was a considerable increase in cell number on day 7 for cells in spinal cord ECM compared to other scaffolds. However on day 14 all the scaffolds facilitated higher proliferation with respect to day 7.

#### Adipose-derived Endothelial and Mesenchymal Stem Cells Enhance Vascular Network Formation *In Vitro* and in Reconstruction Techniques *In Vivo*

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Adipose-derived stem cells comprise one of the most promising stem cell populations identified thus far. Adipose-derived mesenchymal stem cells (MSCs) have demonstrated vascular formation induction, mediated by the support they provide to endothelial cells (ECs), conveyed by the angiogenic factors they secrete. The present study monitors vascularization dynamics within 3D PLLA/PLGA scaffolds embedded with human microvascular ECs (HAMECs) or human umbilical vein ECs (HUVECs), and characterizes the impact of different supporting cells, either MSCs or human neonatal dermal fibroblasts (HNFs), on these dynamics. Tracking cell behavior over time demonstrated that the HAMECs:MSCs combination exhibited the greatest vessel network potential when compared to other cell combinations. The co-culture yielded fully developed vascularized networks within seven days. Moreover, HAMECs:MSCs combination revealed positive vascular properties, such as fast vessel development, complexity and maturity. These characteristics prove that adipose-derived cells obtain strong angiogenic capabilities.

Continuing *in vivo* study was aimed to further move toward clinical application by utilizing potential human clinically applicable cells and engineering large tissue-constructs in a rat model. Furthermore, the optimal cell combination, revealed in the *in vitro* study, served to create an engineered vascularized graft. The extent of vascular network formation *in vivo* has been measured by speckle doppler, showing significantly high blood flow in the co-culture group compared to the controls. The vascularized graft was then transferred as a flap to treat full thickness defects in the hind limb or in the abdomen. After their transfer, the engineered flaps were highly vascularized and well integrated with the surrounding tissue.

#### Preparation of a Decellularized Scaffold from Hare Carotid Artery for Vascular Tissue Engineering Applications

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Tissue engineering is a promising approach for creating small diameter vessels by combining autologous vascular cells with a natural and/or synthetic scaffold [1]. The suitable scaffold was the first key factor. Natural biologic scaffolds are commonly generated by decellularization of tissues and organs. In this study, to establish an *in vivo* small-animal model, we chose rabbit arteria carotis as the origin of the ECM scaffold, which diameter was around 2 mm ideally. Carotid arteries from adult rabbit (New Zealand white rabbits, 6 months old, male, 2.0–2.5 kg from Xiamen University Animal Center) were used for this trial. We access the preparation protocols in five parallel groups by the different combinations of enzyme, non-ionic detergent and physical method. These protocols were improved from pre-research [2], but changed to get better results for 2 mm diameter vessels, 4°C for the whole course. The fresh was used as a negative control, placed on an orbital shaker with double distilled water (ddw) for the whole course. We comprehensively applied SEM, histological analysis, MTS assay, mechanical testings, and biocompatibility to evaluate the protocols of decellularization in detail with 5 groups through different combinations. We finally found that in spite of all the protocols would make the decellularized scaffolds, but the protocol of Group IV was the suitable one for further research which had the lowest cytotoxicity, the highest mechanical capability. With this scaffold and the seeding cells from the patient himself—autologous stem cells or endothelial cells, the TEVG could be an xenograft with less thrombogenicity.

#### Over-expression of Sirt1 Restores Endothelial Phenotype and Extends Cell Lifespan in Induced Pluripotent Stem Cell-derived Endothelial cells (iPSC-ECs)

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**Introduction:** Endothelial cells (ECs) differentiated from induced pluripotent stem cell (iPSC) are a novel cell source for developing vascularized engineered tissues. However, a number of technical challenges have been reported when culturing iPSC-ECs, namely early senescence, limited cell proliferation, and instability of the differentiated phenotype. Sirt1 is a deacetylase that promotes proliferation, prevents senescence and regulates EC functions. We hypothesize that sirt1 overexpression will overcome the described challenges to iPSC-ECs culture *in vitro*.

**Methods:** iPSC-ECs were cultured from passage 1 to 6. Morphology and phenotype at each passage was monitored via phase contrast microscopy and flow cytometry. Sirt1 was over-expressed in iPSC-ECs using lentivirus. Cell morphology, phenotype, and functions were analyzed and compared against no-virus, empty-virus, and sirt1-mutant virus controls.

**Results:** Under normal culture condition, iPSC-ECs gradually lose EC cobble-stone morphology (passage 1) and become fibroblast-like (passage 6). Expression of EC marker, such as CD31, decreases from ~80% (passage 1) to ~30% (passage 6). Sirt1 over-expressing cells exhibit more EC-like morphology, higher percentage of EC markers, decreased senescence, higher proliferation rate and more nitric oxide production relative to control cells. Moreover, the effect is long-lasting, as per the percentages of expressed markers at later passages (CD31 + 60.5%, 89% and 96.3% at the end of passages 5, 7, and 9 respectively). iPSC-ECs in control groups proliferate slowly with low purity after passage 5.

**Conclusion:** In conclusion, sirt1 overexpression in iPSC-ECs restores EC phenotype, improves EC functions and extends cell lifespan, overcoming critical hurdles for using iPSC-ECs in translational research.

#### Magnesium Ions Enriched Decellularized Bone Allografts for Bone Tissue Engineering

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Bone allografts have been widely used to treat large segmental bone defects clinically for a period of time. However, due to the insufficient osteoconductivity after stringent sterilization, the allografts would take pretty long time to integrate with the host bone[1]. Hence, the enhancement of osseointegration between allograft and host bone is considerably important. In previous work[2, 3], we demonstrated that a specific range of magnesium ions could significantly promote osteoblast activity and new bone formation locally without any side effect. This study thus aims at enhancing the osteoconductivity of allograft by incorporating additional magnesium ions into the bone matrix through ion-exchange approach. The decellularized bone slices measured in 10 mm\*10 mm\*1 mm were treated in magnesium chloride (MgCl) solution after gamma irradiation using custom protocols. The samples were cultured with human immortalized MSCs for *in vitro* biological analyses. By optimizing the reaction time, temperature and magnesium ions concentration, the magnesium ion concentration within bone matrix could be increased from 0.41% to about 1.15%. The results obtained from MTT and BrdU-labeling assay suggested that higher cell viability and proliferation rate were found on the sample of 1.15% magnesium ion containing bone matrix as compared with the control ( $p < 0.05$ ). Also, the ALP activity in magnesium-enriched sample was significantly up-regulated. Based on these observations, we proposed that the osteoconductivity of bone allograft could be enhanced by adopting this simple and economical approach. However, animal study is required prior to human clinical trial.

#### A Computational Analysis of Cell-Mediated Compaction and Collagen Remodeling in Tissue-Engineered Heart Valves

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The *in vivo* functionality of tissue-engineered heart valves (TEHVs) often decreases over time due to cellular forces and collagen remodeling, which can lead to leaflet retraction. The goal of this study was to predict cell-mediated compaction and collagen remodeling in TEHVs exposed to either pulmonary or aortic dynamic pressure conditions using computational modeling. In addition, cell contractility was varied in the model to assess the effects of different cell phenotypes. The tissue was modeled as a mixture of contractile stress fibers, collagen fibers, and isotropic tissue components. Collagen remodeling consisted of strain-dependent degradation and oriented production. Tissue compaction was modeled by including cell-mediated contraction of the collagen fibers, where the reference length of the fibers was changed as a function of the cell stress. For pulmonary pressure conditions, our model predicted that valvular insufficiency can only be prevented when cell contractility is very low. In contrast, sufficient valve closure was always predicted for TEHVs exposed to aortic pressure conditions, regardless of the degree of cell contractility. Our simulations therefore indicate that (1) it may be crucial to attract cells with a low intrinsic contractility to preserve the functionality of TEHVs implanted in the pulmonary position, and (2) valvular insufficiency is less likely to occur when TEHVs are implanted in the aortic position.

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#### Engineering an Infectious Treg Biomimetic through Chemoselective Tethering of TGF- $\beta$ 1 to PEG Brush Surfaces

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Modulation of immunological responses to allografts following transplantation is of pivotal importance to improving graft outcome and duration. Of the many approaches, harnessing the dominant tolerance induced by regulatory T cells (Treg) holds tremendous promise. Recent studies have highlighted the unique potency of cell surface-bound TGF- $\beta$ 1 on Treg for promoting infectious tolerance, i.e. to confer suppressive capacity from one cell to another. To mimic this characteristic, TGF- $\beta$ 1 was chemoselectively tethered to inert and viable encapsulation platforms using Staudinger ligation. We report the synthesis and functional characterization of these engineered TGF- $\beta$ 1 surfaces. Inert beads tethered with TGF- $\beta$ 1 were capable of efficiently converting naïve CD4+CD62Lhi T cells to Treg. Concordantly, translation of conjugation scheme from inert surfaces to cell encapsulation platforms on viable cells also led to Treg conversion. Further, antigen-specific Treg could be generated using these platforms. These findings demonstrate the unique faculty of tethered TGF- $\beta$ 1 encapsulation platforms to function as an "infectious" Treg and provide a compelling approach for generating tolerogenic microenvironments for allograft transplantation.

#### A Gene Expression Profiling of Umbilical cord Human Wharton's Jelly Stem Cells Transdifferentiated to Cornea Epithelial Cells

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**Objective:** Human Wharton's jelly mesenchymal stem cells (HWJSC) previously demonstrated *ex vivo* differentiation potential to cornea epithelial cells. In this work, we carried out a gene expression analysis of HWJSC differentiated to cornea epithelium in order to shed light on the genetic mechanisms associated to the transdifferentiation process.

**Methods:** Primary cell cultures of HWJSC, cornea epithelial cells and stromal keratocytes were established from human tissue biopsies. Then, a bioengineered model of orthotypical human artificial cornea (OHAC) was constructed using fibrin-agarose scaffolds with keratocytes immersed within and epithelial cells on top. A heterotypical model (HHAC) was generated using HWJSC on top. Total RNA was extracted from artificial corneas after 7, 14, 21 and 28 days of development in culture and the expression of 45 genes with a role in epithelial differentiation and extracellular matrix (ECM) synthesis was quantified by RT-PCR.

**Results:** Both artificial cornea models showed upregulation of relevant differentiation genes, although some genes were expressed with higher intensity in OHAC, including the intercellular adhesion-related genes DSC1, DSG1, DSP, GJA1, JUP, and cytokeratins KRT3, KRT5, KRT12 and laminins LAMA1, LAMA3. High expression of GJA4, KRT8, LAMB1, LAMC1, was found in HHAC. Cluster analysis tended to separate OHAC and HHAC.

**Conclusions:** These results confirm the differentiation potential of HWJSC, although relevant genes and gene functions could not be fully upregulated *ex vivo* as compared to OHAC.

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### An External Shape Memory Stent to Prevent Vein Failure

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Hemodialysis is the primary lifeline for patients with end-stage renal disease (ESRD), but arteriovenous graft (AVG) failure imposes significant morbidity, mortality, and financial impositions. Failure rates of 50% after 1 year and 75% after 2 years are reported in hemodialysis patients that utilize polytetrafluoroethylene (PTFE) dialysis grafts. Stenosis at the venous anastomosis ultimately leads to compromised blood flow, necessitating vascular interventions (e.g. balloon angioplasty or stents) or re-do access surgeries. Major financial impositions ensue for patients, insurers, and dialysis clinics, while blemishing hospital records with unwanted, expensive patient readmissions.

The leading cause of failure at the venous anastomosis is neointimal formation triggered by venous responses to surgical injury resulting from PTFE implantation and arterial flow.

We are developing an external stent that can minimize neointimal formation by eluting anti-neointimal therapeutics, providing mechanical support, and promoting outward instead of inward vein remodeling in the arterial circulation. Existing external mesh supports applied in other settings, such as to saphenous vein grafts in heart bypass grafting surgeries, have demonstrated some promise but cannot be applied in hemodialysis vascular access surgeries because they cannot be applied to the foci of neointimal formation at the venous anastomosis. Our novel shape memory polymers (SMPs) enable facile wrapping of the external support around the venous anastomosis that is most critical to maintaining vein patency. Preliminary data in an *ex vivo* AVG model with human saphenous veins (HSV) indicates an ability of these external supports to reduce neointimal formation and, in turn, obviate the subsequent adverse clinical repercussions.

### The Origin of Pericytes and Smooth Muscle Cells from a Clonal Mesodermal Precursor Mesenchymoangioblast

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Recent advances in human pluripotent stem cell (hPSC) technologies made it possible to generate all types of vascular cells (endothelial cells, pericytes (PCs) and smooth muscle cells (SMCs)) *ex vivo* for the study of vascular biology and diseases, and vessel engineering. However, the understanding of vasculogenic cell development in hPSC cultures and the use of hPSC-based progenitor cells for vascular therapies and engineering, are hampered by the lack of knowledge concerning the hierarchy of vasculogenic progenitors and markers, which can be used to discriminate PCs, SMCs, mesenchymal stem cells (MSCs) and their ancestors. In our previous studies, we showed that the onset of mesenchymo- and vasculogenesis from hPSCs is defined by the emergence of the clonal mesodermal precursor for endothelial cells and MSCs, mesenchymoangioblast (MAB) (Vodyanik *et al.*, 2010). Here, we report that MABs have the capacity to produce vascular SMCs and PCs, which can be further specified to types 1 and 2 PCs with either a proinflammatory or contractile phenotype, respectively. Discovery of the vasculogenic potential of MABs, coupled with transcriptomic analysis of MABs and their derivatives, allowed us to identify stage- and lineage-specific markers and establish the lineage tree of vasculogenic progenitors in hPSC cultures. Overall, these studies provide the platform for applying well-defined vascular progenitors for vasculature engineering, and elucidation of the molecular mechanisms regulating development of vasculogenic cells in hPSC cultures.

### Feasibility of Xenotransplantation of Bone Tissues from Alpha 1,3 Galactosyl Transferase Knockout Pig

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In this study we investigated whether lack of Gal expression is associated with reduced immune responses of human PBMC against pig bone extracts. When human PBMC obtained from heparinized blood of healthy volunteers was stimulated with bone extracts from  $\alpha$ -gal knockout (KO) pigs, proliferation of human PBMC and production of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  were significantly lower than those of bone extracts from wild type (WT) pigs. In addition, there was less activation of CD4<sup>+</sup> helper T cells and less production of IL-2, IFN- $\gamma$ , and IL-17 in response to bone tissue extracts from  $\alpha$ -gal KO pigs. Reduced immune responses against bone tissue extracts from  $\alpha$ -gal KO pigs are possibly due to lower levels of activation of signal transduction pathways such as NF- $\kappa$ B, p38, ERK and JNK. Current findings can be used for subsequent studies to evaluate the compatibility of bone tissues from  $\alpha$ -Gal KO pigs intended for human bone grafting, thereby increasing the feasibility of clinical application in future.

**Key words:**  $\alpha$ -gal knockout, pig, xenotransplantation, immune rejection, human PBMC.

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### In Vitro Evaluations of 3-D Bioprinted Cardiac Tissue Constructs

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Bioengineering of the functional cardiac tissue constructs combined with cardiomyocytes and scaffolding offers a great promise of creating viable myocardial tissues. However, the complexity of myocardium, structurally and functionally, presents many challenges for future applications. The cardiac tissue possesses a highly organized structure with unique physiological and mechanical properties. To overcome these challenges, we applied the 3-D organ printing strategy to fabricate a contractile cardiac tissue construct *in vitro*. Primary cardiomyocytes, isolated from rat neonatal heart tissue, were suspended in a fibrin-based hydrogel bio-ink. The cell-laden hydrogel bio-ink containing cardiomyocytes was printed through a pneumatic pressured nozzle. The printed cardiac constructs were cultured and matured *in vitro*. The bioprinted cardiac constructs displayed spontaneous contractions at 3 days after bioprinting, and gradually increased in contractile strength and synchroization over a period of up to 4 weeks, indicating evidence of cardiac tissue development and maturation. The cardiac tissue constructs were also examined by immunostaining for  $\alpha$ -actinin and connexin 43. Our results demonstrate the feasibility of using a 3-D printing strategy for engineering functional cardiac tissue constructs. This approach has a great potential to precisely generate functional cardiac tissues for use in pharmaceutical and regenerative medicine applications.

### Application of Tissue-Engineered Humanized Bone Models Reveals New Mechanistic Insights of $\beta$ 1 Integrins in Breast Cancer Bone Colonization

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The skeleton is a preferred site for breast cancer (BC) metastasis. The molecular mechanisms during bone colonization are still not fully understood, which may be attributed to the lack of suitable models to interrogate interactions between human BC



cells and the bone microenvironment.  $\beta 1$  integrins have been identified as a prognostic marker of invasive BC but their role in the establishment of metastases in the skeleton remains unclear. Here, the role of  $\beta 1$  integrins in bone colonization was investigated using tissue-engineered humanized *in vitro* and *in vivo* bone models. *In vitro*, bone-metastatic BC cells with suppressed integrin  $\beta 1$  expression showed reduced attachment, spreading, and migration within decellularized human bone matrices compared to control cells, but  $\beta 1$  integrin knockdown had no effect on cell proliferation. *In vivo*, tumor development within tissue-engineered humanized bone microenvironments was significantly inhibited upon  $\beta 1$  integrin suppression, as revealed by *in vivo* fluorescence imaging and histological analysis. Tumor cells invaded bone marrow spaces in the humanized bone, forming osteolytic lesions characteristic of BC. Despite their role in modulating tumor cell proliferation in the bone,  $\beta 1$  integrins did not influence osteoclast activation and bone resorption. Using a tissue micro-array assembled from a cohort of 22 patients,  $\beta 1$  integrin expression was detected in clinical bone metastatic tissue across different BC subtypes. Taken together, using unique tissue-engineered humanized bone models we demonstrate key roles of  $\beta 1$  integrins during bone colonization by BC cells, suggesting that  $\beta 1$  integrins may be potential targets in the treatment of bone-residing tumors.

#### Tropoelastin-Silk Hybrid Films as High Performance Candidates for Corneal Tissue Replacement

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Damaged corneas are surgically replaced by transplantation to restore vision and eliminate blindness. Due to the worldwide shortage of donor corneas and risk of graft rejection, there is a tremendous unmet demand for a robust corneal replacement. Here we assess the utility and functional value of tropoelastin-silk hybrid materials as a corneal replacement platform.

Tropoelastin is predominantly used to build elastin. Elastin provides protein-based, mechanical integrity to tissues and has critical functions in the regulation of cell performance. Silk fibroin is a fibrous protein that has been widely used in biomedical materials that benefit from its strength, relative stability and slow degradation. Hybrid protein alloys comprising recombinant human tropoelastin and silk fibroin are attractive implantable scaffolds because of their combined contributions to biotolerance, cell interactions and mechanical compatibility. We are fabricating stabilized blends of water-soluble tropoelastin and silk fibroin with a novel methodology that generates densely packed biocompatible films.

Our hybrid films show many desirable features for a suitable corneal replacement. They display a remarkable combination of physical properties encompassing flexibility, elasticity and suturability. These benefits are enhanced by mechanical stability and biological compatibility. The fabricated films have similar properties to the natural cornea, including optical clarity, refractive index, glucose permeability and mechanical performance. Furthermore, these films support the growth and function of the two main corneal cell types, epithelial and endothelial cells, and overall biological utility. Our results point to the value of this biomaterial in reconstructing tissues with clinical value for corneal replacement.

#### A Study on Inflammatory Response of PLGA Polyoxalate Scaffold

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Abstract: Poly(lactic-co-glycolic acid) (PLGA) as a biocompatible material is approved by FDA. However, PLGA is not a desirable

polymer for fast release of small water soluble molecules owing to its long degradability during 30 days. PLGA causes an inflammatory. So, we can adjust the release rate of POX because the degradable rate of POX is faster than PLGA. Also, two polymers is a biocompatible synthetic polymer with acceptable mechanical strength. We prepared Polyoxalate loaded PLGA films from various preparation ratios for film physicochemical, biological characteristic, physics chemical properties such as thickness measurement, surface morphology, transparency and DSC, FT-IR, SEM were carried out. Cell proliferation and viability were measured via MTT assay and SEM. We analyzed RT-PCR, histology (H&E, ED-1) for anti-inflammatory effect. Though these results, PLGA/POX hybrid scaffold have low inflammatory reaction compared with the PLGA. we concluded POX can be improve PLGA film's characteristic and POX/PLGA film can be applied to diverse tissue engineering application.

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#### Tensile Forces Regulate the Formation and Orientation of 3D Vascular Networks

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Techniques for generating vascularized tissues are expected to bear significant clinical value, as they may ensure sufficient oxygen and nutrient supply within the host tissue, cardinal to transplant integration and survival. Recent works have attempted to manipulate blood vessel network properties, such as geometry, maturity and stability, by supplementing cultures with biological factors, biomaterials and geometrical constraints. However, although mechanical forces play a central role in all biological processes, they have yet to be further investigated in relation to vascular network assembly. The present study assessed the effect of uniaxial cell-induced and/or externally applied tensile forces on the morphology of three-dimensional vascular networks. Mature and stable vascular networks were obtained within three-dimensional polymeric constructs co-seeded with fibroblasts and endothelial cells. Bioreactors were used to examine tensile forces effects along with examination of force measurements, immunofluorescence staining and analysis of angiogenic protein expression. The intensity of cell-induced forces correlated with the network quality, revealing related elements of the angiogenesis mechanism. Different orientations of endothelial vessel structures were observed under static and cyclic uniaxial tensile forces along with specific angiogenic factor secretion. Implantation of scaffolds bearing networks oriented to match those of the host tissue improved the graft's vascularization when implanted in mouse abdominal muscle model. Our results shed light on the involvement of tensile forces in vascularization and on the mechanism underlying force buildup during network assembly. The approach we used monitored tissue structural responses, rather than the single-cell responses, enhancing the understanding of coordinated multicellular responses to environmental signals.

#### Reestablishment of Perfusion in Critical Limb Ischemia Model with Pulsed Focused Ultrasound (pfus) and Mesenchymal Stem Cells in Aged Mice

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Chronic peripheral arterial disease (PAD) manifests as critical limb ischemia (CLI) with a 5-year mortality rate >70%. Mesenchymal stem cells (MSC) show promise to minimize CLI progression and restore perfusion in experimental models, but approaches suffer

from minimal homing to ischemic tissue and require direct injections of cells. This study investigated if pulsed focused ultrasound (pFUS) could enhance MSC homing in an aged CLI model and improve hindlimb perfusion.

CLI was induced by cauterizing the external iliac artery in C3H mice (9–12 months). To assess MSC homing, mice were euthanized at 15-days post-CLI (24 hr post-treatment). To assess perfusion, 14-days post-CLI mice in various cohorts received 3 consecutive days of treatment (i.e., 3×-saline, 3×pFUS, 3×MSC, 3×MSC+pFUS). pFUS was applied to ischemic hamstrings at 40 W (5% duty cycle, 100 pulses) and 106 MSC ~ 1 hr pre-pFUS were IV-injected via the lateral tail vein. Laser Doppler perfusion imaging was performed weekly for 7–8 weeks.

Significantly more MSC homed to pFUS-treated ischemic hamstrings than ischemic hamstrings without pFUS ( $p < 0.01$ ). LDPI revealed that restoration of limb perfusion was significantly greater in the pFUS+MSC mice beginning 2-weeks post-treatment, while other groups remained near baseline levels. When treatment is delayed (14-days post-CLI, during sub-acute inflammation) in aged mice, IV-injected MSC largely failed to home to ischemic muscle and have limited therapeutic potential. However, pFUS can noninvasively establish a gradient of locally upregulated chemoattractants to enhance MSC homing and help restore perfusion to ischemic limbs. pFUS guidance of MSC may be critical to develop effective cell therapies in PAD.

#### Alternate Modes of BMP-2 Loading in Clay Gels Induce Alternate Modes of Ectopic Ossification

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**Introduction:** Bone Morphogenic Proteins (BMPs) induce ectopic bone. This presents significant opportunities for bone regeneration strategies but also entails significant challenges. Key to harnessing BMPs will be the ability to localise and sustain activity at a target site. Clay (Laponite) hydrogels (LG) can bind growth factors for localised efficacy. Our study investigated whether LG localisation could enhance BMP-2 induced ossification.

**Methods:** *In vitro*, LG localisation of BMP-2 activity was assessed using dylight633 labelled BMP-2, and assays for C2C12 cell alkaline phosphatase activity (APA). *In vivo*, non-viable trabecular bone matrix (TBM) was perfused with LG alone (A) or LG 'pre-mixed' with BMP-2 (B). Alternatively, BMP-2 solution was applied exogenously to LG perfused TBM (C) or TBM alone (D) at point of implantation. Bone formation was assessed after 28 days by histology and micro-computed tomography.

**Results:** LG localised pre-mixed and exogenously applied dylight633-BMP-2. C2C12 cells displayed localised enhancement of APA in association with LG when exposed to exogenous, but not pre-mixed, BMP-2 ( $P < 0.0001$ ). *In vivo*, increased bone volume ( $p < 0.05$ ) was observed in LG+BMP-2 treatments (B&C) versus BMP-2 alone (D). Endochondral ossification was observed, localised within LG, in response to pre-mixed BMP-2 (B). This contrasted with direct, appositional bone formation observed upon TBM surfaces (and enhanced by LG) in response to exogenously applied BMP-2 (C).

**Conclusions:** LG control BMP-2 distribution to enhance ectopic bone formation and induce alternate modes of ossification. This has significant implications for clinical translation of BMP osteoinduction.

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#### A Novel Bioreactor and Culture Method Drives High Yields of Platelets from Stem Cells

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**Background:** Platelet transfusion is the primary treatment for thrombocytopenia. Platelets are obtained exclusively from volunteer donors, and the platelet product has only a five day shelf life, which

can limit supply and result in platelet shortages. Platelets derived from stem cells could help to fill this clinical need. Current culture methods yield far too few platelets for clinical application. We have developed a defined, serum-free culture method using a novel bioreactor to increase the yield of platelets from stem cell-derived megakaryocytes.

**Methods:** CD34+ cells isolated from umbilical cord blood were expanded on a nanofiber membrane using serum-free medium. These cells were then differentiated into megakaryocytic lineage by culturing with thrombopoietin and stem cell factor in serum-free conditions. Polyploidy was then induced by addition of Rho kinase inhibitor or actin inhibitor. A novel bioreactor was developed to recapitulate aspects of the bone marrow vascular niche. Polyploid megakaryocytes were subjected to flow in the bioreactor and induced to shed platelets. Platelet production from the bioreactor was confirmed by light microscopy, fluorescence imaging, and flow cytometry.

**Results:** CD34+ cells were expanded 200-fold. CD41+ cells were expanded 100-fold. Up to 100 platelets per input megakaryocyte were produced from the bioreactor, for an overall yield of  $10^6$  platelets per input

CD34+ cell. The platelets externalized p-selectin following activation.

**Discussion:** Altogether, our results show that functional platelets can be produced *ex vivo* on a clinically relevant scale using serum-free culture conditions with a novel step-wise approach and an innovative bioreactor.

#### Extracellular Matrix Hydrogel Scaffolds Developed for Neural Tissue Reconstruction

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**Introduction:** Efficient regenerative reconstruction of injured CNS has not yet been established despite intensive research. Biologic scaffolds composed of native extracellular matrix (ECM) possess unique properties that support constructive remodelling of various tissues. In this study, we characterized ECM-derived hydrogels prepared from CNS- and non CNS-tissues with respect to their composition and their *in vitro* effects on neurite outgrowth and proliferation as well as migration of human mesenchymal stromal cells (MSCs).

**Materials and Methods:** The ECM was extracted from porcine brain (BM); spinal cord (SCM) and urinary bladder (UBM) using tissue decellularization. All materials were characterized in terms of residual DNA concentration and collagen and glycosaminoglycans content. Viability and migration of MSCs isolated from bone marrow (BM MSCs), adipose tissue (ASCs) and Wharton jelly (WJ MSCs) were evaluated using a WST assay and xCELLigence system. The neurotrophic properties of ECM hydrogels were assessed on dorsal root ganglion (DRG) explant cultures.

**Results:** Scanning electron microscopy showed the ECM hydrogels to have a nanofibrous structure similar to that of the native ECM. The highest amount of glycosaminoglycans was found for BM, while UBM contained the highest amount of collagen. All types of ECM hydrogels promoted proliferation and migration of hMSCs. Moreover, all types of ECM also promoted massive neurite sprouting out of DRG explants. In conclusion, ECM scaffolds are a promising bioactive biomaterial for neural tissue reconstruction.

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#### Autologous Regenerative Therapies: Rapid Concentration of Progenitor Cells, Growth Factors, and Proteins at the Point-Of-Care

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Clinical translation of regenerative medicine technologies requires a source of stem and progenitor cells. The Autologous Regenerative Therapies Bone Marrow Concentration (ART-BMC) system provides a rapid concentration (>90%) of mesenchymal and hematopoietic stem cells and soluble growth factors from human bone marrow aspirate (BMA) at the point-of-care. An adjustable collection port allows for selection of user-specified fractions of centrifuged BMA. An integrated hollow, nano-porous fiber filter concentrates autologous proteins and growth factors (e.g. fibrinogen, alpha-2-macroglobulin, PDGF, VEGF, TGF- $\beta$ ) larger than 25 kDa in a total processing time of less than 20 minutes. Cellular analysis was performed on 20 human BMA samples by total nucleated cell count, colony forming unit-fibroblast (CFU-F) assay, osteogenic differentiation assay, and flow cytometry for mesenchymal and hematopoietic markers. All protein assays were performed by ELISA. Concentrated plasma has demonstrated proportional enrichment of proteins based on volume reduction, significantly improved cell binding kinetics and retention to coated substrates (hydroxyapatite scaffolds and allograft bone), and faster setting times for autologous fibrin hydrogels.

ART-BMC offers an FDA-compliant system for concentrating autologous stem and progenitor cells, platelets, and growth factors to be used independently or combined with biomaterials in injection or surgical therapies. Similar commercial systems recover no greater than 56% of mesenchymal cells and no protein/growth factor concentration. The ART-BMC represents the next step in clinical translation of regenerative medicine and is suitable for processing cell-based fluids during a single surgical event.

#### Artificial Chaperones Guide Ultra-structure Formation in Biomimetic Collagen Membranes

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A paradigm in regenerative medicine is the goal recreating native tissue architectures. Biomimetic structures in collagen-related materials can be achieved by electrospinning of nanofibers or by self-assembly of pre-programmed peptides (1, 2). However, the clinical translation is limited by scalability and cost-effectiveness.

In an inspiring example, corneal collagen shows a lamellar organization allowing for transparency in the eye (3). This assembly is directed by chaperones such as lumican and decorin (4, 5). Here, we recapitulated this ultra-structure employing cyclodextrins (CD) as artificial chaperones: Membranes featured lamellae of 1.5 – 2.0  $\mu\text{m}$  thickness and fibril diameter of 10 nm as determined by electron-microscopy. The biomolecular mechanism of the interaction was investigated. The triple-helical structure in presence of CDs was confirmed by rotational peaks at  $\lambda_{\text{min}} = 195 \text{ nm}$  and  $\lambda_{\text{max}} = 220 \text{ nm}$  at 25 °C by circular-dichroism spectroscopy. Functionalized  $\alpha$ -CD contributed to helical stability as the melting point increased from  $M_p = 45 \text{ }^\circ\text{C}$  to  $M_p = 50 \text{ }^\circ\text{C}$ , respectively. In turn,  $\gamma$ -CD had a destabilizing effect with  $M_p = 42 \text{ }^\circ\text{C}$ . Strikingly, collagen fibrils were induced by CDs in non-fibrillogenic buffer as measured by turbidometric analysis (UV/Vis). Phenylalanine residues within triple-helical domains of the subunits Collagen  $\alpha 1(I)$  and Collagen  $\alpha 2(I)$  (CO1A1 and CO1A2, NCBI) are putative targets for CD identified by primary structure analysis.

In conclusion, the use of artificial chaperones describes a promising route to unlock the inherent self-organization potential of naturally derived biomaterials. Exemplified on cornea-mimetic membranes, CD drives assembly of minimally processed and readily available collagen.

#### Assessing Differences between Mesenchymal Stem Cells and Trabecular Meshwork Cells

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**Introduction:** Glaucoma affects over 70 million people worldwide and is normally associated with elevated intraocular pressure, which is largely controlled by the trabecular meshwork (TM). In glaucoma, TM cellularity decreases. Thus, stem cells could offer therapeutic benefit if properly differentiated toward a TM lineage. To assess differentiation, functional and genetic characterization methods are required. Here we evaluate techniques to assess differences between mesenchymal stem cells (MSCs) and TM cells prior to differentiation.

**Methods:** Three human adipose-derived MSC and four human TM cell strains were characterized using three approaches. First, qRT-PCR was performed to profile expression patterns of candidate mRNAs. Second, myocilin expression was detected with western blots after 1 week of dexamethasone exposure. Finally, phagocytic activity was assessed after exposure of cells to heat-killed *E. coli* particles for 24-hours, normalizing particle counts to cell counts.

**Results:** Differentially expressed mRNAs were observed, with MGP, MYOC and tPA message levels higher in most TM strains. Dexamethasone led to higher MYOC protein expression in TM cells vs. MSCs. With the phagocytosis assay, TM cells engulfed 2.5-fold more particles vs. MSCs.

**Conclusions:** qRT-PCR identified a number of markers upregulated in TM cells vs. MSCs. However, cell strains were heterogeneous in expression, so subsequent studies should use multiple cell strains to account for this variability. Dexamethasone induction of MYOC was confirmed as a robust but not rapid approach. Also, TM cells consistently had higher phagocytic capacity vs. MSCs. Next steps will expose MSCs to relevant stimuli to establish a differentiation protocol to a TM lineage.

#### Human Macrophage Activation and Polarization in Response to Cryopreserved Cardiovascular Matrices

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Cryopreservation of cardiovascular matrices is a common method in transplant medicine, but conventional frozen cryopreserved (CFC) transplants can show inflammatory processes, calcification and destruction. An alternative method of ice-free cryopreservation (IFC) improved preservation of matrix structures and reduced immune cell infiltration after heart valve implantation in a sheep model. To clarify the underlying mechanisms, the objective of this study was to investigate early events in immune responses to cryopreserved matrices like macrophage polarization.

Human peripheral blood mononuclear cells were separated from buffy coats and CD14+ monocytes were isolated. Monocytes were then differentiated to macrophages for 7 days with M-CSF. In co-cultures with human and porcine CFC or IFC treated cardiovascular tissue, their polarization was analyzed by examining the surface marker expression level (e.g. CD80, HLA-DR, CD206, CD16) with flow cytometry and their cytokine release by ELISA.

In our simple *in vitro* assay, polarization controls could verify enhanced CD80 and HLA-DR expression and the induction of IL-6 and IL-10 as reliable markers for M1-macrophages. In contrast, for the M2-macrophages generated, none of these characteristics were up-regulated. Similar expression with either type of cryopreserved matrices was observed for M1-marker CD80 and M2-marker CD206 on macrophages. Interestingly, differential expression of CD16 was detected in co-cultures with CFC versus IFC tissue and IL-6 levels were reduced in IFC tissue co-culture.

IFC treatment of cardiovascular tissues partially modulates macrophage features particularly with regard to reduced tissue cytokine release and opens new vistas for tissue preservation.

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### Collagen Based Tubular Structures for Urethral Repair in a Rabbit Model

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Actual surgical procedures using existing autologous tissues for the treatment of congenital malformations or injuries of the urethra are associated with post-operative stenosis and fistula. Tissue engineered collagen tubes can be a promising alternative in this reconstructive surgery to the often hampered use of existing tissues. In our previous work we could demonstrate in a rabbit model that there is spontaneous regrowth of urothelium using high-density collagen gel tubes. We have developed a novel manufacturing procedure resulting in acellular collagen gel tubes with enhanced mechanical properties, allowing better handling of the graft. Burst pressure, stress-strain and suture resistance measurements proved better mechanical properties of these collagen tubes. The tubes were used as urethral grafts and sutured between the native prostatic and the very distal urethra following subtotal excision of more than 80% of the total urethral length. No catheter was placed postoperatively. This procedure was applied in 36 male New Zealand white rabbits. At 1, 3, 6, and 9 months the animals were macroscopically evaluated and contrast voiding cysto-urethrography was performed. After sacrifice histological examination was done. This multi-centric study revealed spontaneous urothelial coverage of the grafts and time-dependent smooth muscle cell migration could be observed in all grafts. These novel collagen based tubes are suitable for surgical reconstruction and may well become an alternative to existing treatments.

### Encapsulation and Bioprinting of Hepatocyte Aggregates in Modified Gelatin

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Upon isolation from their native microenvironment and 2D culture, hepatocytes rapidly dedifferentiate and lose liver specific functions.

Therefore, we investigated the hepatocyte performance in 3D aggregates, keeping in mind the criteria needed to make them applicable to create 3D bioprinted constructs.

A high-throughput agarose microchip system was used to generate aggregates with uniform and predictable dimensions. HepG2 cells as well as primary derived mouse hepatocytes were used. For 2D cultures, cells were plated in collagen coated wells; for 3D cultures, cells were immediately seeded in the agarose micro-wells. Micro-wells with diameters of 200 and 400 µm were used. Different cell concentrations (ranging from 75000 to 1000000) were seeded on each microchip. Cultures were performed for up to 2 weeks.

For both cell types, stable and uniform aggregates were formed within 3 days. Aggregates of different dimensions could be obtained by varying parameters such as micro-well diameter, cell number and cell type. Best results for cell viability and hepatocyte performance were obtained in aggregates with a diameter of 100 µm. For the primary cells, HNF4α expression was comparable to freshly isolated

hepatocytes. The expression of Cyp3A4 was decreased to about 50%, which is significantly less than in 2D cultures.

Encapsulation of the aggregates in a 3D printable modified gelatin (photo-curable gelatin methacrylamide) did not compromise cell viability and gene expression profiles. The modified gelatin was used to create 3D printed macroporous gelatin/aggregate constructs. Long-term co-cultures could be obtained by seeding the printed constructs with endothelial cells.

### Non-diabetic MSCs Support Diabetic Femoral Fracture Healing

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Fragility fractures and mal-union repair are a complication of diabetes mellitus resulting from impaired osteoblast and osteocyte function. It is therefore hypothesized that faulty diabetic fracture repair results from an aberrant population of host bone marrow (BM) progenitor cells (MSCs). The therapeutic efficacy of locally administered non-diabetic human MSCs to support femoral fracture repair in a murine model of diabetes was investigated.

Diabetes was induced in 10 wk old male mice, followed by femoral fracture creation. Healthy human BM-derived MSCs were locally administered directly to the fracture while control mice received saline. Fifty six days after fracture, the mice were sacrificed. Regardless of treatment group, all animals maintained their weight and elevated blood glucose levels. The onset of diabetes resulted in mal-union repair, although those groups receiving MSCs had a reduced callus volume as compared to controls. Four point flexure testing demonstrated a significant increase in flexure strength and modulus in MSC-treated fractures. Corroborating microCT analysis indicates an increase in bone volume (BV), a statistically significant increase in bone mineral density and trabecular thickness and a statistically significant decrease in the ratio of bone surface area to BV in animals treated with MSCs.

Although the administration of human MSCs did not alter the diabetic condition, treatment of the diabetic fracture with MSCs resulted in enhanced callus resorption, increased flexure strength, increased repair tissue mineral density and trabecular structure. Therefore, the administration of MSCs to diabetic fractures resulted in the creation of higher quality reparative bone.

### Label-Free Analysis of Single Hematopoietic Stem and Progenitor Cells

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Hematopoietic stem cells (HSC) are adult stem cells responsible for life-long hematopoiesis. Although they have been actively utilized in research studies and clinical settings for the past several decades, our ability to control their fate decisions *in vitro* is limited. Major challenges include limited functional assays for monitoring the time-resolved behavior of individual HSCs and hematopoietic progenitors in a label-free fashion. Current techniques require fixation, long-incubation times, or fluorescence labeling that render *in situ* analysis of single cells almost impossible. To overcome these technical hurdles, we employ Raman spectroscopy and photonic crystal enhanced microscopy (PCEM) to collect chemical and adhesive signatures of single HSCs and their progeny to establish new functional metrics for hematopoiesis. We demonstrated that Raman spectroscopy is capable of identifying primary HSCs (long-term vs. short-term repopulating), lineage-committed progenitors, and terminally differentiated cells based solely on their corresponding Raman peaks. We have also shown the use of PCEM as a label-free

platform for collecting subcellular adhesion profiles of individual cells from larger populations. Combining these analytical tools, we traced functional changes in single hematopoietic progenitor cells during granulocytic differentiation, characterizing both changes in cell differentiation state (Raman) as well as changes in adhesive and motile phenotype (PCEM) during these differentiation events. Our data suggest dual Raman/PCEM analysis is a promising alternative approach for monitoring the functional phenotype of hematopoietic cells in a label-free, time-resolved manner.

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### Extracellular Matrix Stiffness causes Systematic Variations in Proliferation and Response to Drugs against Myeloid Leukemias

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The bone marrow microenvironment contributes to persistence of myeloid leukemia cells after chemotherapy, which may lead to disease recurrence. However, it remains unclear how biophysical interactions between leukemia cells and the extracellular matrix regulate their growth and resistance to drugs. Here, we show that myeloid leukemia cells with distinct genetic mutations show differential proliferative responses to a range of pathophysiological 3D matrix mechanics relevant to the hematopoietic system. Myeloid leukemia cells generally become resistant to a number of existing drugs by matrix softening, but we found distinct subsets of targets that can be perturbed to modulate proliferation with the same potency regardless of matrix stiffness. Surprisingly, there is no correlation between how matrix stiffness regulates cell proliferation and how it modulates drug sensitivity, providing evidence against the mitotoxicity hypothesis. This approach may thus be used to design personalized drug regimens to enhance clearance of residual disease for myeloid leukemias.

### Cancer-Associated Fibroblasts Support Angiogenesis Despite VEGF Inhibition

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Recent work has demonstrated that carcinoma/cancer-associated fibroblasts (CAFs) are important regulators of the tumor microenvironment, secreting factors, including vascular endothelial growth factor (VEGF), which control tumor progression. We hypothesized that these cells also contribute biomechanical strain to the matrix surrounding tumors to promote angiogenesis. Our lab has developed a unique 3D *in vitro* model of vascularized tumor tissue that will permit elucidation of CAF-mediated biomechanical factors that influence angiogenesis. Fibrin gels were synthesized containing endothelial cells derived from cord blood and either CAFs or normal breast fibroblasts (NBFs). After 7d, samples containing CAFs demonstrated significantly more vascularization compared to NBFs ( $0.0103 \pm 0.0008$  vs.  $0.0018 \pm 0.0002$  total vessel length per area ( $\mu\text{m}^{-1}$ ),  $p < 0.001$ ). Furthermore, results indicate that CAF-conditioned media does not fully rescue angiogenesis in NBF samples ( $0.005 \pm 0.0007$  total vessel length per area ( $\mu\text{m}^{-1}$ ),  $p < 0.001$ ). Secreted VEGF was significantly increased in CAF specimens compared to NBFs ( $506 \pm 18$  vs.  $1021 \pm 61$  pg/mL,  $p < 0.001$ ). However, when a VEGFR inhibitor was given to gels containing ECs/CAFs, vessel formation was not completely suppressed and was higher than NBF samples. A series of contraction studies demonstrate that CAFs induce larger deformations in fibrin gels compared to normal cells ( $3.3 \pm 0.7 \mu\text{m}$  vs  $4.3 \pm 0.6 \mu\text{m}$ ). These data demonstrate that CAF-mediate angiogenesis in the tumor microenvironment is not wholly dependent on VEGF signaling and may be, in part, due to increases in biomechanical strains generated by CAFs. Future work will determine the molecular mechanism by which CAF-associated biomechanical induces angiogenesis to improve our understanding of the tumor microenvironment.

### Reconstruction of Gut Muscle Layer: Mesoangioblasts' Delivery Optimization in Decellularised Scaffolds

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In the field of bioengineered gastrointestinal conduits, smooth muscle cell delivery into synthetic polymers or biologic scaffolds remains limited to a surface seeding approach. In this study, we optimized cell delivery and engraftment within an oesophageal acellular matrix to develop a functional smooth muscle layer for gut regenerative medicine. A comparison of different cell seeding techniques was performed to obtain uniform cell distribution and colonization of the natural scaffold. Human mesoangioblasts were seeded into decellularised rat oesophagi using a variety of methods.

Cells delivered through luminal flow attached to the internal side of the scaffold, but showed limited migration in underlying layers. Cells delivered by surface cell-seeding remained on the external surface of the scaffold however optimised cell delivery demonstrated comparable results in terms of cell engraftment and proliferation with the addition of engraftment inside the muscle layer of the decellularized matrix. After nine days of culture, scaffolds with optimised cell delivery showed cells spreading from the delivery site along all the thickness, displaying homogeneous distribution. In addition, engrafted cells differentiated towards smooth muscle fate (confirmed by SM22 expression) also maintaining a pool of proliferating precursor cells (Ki67+ cells).

In conclusion, we have demonstrated optimised delivery of cells within the muscle layer of a decellularized oesophagus as a successful seeding method for tissue re-colonization. This finding is a pivotal step in the regeneration of a functional muscle gut layer.

### Altered Gene Expression after Ventricular Stabilization with a Decellularized Ecm-based Customized Scaffold in a Rodent Lad-ligation Model

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**Introduction:** Tissue engineered scaffolds are widely used to support damaged myocardium in pre-clinical *in vivo* models. Here, we evaluated the gene-expression of ventricular samples with or without ventricular stabilization by a decellularized ECM-based customized scaffold (cdECM-scaffold) after myocardial infarction (MI) in a standardized rodent LAD-ligation model.

**Methods:** MI was induced by standard LAD-ligation in male Wistar rats. After 14 days MI was echocardiographically confirmed (FAS < 40%) and cdECM-scaffold based on cardiac-ECM from donor rats was epicardially implanted for ventricular stabilization. After 4 weeks hearts were explanted and assessed by standard histology (HE, Movat, Masson Trichrom). Gene expression of anterior left ventricular wall tissue samples was analyzed by quantitative real-time PCR for immunomodulatory (IL 10, Tgfb2, TNF $\alpha$ ), pro-angiogenic (Vegfa, FGF2, Pgf, Pdgfb), pro-survival (hgf, SDF1, IGF1, Akt1), remodeling (Timp1, MMP2, MMP9) and infarction-specific (Nppa, Nppb) markers.

**Results:** Histology showed integration of the cdECM-scaffolds into the myocardial scar with high degree of cellular infiltration. Further, ventricular stabilization via cdECM-scaffold significantly reversed the MI-induced expression pattern of Nppa/Nppb ( $0.37 \pm 0.29$  vs.  $0.71 \pm 0.45$ ;  $0.5 \pm 0.27$  vs.  $0.9 \pm 0.33$ ;  $p < 0.05$ ) as well as of TNF $\alpha$  and Tgfb2 ( $0.96 \pm 0.41$  vs.  $1.36 \pm 0.47$ ;  $0.67 \pm 0.18$  vs.  $0.88 \pm 0.16$ ;  $p < 0.05$ ) while showing a clear trend towards decreased gene expression of remodeling factors and increased expression of

pro-survival factors as compared to the control group (n=7, respectively).

**Conclusion:** Ventricular stabilization after MI via a cdECM-scaffold showed biological integration leading to an altered gene expression pattern, possibly reducing chronic deteriorating effects on infarcted myocardium such as tissue inflammation and remodeling while increasing potential for myocardial regeneration.

#### A Small-diameter Decellularized Vascular Graft Covered with Electrospun Fibers

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For cardiovascular disease treatment, it is expected to develop small-diameter vascular grafts. In this study, a decellularized aorta was fabricated to make a small-diameter vascular graft having long length. The intima-media of aorta was peeled and decellularized using high hydrostatic pressure (HHP) method. The HHP decellularized intima-media was formed cylindrically and covered with segmented polyurethane (SPU) by using our original electrospinning apparatus. The small-diameter decellularized vascular graft covered with SPU fibers was obtained. For SEM observation of the obtained decellularized vascular graft with SPU fibers, the inner layer was decellularized intima-media and the outer layer was SPU fibers. The SPU fibers layer shows a porous 3-dimension structure. The SPU fibers were covered on the intima-media surface with the homogeneous thickness. The thickness of SPU fibers layer was controlled by electrospinning time and increased with prolonging time. The physical properties, such as rupture pressure, stiffness and vasodilation of the small diameter decellularized vascular grafts covered with SPU fibers were similar to that of native blood vessels by controlling the outer layer of SPU fibers.

#### A Novel Endoscopic Device for Transplantation of Tissue-engineered Cell Sheets

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Esophageal stricture is one of the major problem associated with an extensive endoscopic submucosal dissection (ESD) for superficial esophageal neoplasms. In our previous clinical study, tissue-engineered epithelial cell sheets derived the patient's own oral mucosal epithelial cells by using a temperature responsive culture dish were transplanted onto the ulcer surface following ESD in 10 patients (1). The result of this study, autologous epithelial cell sheets promoted early re-epithelialization and derived safety outcome. However, their regenerative approaches using the tissue engineering like the cell sheet had several problems. One of the crucial problem was that the cell sheet transplantation technique was not easy procedure without the dedicated device, how to transport and transplant properly in such the intestinal lumen.

Encouraged by this result and challenge, we developed the endoscopic device by 3D printer for the cell sheet transplantation and transplanted autologous epithelial cell sheets with this combination device endoscopically in a porcine.

**Results:** 2-3 pieces of the epidermal cell sheets (20mm in diameter) were safely and properly transplanted onto the ulcer site after esophageal ESD.

**Conclusion:** a novel endoscopic device would enable easily transplantation of cell sheets in the lumen of the esophagus.

(1) Ohki T, Yamato M, Ota M, *et al.* Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. *Gastroenterology*. 2012.

#### Endochondrally Priming of Human MSCs *In Vitro* Enhances their Mineralisation Potential without the Addition of Osteogenic Growth Factors

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In this study, we test the hypothesis that an *in vitro* bone regeneration strategy that mimics the endochondral ossification process, specifically the formation of the cartilage template and subsequent vascularisation of that template, will enhance the mineralisation potential of human MSCs without the use of osteogenic growth factors.

Human MSC aggregates (250,000 cells) were created by centrifugation for 5 mins at 400 g. These aggregates were primed with chondrogenic media for 21 days, to produce a cartilaginous aggregate, after which they were co-cultured with MSCs and Human Umbilical Vein Endothelial cells (HUVECs) in endothelial growth medium for another 21 days to allow for vascularisation. Biochemical assays (DNA, Alkaline Phosphatase (ALP), Calcium, Vessel Endothelial Growth Factor (VEGF)) and histological staining (Alcian Blue, Alizarin Red and CD31 +) were performed after 3 weeks of co-culture.

The results of this study show that three weeks after the addition of both HUVECs and human MSCs to an already formed cartilage template there was significantly higher ALP, Calcium and VEGF than the non-vascularised groups. Moreover, there was significantly higher ALP and VEGF, but no significant difference in calcium, compared the same group cultured in osteogenic media. Most interestingly, the formation of rudimentary vessels (detected by CD31 + staining) was observed in all the prevascularised aggregates. Taken together, this study suggests that the application of both chondrogenic and vascular priming of human MSCs can obviate the need for osteogenic growth factors to induce osteogenesis by human MSCs, whilst allowing for the formation of rudimentary vessels *in vitro*.

#### Construction and Transplantation of a Tissue Engineered Corneal Graft by Combination Cell 3d Printing and Compressed Hydrogel Scaffold

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Corneal transplantation is a major part of the strategies currently used to correct corneal blindness. The construction of a tissue engineered cornea for use in the replacement of dysfunctional corneal tissue would resolve the problem of the shortage of corneal graft material. At this study, first the keratocytes were mixed with collagen/gelatin/alginate, through the 3D printer (based on the principle of rapid prototyping), the mixed biomaterials was printed layer by layer to construct the 3D hydrogel scaffolds. Second, through the controllable compression technology (mechanical compression combined with capillary principle) to compress the 3D hydrogel and used the low concentration of calcium chloride to crosslink the compressed scaffold. Then, seeded the isolated corneal epithelial stem cells on scaffold and used cytokines to promote the cells to adhere, proliferate and stratify on the scaffold. Finally, the constructed corneal lamellar was transplanted to reconstruct the damaged cornea. This study provides the first line of evidence that the keratocytes printed scaffold can adequately support limbal epithelial cell expansion, stratification, and differentiation and have potential application in tissue engineering cornea.

#### Alteration of Electrospun Scaffold Properties by Silver Nanoparticle Incorporation: Evaluation for Blood Vessel Tissue Engineering

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Biodegradable electrospun matrices provide cellular homing sites that closely mimic native extracellular matrix (ECM) environment present in tissues. The feasibility of incorporating silver nanoparticles (SNP) into poly( $\epsilon$ -caprolactone) (PCL) scaffolds for imparting antimicrobial activity and their effect upon scaffold properties meant for blood vessel tissue engineering application is investigated. PEG protected SNP solution (0.1%) was incorporated into PCL solution which was electrospun into fine fibres. Uniform distribution of SNPs was confirmed within electrospun matrix and about ~31% release was observed within five days immersion into media in a sustained manner. Shift in fiber diameter to lower range was noticed which resulted in narrowing of pore size distribution. Micro CT examination showed bare PCL scaffolds to possess 92% pores in 12–60  $\mu$ m range whereas PCL-SNP scaffolds showed 98% pores in 12–36  $\mu$ m size. Crystalline behaviour of scaffolds was found to improve after SNP addition. Effect on suture retention strength, burst strength and tensile properties were also investigated. Scaffolds were found to be cytocompatible and PCL-SNP mats showed uniform endothelial cell coverage with well spread actin filaments after 5 days of culture compared to bare scaffold. These findings could also be extended to the generation of bi-layered scaffold with PCL-SNP as luminal layer and bare PCL scaffold as abluminal layer where uniform endothelial coverage, antimicrobial activity and required mechanical properties can be combined for blood vessel tissue engineering.

#### Contractile Force Measurement of Human iPS Cell-Derived Myocardial Sheet

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We have developed temperature-responsive cell culture surfaces on which cells can adhere at 37 °C but cannot adhere below 32 °C. When cells are cultured and reach confluence on this surface, we can harvest a cell sheet from the surface by decreasing temperature. Utilizing this technology, in the present study, we created human iPS cell-derived myocardial sheets and investigated their contractile properties. We used a human iPS cell line in which a cardiac  $\alpha$ -myosin heavy chain promoter-driven puromycin resistance gene was introduced. Cardiac differentiation was induced by a bioreactor-based method. The differentiated cardiomyocytes were enriched by puromycin selection and plated onto the temperature-responsive surfaces. When the cells reached confluence, a fibrin gel sheet was put on the cells and temperature was decreased to 20 °C, which allowed the myocardial sheet to transfer from the temperature-responsive surface to the fibrin gel sheet. The sample was fixed to a force-measuring device and its contractile force was measured successfully. In conclusion, the present method is useful for the evaluation of myocardial sheet contractility and for *in vitro* drug testing.

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**Disclosures:** Teruo Okano, Ph.D. is a founder and a member of the board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University.

#### Cardiac Patch Design with Neural Growth Factor Impregnated Polyurethane Nanofibers and Mesenchymal Stem Cells

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Nanofibrous scaffolds have recently draw attention for potential applications in cardiac tissue engineering. Recently, it has been reported that neural growth factor (NGF) stimulates the angiogenic activity and supports the viability of cardiomyocytes in acute myocardial ischemia. In this study, in order to create cardiac patch, biocompatible polyurethane (PU) nanofiber polymer was developed by means of electrospinning method. NGF was impregnated on this material and cardiomyocytes differentiated from human bone marrow mesenchymal stem cells (BM MSCs) were performed for seven days. Isolated MSCs homogeneity was determined by flow cytometry using specific markers (PE-anti-CD90, FITC-anti-CD73, anti-CD105-APC). Cardiomyogenic differentiation was performed by 5-azacytidine and differentiation was tested with Troponin by immunofluorescence staining. BM MSCs and cardiomyocyte survival/proliferation were evaluated with WST-1 kit on the 3th and 7th days. As a result, the proliferation capacity of MSCs on PU was higher than non-coated surfaces (% 70.6  $\pm$  1.0 vs. 60.6  $\pm$  2.0,  $p=0.02$ ) at day-3. This result demonstrated that PU was biocompatible and supported stem cell proliferation. However, cardiomyocyte differentiation on PU nanofibers changed depended on NGF. There was a slight increase in cardiomyocyte proliferation on NGF impregnated PU nanofiber compared to PU alone surfaces (71.1  $\pm$  2.4 vs. 60.0  $\pm$  8.4,  $p=0.2$ ) at day-7. NGF's effect on cardiomyocyte proliferation was investigated with  $\beta$ -adrenergic signalling pathways by using anti-p70 S6 Kinase MAb. Flow cytometry analysis confirmed that NGF activated  $\beta$ -adrenergic signalling pathways via p70 S6 kinase in cardiomyocytes (expression rate; NGF (+) %1.8 vs. NGF (-) % 0). This work was supported by Hacettepe University, Ankara, Turkey (Project Number: 013D11102001).

#### Long-term Sustained Delivery of Zinc-doped Biomimetic System Improves Osteoporosis Condition

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The development of controlled drug delivery system based on marine-derived materials and structure has long shown potentials in bone tissue engineering. In this study, calcareous exoskeleton (foraminifera) was converted to beta-tricalcium phosphate along with zinc ions and implanted intramuscularly in osteoporotic conditioned rats for three months. The aim of this study is to determine if the combination release of zinc, calcium and phosphate from the delivery system can prevent bone loss at the local implant site and the overall systemic bone recovery effect.

Micro-computed tomography analysis showed improvements of bone regeneration at the local implant site with increase in bone mineral content and bone mineral density. When examining the overall systemic recovery of bone it was shown that bone mineral content was statistically higher in the +zinc group compared with controls while bone mineral density was maintained. Mechanical loading of excised bones also showed increase bone mechanical strength in the +zinc group. Corresponding *in vitro* assays showed +zinc enhanced bone marrow stromal cells numbers, proliferation activities isolated from osteoporotic rats. Furthermore, gene expression profiles showed increase levels of BMP-2, TGF- $\beta$  and VEGF, which correlate the *in vivo* results.

These results suggest that zinc doped biomimetic carriers do have beneficial effect on osteoporosis treatment but the complexity of the disease would require a more prolonged treatment possibly in combination with other bioactive compounds.

#### Keratin-Alginate Sponges for Tissue Engineering

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Hair keratin has shown good potential as an alternative functional biomaterial for tissue engineering applications. However, using keratin alone resulted in matrices with poor mechanical properties. In our recent study, we investigated the feasibility of producing keratin-alginate composite sponges and explored the suitability of using these as 3D cell carriers. Hair keratin was extracted from human hair in reducing conditions using established techniques. This was crosslinked with alginate using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to produce peptide linkages. The crosslinked mixture was cast and freeze-dried to produce 3D sponges. These sponges were extensively characterized for various physical, chemical and mechanical properties. Crosslinking was confirmed using Fourier Transformed Infrared Spectroscopy and biochemical assays. The tensile, compressive and flexural moduli of the sponges increased with crosslinking degree. Water vapour transmission rate across the crosslinked keratin-alginate sponges were found to be comparable with commercially available foam dressings. The keratin-alginate sponges were able to support the attachment and growth of mouse fibroblasts, such that increasing keratin content resulted in more significant cell proliferation. Cytokine secretion by fibroblasts was also supported in these matrices. In summary, we have produced a novel keratin-alginate composite sponge and showed that these have desirable properties for tissue engineering applications. Given that there are currently no other human based material that can be used on the scale that hair keratin offers, this approach could find clinically relevant applications in the future.

#### E-Valve Development of a Tissue-Engineered Elastin-Based Heart Valve Prosthesis

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Cell-based tissue-engineered heart valves (TEHVs) have been produced with a variety of materials and fabrication techniques, commonly resulting in *in-vitro* production of abundant collagen and scarce, if any, elastin. Elastin is essential for the long-term functionality of the valve as it is responsible for its cyclic recoil. Here we propose the use of elastin-like recombinamers (ELRs) for HV tissue engineering. ELRs are genetically engineered polymers consisting of the repetition of pentaaminoacid sequences typical of the human elastin, with the possibility of adding desired (bio)molecules. Specifically, we employed ELRs chemically modified with cyclooctine and azine groups to obtain gels by catalyst-free click chemistry under cell-friendly conditions. The HV scaffold was fabricated by injection molding technique in the presence of smooth muscle cells isolated from the human umbilical cord vein and was conditioned dynamically for 21 days in a bioreactor. The cells were homogeneously distributed within the ELR matrix and were able to produce abundant and aligned collagen as revealed by immunohistochemistry. The valve showed complete closure and unobstructed opening with no appreciable changes of its 3D geometry during *in-vitro* remodeling as confirmed by ultrasound imaging. The ease of fabrication and the tunability of the polymerization process allow us to mold valves with different designs and porosity. In this way, cell-free scaffolds can be infiltrated by cells, showing the potential of these materials for *in-situ* tissue engineering. To this end, the non-thrombogenicity of ELRs and their tunable mechanical properties are crucial aspects.

#### Endogenous BMP Activity is Necessary for Bone Marrow-Derived MSC Osteogenesis

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Bone marrow-derived MSCs undergo *in vitro* osteogenesis in media supplemented with dexamethasone, ascorbic acid and beta-glycerophosphate, without the need for exogenous bone morphoge-

netic protein (BMP) stimulation. This study was carried out to determine whether endogenous BMP secretion is necessary for BM-MSC osteogenesis.

Bone marrow aspirates were collected from healthy adult horses, then were cultured as monolayers through two passages to enrich for MSCs. The expanded cells were re-seeded in basal medium or osteogenic medium for up to 10 days. The effect of osteogenic medium on BMP-2, -4, -6 and -7 mRNA levels was determined by QPCR. Endogenous BMP activity was inhibited by co-administering BMP signaling antagonists Noggin (50 and 500 ng/ml), K0288 and DMH-1 (10, 100 and 500 nM). Osteogenesis was monitored on days 3, 7 and 10 by staining monolayers with alizarin red, measuring calcium levels and alkaline phosphatase (ALP) activities, and assessing Runx2 and Osterix (OSX) mRNA expression by QPCR.

BMP-4 transcript levels were increased over the first 7 days in culture. BMPs-2, 6 and 7 mRNA levels were unchanged. All three BMP inhibitors reduced Alizarin Red staining. Calcium deposition and ALP activity were also dose-dependently reduced by all three BMP inhibitors. Runx2 mRNA up-regulation was not affected by BMP inhibitors but OSX expression was significantly suppressed.

The significant inhibition of osteogenesis by all three BMP signaling inhibitors indicates that endogenous BMP activity is necessary for BM-MSC osteogenesis. OSX expression was significantly and dose-dependently inhibited by all three inhibitors, suggesting that OSX mediates endogenous BMP signaling during osteogenesis.

#### Biomaterials of Biological Origin in Tissue Engineering: an Overview

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The present work intends to overview a wide range of biomaterials of biological origin that are being used in research with applications in the tissue engineering and regenerative medicine. Biologically derived biomaterials have been demonstrated to show several advantages compared to synthetic biomaterials [1]. These are biocompatibility, biodegradability and remodeling. In addition, biomaterials of biological origin are very attractive due to their similarities with the extracellular matrix (ECM), chemical versatility as well as typically good biological performance [1]. Therefore, these biomaterials could be applied in the repair or replacement of damaged human tissues and organs. The use of biomaterials of biological origin is currently undergoing a renaissance in the biomedical field [2]. Biomaterials of biological origin can be classified into many groups including protein-based biomaterials (collagen, gelatin, silk), polysaccharide-based biomaterials (cellulose, chitin/chitosan), decellularized tissue-derived biomaterials (decellularized heart valves, blood vessels, liver) and biomaterials of marine origin (biocoral, marine sponge). Three main areas of applications of biomaterials of biological origin as materials in medicine were identified: wound management products, drug delivery systems, and tissue engineering [2]. The various types of natural products and forms of biomaterials of biological origin are highlighted. This work presents a brief history of natural products as biomaterials, various types of natural biomaterials, properties, demand and economic importance, and the area of application of biomaterials of biological origin in recent times.

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#### A High-Throughput Screening Platform for Mesenchymal Stem Cells-Supported Expansion of Hematopoietic Stem Cells

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**Introduction:** Hematopoietic Stem Cells (HSC) *ex-vivo* expansion is a promising strategy that could revolutionize Umbilical Cord Blood (UCB)-derived HSC transplantation. However, this has not been



clinically implemented due to lack of a protocol that efficaciously multiplies the cells while preserving their stemness. The usefulness of high-throughput screening (HTS) systems to identify optimal expansion conditions is indisputable. Here we propose to develop a HTS platform capable of incorporating bone-marrow niche elements and screen for compounds that expand HSC while maintaining their stemness.

**Methods:** Wharton's Jelly-Mesenchymal Stem Cells (WJ-MSC) were co-cultured with KG1a cells (CD34+ leukemia cell line). Number of cells in control (without WJ-MSC) and co-culture wells were assessed after 5 days using anti CD34-PE. Images were acquired with a high content image microscope and analyzed with an intensity based segmentation method using the IN Cell Investigator software.

**Results:** WJ-MSC enhanced proliferation of KG1a cells by 5-fold compared to control conditions, confirming the beneficial effect of an WJ-MSC feeder layer on expansion of CD34+ expressing cells.

**Future work:** development of an HTS system using WJ-MSC as feeder layer for UCB-derived CD34+HSC to screen for small molecules that can enhance HSC expansion.

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## Extracellular Matrix Sheets Enhanced the Properties of Transplantable Cardiomyocytes

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Tissue-engineered cardiac biomaterials are among the major targets of tissue engineering strategies due to the persistent high incidence of cardiovascular diseases as one of the worldwide major causes of mortality. Although various approaches to promote post-infarction cardiac regeneration have been developed, a number of limitations remain to be overcome prior to their clinical applications. Many biosynthetic biomaterials have exhibited limitations for cardiac regeneration. For these reasons, development of natural biomaterials to support regeneration processes in the myocardium is vital. The extracellular matrix (ECM) components are generally functional for mechanical support as well as for the induction and preservation of the suitable cellular phenotypic and physiological characteristics. Hence, this study was purposively designed to investigate the effects of natural cardiac ECM sheet to the proliferation rate and phenotype maintenance of cardiomyocytes. We generated the ECM sheets from decellularized cardiac sections as a novel biomaterial for expanding cardiomyocytes. The cardiomyocytes were cultured with or without these ECM sheets. We employed automatic cell counting and MTT assay, microscopy, RT-PCR, Real-time PCR, and western blot assay to assess the proliferation rates, phenotypes, and cardiac gene and protein expressions, respectively. Cardiomyocytes, expanded under cardiac ECM sheets, exhibited higher proliferation rates, greater cardiac gene and protein expressions, and maintained cardiac phenotypes than those cells were grown in the absence of natural cardiac ECM sheets. In conclusion, we demonstrated that the cardiac sheets can be novel *in vitro* models for investigating cell and ECM interactions and consequently provide significant foundation for further advancement of cardiac transplantation strategies.

## Generation of an Engineered, Vascularized, iPSC-derived Hepatic Module to Mitigate Genetic Disorders

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The lack of donor livers for transplantation has prompted studies to generate autologous tissue-engineered liver constructs. We developed an engineered hepatic module containing hepatocytes and adipose-derived stromal vascular fraction cells (SVF), and are evaluating the use of this module to functionally mitigate the genetic defect found in the heritable disease Familial Hypercholesterolemia (FH; characterized by low-density lipoprotein cholesterol receptor (LDLR) deficiency. We reprogrammed FH patient fibroblasts into iPSC (FH-iPSC) via transient transfection with synthetic mRNA and subsequently verified iPSC pluripotency. FH-iPSC were then electroporated with a 31 kb episomal *LDLR*-plasmid (forming FH-iPSC-LDLR) containing genomic regulatory sequences (conferring physiological cholesterol sensitivity), an *EBNA1* nuclear-targeting sequence with *OriP*, and a hygromycin-resistance cassette. FH-iPSC +/- LDLR were subsequently differentiated into hepatoblasts, which expressed AFP and Albumin, stored lipids, and metabolized Cardiogreen. To validate the functional restoration of the LDLR, hepatoblasts and FH-iPSC-derived mesenchymal cells (FH-MC +/- LDLR; used to facilitate quantification) were starved overnight in lipoprotein-deficient serum with excess sterols, Lovastatin, or ethanol (control). DiI-LDL excess unlabeled LDL was administered for 5 h the following day. With Lovastatin, FH-MC-LDLR internalized ~2-fold more DiI-LDL than FH-MC. When FH-HB +/- SVF modules were implanted in immunodeficient mice for 2 w, the modules exhibited increased albumin synthesis compared to modules containing only FH-HB, suggesting that SVF is vital to hepatoblast survival and engraftment. A module combining functional iPSC-derived cells with SVF could serve as a therapeutic system for treating FH as well as a platform for treating other genetic disorders.

## Tissue Engineering of Heart Valves: Valvular or Pericardial Matrix?

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To date, an optimal decellularization of heart valve leaflets (HVL) and pericardia (PER) with an adequate preservation of the extracellular matrix (ECM) is still lacking. In this study the efficacy of two detergent-based decellularization methods and their effect on ECM components were evaluated. In addition, decellularized HVL and PER were compared for their use in heart valve tissue engineering.

Porcine tissues were decellularized using different concentrations of sodium deoxycholate (SDC) or Triton X-100. Decellularized and non-treated tissues were processed for histological, biochemical and mechanical analysis to evaluate the effect of the decellularization agents on the structural components of the ECM.

The SDC treated tissues still showed cell remnants, whereas tissues treated with Triton X-100 were completely cell free. For both decellularized tissues, an almost complete washout of glycosaminoglycans and a reduction of collagen was observed. Interestingly, only the elastic fibers of PER were affected and this tissue tended to rupture faster. In this study, it is shown that Triton X-100 and trypsin as well as SDC altered the surface ultrastructure of decellularized porcine tissues, resulting in an irregular mesh of ECM fibers with small or large pore sizes.

It is clear that Triton X-100 is superior to SDC to generate complete cell free bioscaffolds from porcine cardiac tissues. Our study clearly demonstrated that the decellularization agents have more negative impact on PER than on HVL. Thus, for the purpose of tissue engineering of heart valves, it is advisable to use valvular rather than pericardial matrices.

**Vasculogenic Dynamics in 3D Engineered Tissue Constructs**Y. Blinder<sup>1,2</sup>, N. Raindel<sup>1</sup>, D. J. Mooney<sup>3,2</sup>, S. Levenberg<sup>1</sup>;<sup>1</sup>Bio-Medical Engineering, Technion - Israel Institute of Technology, Haifa, ISRAEL, <sup>2</sup>Wyss Institute for Biologically Inspired Engineering, Cambridge, MA, <sup>3</sup>School of Engineer and Applied Sciences, Harvard University, Cambridge, MA.

Vascularization of implantable 3D engineered tissue constructs can be achieved by co-culturing endothelial and fibroblast cells on macroporous scaffolds. Here we show a novel use of these constructs as in-vitro assays for studying the dynamics of neovascular formation, using a combination of live confocal imaging and an array of image processing and analysis tools. We show this neovascularization to be a multi-stage process involving both vasculogenic and angiogenic elements, including an initial endothelial multicellular cluster formation followed by rapid extensive sprouting, ultimately resulting in a stable interconnected endothelial network morphology. An extensive extra-cellular matrix environment is shown to be deposited throughout the constructs in a time-correlated manner with vascular morphogenesis. This approach further reveals that vascular network nodes are formed by two separate morphogenic mechanisms of anastomosis and cluster thinning. Finally, we show a proof-of-concept for this approach as a possible drug screening method, by studying the effect of an anti-angiogenic agent on vascular morphology. This novel approach provides new and important abilities to researchers in any field in which vascular morphogenesis is relevant, including but not limited to regenerative medicine, cancer, diabetes and general wound healing research.

**Electrospun Poly(caprolactone)/Poly(lactide-co-trimethylene carbonate) Composite Tubes for Small Diameter Vessel Regeneration**I. Stefani<sup>1,2</sup>, M. A. Asnaghi<sup>1,3</sup>, S. Mantero<sup>1</sup>, J. J. Cooper-White<sup>2,4</sup>;<sup>1</sup>Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta", Politecnico di Milano, Milano, ITALY, <sup>2</sup>Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, AUSTRALIA, <sup>3</sup>Department of Biomedicine, University Hospital Basel, Basel, SWITZERLAND, <sup>4</sup>Biomedical Manufacturing, Manufacturing Flagship, CSIRO, Clayton, AUSTRALIA.

Autologous grafts are commonly used for replacement of damaged small diameter hollow tissues, such as blood vessels and urethra. However, such approaches are hampered by scarce availability of suitable graft tissue and poor long-term stability. In an attempt to address these limitations, we developed an innovative methodology to co-electrospin mixtures of Poly(caprolactone) (PCL) and Poly(lactide-co-trimethylene-carbonate) (PLA-TMC) to create nano/microporous tubular scaffolds of tunable internal diameter and wall thickness. The produced matrices were chemically (GPC, Raman spectroscopy, AFM) and mechanically characterized (Burst Pressure =  $3425 \pm 354$  mmHg, Suture retention strength =  $5.6 \pm 0.9$  N), and demonstrated properties comparable to those of native vessels. A validated multifunctional dual-phase (air/aqueous) bioreactor, designed to both rotate and perfuse tubular scaffolds, was utilised to biologically assess the electrospun matrix. By combining the bioreactor system with the PCL/PLA-TMC scaffolds, a suitable 3D environment for human mesenchymal stem cell (hMSC) growth and differentiation was achieved. By facilitating nutrient supply/catabolites removal and enhancing medium flow through the scaffold wall, cell migration and colonization throughout the wall thickness were favoured (electron microscopy). Moreover, histological analyses showed how the dual-phase culture improved extracellular matrix deposition throughout the porous structure, crucial for the formation of a functional neo-tunica media. Finally, the correct commitment of cultured hMSC was confirmed by Lillie allocrome staining. The combination of our novel matrix and dynamic culture system created an environment that promoted hMSC growth and differentiation into applicable cell types to generate a functional vessel, a concrete step towards our goal of creating a mature vascular tissue for implantation.

**Characterizing the Role of Dextran in the Decellularization of Porcine Corneas**A. P. Lynch<sup>1,2</sup>, S. L. Wilson<sup>3</sup>, M. Ahearne<sup>1,2</sup>;<sup>1</sup>Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, IRELAND, <sup>2</sup>Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin, IRELAND, <sup>3</sup>Academic Ophthalmology, Division of Clinical Neuroscience, Queen's Medical Centre Campus, University of Nottingham, Nottingham, UNITED KINGDOM.

In recent years, corneal decellularization has emerged as a promising alternative to traditional tissue-engineered methods to create a corneal replacement for transplantation. One of the significant problems arising from the decellularization of porcine corneas is that of corneal swelling, limiting its potential use as a scaffold for corneal tissue engineering. Here, we propose the use of the complex polysaccharide dextran during decellularization to combat this undesirable effect. To achieve this, corneas were treated with detergents and nucleases under constant rotation followed by a washing step. A dextran solution was added to one group throughout the decellularization process and to a second group during the washing cycle. A final group consisted of corneas decellularized by several freeze/thaw cycles. The resultant scaffolds were extensively characterized by histological and biochemical analyses in addition to examining the ultrastructure of the cornea by transmission electron microscopy (TEM). Results revealed that a combination of detergents and nucleases effectively removed the majority of cellular material from the cornea. The addition of dextran prevented significant swelling when used throughout the protocol or during the washing process alone. A degree of transparency was restored to all groups by placing the scaffolds in glycerol, suggesting maintenance of the extracellular matrix. However, TEM analysis revealed that dextran must be present throughout the decellularization process to preserve the native ultrastructure of the cornea. This data suggests that dextran could be a useful addition for the decellularization of porcine corneas for corneal tissue engineering.

**Autophagy is Essential During the Differentiation of Adipose Derived Stem Cells to Functional Smooth Muscle Cells for use in Tissue Engineering**

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Tissue engineering using smooth muscle cells may provide a treatment option for diseases with smooth muscle pathology such as bladder dysfunction, urinary incontinence, and erectile dysfunction. As autologous smooth muscle cells (SMC) should not be harvested from organs with end-stage disease there is a need for other cell sources. Adipose derived stem cells (ADSC) can be easily harvested and differentiated into SM tissue. We have shown that autophagy, a conserved lysosomal degradation pathway, is required for cell survival and differentiation of stem cells. ADSC undergoing differentiation to SMC efficiently remodel their cytoskeleton and shape in an energy-consuming process. We investigated functional role of autophagy during differentiation and remodeling of ADSCs to SMC *in vitro*. Rat ADSCs were characterized and induced towards SMC using induction medium for 1 to 6 weeks. The changes in gene and protein expression level for SMC markers: calponin, smoothelin,  $\alpha$ -SMA, MyH11; and autophagy: LC3, Atg5, Beclin1, were investigated by ICC and WB. Upon induction, up-regulation of Atg5-Atg12 and free Atg5 was observed during 4–6 weeks. This was supported by an increase in conversion of cytosolic LC3I to membrane-bound LC3II protein. At the same time the contractile proteins calponin, MyH11 and smoothelin were up regulated during 1–3 weeks and decreased after 5–6 weeks of differentiation detected by WB and ICC. Our study demonstrates that autophagy plays an important role in ADSC differentiation to SMC. This finding might lead to novel supporting strategies for ADSC use in clinics, since autophagy can be easily influenced by FDA approved drugs.

### Synergistic Effects of Combining Undifferentiated Adult Stem Cells and Differentiated Cells for the Engineering of Functional Bladder Smooth Muscle Tissue

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Tissue engineering using a combination of cells may offer an approach for functional reconstruction. Adipose derived stem cells (ADSC) are investigated as an alternative cell type to bioengineer contractile bladder tissue when differentiated to smooth muscle cells (SMC). However, it is uncertain whether these cells maintain their phenotype long term *in vivo*. It is our aim to evaluate different combinations of cells to improve the smooth muscle formation, by improving the microenvironment and cell-to-cell interactions. Rat ADSCs were characterised and differentiated to SMC (3 weeks) prior to subcutaneous injection into nude mice. Cells were injected in different combinations (ADSC, ADSC+differentiated ADSC, SMC, differentiated ADSC + SMC). Tissue formation was followed by MRI and PKH labelling. Formed tissues were analysed for gene and protein expression by RT-PCR, Western Blot and immunohistochemistry. In all experimental conditions the PKH-positive cells were detected after 4 weeks, indicating the presence and survival of engineered tissues *in vivo*. Using MRI we were able to visualize the engineered SM tissue over the study period. Tissue size differed between the experimental conditions with tissues grown from cells with 3 weeks differentiation + ADSC showing largest constructs with good correlation in histology. Differentiated ADSC combined with ADSC or SMC showed positive upregulation of smooth muscle makers calponin, smoothelin, MYH11 and  $\alpha$ SMA similar to bladder derived SMC. Our research offers key information on survival and functionality of bioengineered smooth muscle tissue grown using differentiated ADSC in combination with differentiated cells. This approach could help to engineering contractile bladder tissue for future clinical application.

### Keratin Nanofiber Scaffold for Vascular Graft

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Current artificial blood vessel made by polyester or polytetrafluoroethylene lacks endothelialization on inner surface after implantation. Keratin is the major structural protein of mammalian hair and nail. Since it can be extracted from the non-vascular tissues, it may not be necessary to consider the biological risks such as BSE. Also, it may be possible to be obtained from the patient's own tissue as safe material for tissue regeneration. In this study, the possibility of keratin nanofibers as scaffold material for vascular graft was evaluated.

Keratin protein was extracted by the deoxidization method from wool fibers and dissolved in formic acid. It was then mixed with PEG and spun to the fiber by electrospinning under 11 kV with injection speed of 0.5 mL/h. They were stacked as the sheet onto the culture slide glass and evaluated for cell adhesion and proliferation. Cultured L929 mouse fibroblasts on the fibers were counted by WST-8 assay and observed by SEM.

L929 cells were well attached and proliferated on nanofibers. Unlike cell culture on the smooth uniform surface like a film, cell growth rate on nanofibers were affected by differences in surface characteristics of fiber diameter and porosity. These results suggest that keratin nanofibers may be the suitable material as scaffold for vascular graft and other tissue regeneration.

### Modulation of Mirnas Expression in Therapy of the Human End-stage Failing Heart

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Myosin is the molecular motor of contraction. In the heart, three distinct myosin heavy chain isoforms coexist in delicate balance. As has been recently shown, the shift from one myosin isoform to other may be one of key factors causing heart failure. In this study we aimed to analyze gene expression of myosin heavy chain isoforms in failing hearts.

We examined samples from left ventricles of 40 patients with end-stage heart failure indicated for heart transplantation. We used quantitative RT-PCR to measure mRNA levels of cardiac myosin heavy chain isoforms (MYH6, MYH7, MYH7B), related transcription factors (GATA4, SRF, NKX, YY1) and microRNAs (miR-1, 133a, 208a, 208b, 499, 29b), based on bioinformatics predictions and databases.

In adult human failing hearts, we found the slow-twitch myosin heavy chain MYH7 (~98%) to be the predominantly expressed isoform whereas fast-twitch MYH6 isoform constitutes just about 1% of all myosin isoforms. This excessive expression of MYH7 is regulated by several transcription factors and microRNA, which expression is also altered. Conclusively, dysregulated gene expression of myosin heavy chains resulting in MYH7 upregulation and MYH6 downregulation might be an adaptation in heart failure and interesting target for future pharmacotherapy.

### Increased Angiogenic Potential of Human Cardiac Progenitor Cells in Growth Factor Sequestering Hyaluronic Acid Hydrogels

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Strategies to increase angiogenesis are at the forefront of tissue engineering and regenerative medicine. Progenitor cells can play a key role in neovascularization of ischemic tissue. However, low survival and poor cell engraftment following implantation limits the potential of progenitor cells to treat ischemic diseases. To overcome these limitations we have developed a family of hyaluronic acid (HyA)-based hydrogels with the ability to retain both endogenous and exogenously added growth factors through the interaction with covalently-linked heparin in the hydrogel network 1. In this work, we assess the ability of these hydrogels to promote an angiogenic response by encapsulating human cardiac progenitor cells (hCPCs) in the presence of exogenously added transforming growth factor beta-1 (TGF- $\beta$ 1). Subsequent to encapsulation of hCPCs within the hydrogel, cell viability, proliferation and vascular-like tube formation were assessed. Encapsulated hCPCs proliferate over time, with enhanced formation of vascular-like networks in the presence of TGF- $\beta$ 1. These results emphasize that TGF- $\beta$ 1 in HyA hydrogel is essential to stimulate the angiogenic response in a CD105-dependent manner. Further detailed analysis of the secretion of a range of angiogenic factors by hCPCs within the hydrogel is ongoing, with the intention of testing the efficacy of the hCPC/HyA hydrogel system in an *in vivo* ischemic model to assess functional angiogenesis.

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### Evaluation of the Effect of Bone Differentiation of Bone Marrow Stem Cells on DC/DBP Sponges

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Biomaterials for bone tissue engineering with high bioactivity and biocompatibility are highly required. Collagen is known to be the most promising materials and have been found diverse application in bone tissue engineering due to their excellent biocompatibility and biodegradability. Duck's feet derived collagen is excellent the adhesion ability of cells and collagen extraction process takes short time. However the duck's feet collagen (DC) has the low mechanical properties. To solve this problem, we mixed with the demineralized bone particle (DBP). DBP is a natural bioactive material which powerfully induces of new bone growth. DBP is composed of collagen, calcium and proteoglycan. In this study, we fabricated natural biomaterial sponges used to make DC/DBP composite powder with different ratio; 3:1, 1:1 and 1:3 DC/DBP. Bone marrow stem cells (BMSc) were seed on DC/DBP sponges and confirmed the effects of adhesion and proliferation. And, we confirmed cell attachment, proliferation and osteogenesis by the SEM, MTT and ALP assay. RT-PCR was performed to confirm the bone differentiation specific genetic marker. To confirm bone forming *in vivo* environment on DC/DBP sponges by histological staining. DC/DBP sponges showed the effect of bone differentiation *in vivo* from H&E staining and von Kossa staining. This results suggest that DC/DBP which natural material, provide suitable environment to BMSc and proper content affected on culture condition to improve bone differentiation. This research was supported by the Brain Korea 21 PLUS Project, NRF and Technology Commercialization Support Program (KMIFAFF 814005-03-1-HD020).

### Human Mesenchymal Stromal Cells for Cell Based Therapy of Stress Urinary Incontinence

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Many people in Western countries (>10%) suffer from stress urinary incontinence (SUI). Loss of muscle cells is associated with SUI. Pre-clinical studies suggest that cell based therapies may cure instead of ameliorate SUI. We therefore investigated human MSC for their myogenic differentiation capacity and for expression of growth factors supporting muscular and neuronal regeneration *in situ*.

MSC were isolated from human bone marrow, adipose tissue and term placenta, enriched by plastic adherence, expanded in GMP-compliant media and characterized according to the ISCT criteria (1,2). Expression of myoregenerative and neuroregenerative factors (bFGF, HGF, IGF-1, IGF-2, PDGF, TGF $\beta$ 1; BDNF, BFGF, G-CSF, GDNF, VEGF) was investigated by qRT-PCR and protein array. Generation of smooth muscle cells was induced by addition of TGF $\beta$ 1, PDGF and ascorbate to MSC. Differentiation was monitored over time by qRT-PCR, immunoblot, and immunocytochemistry.

After expansion in GMP-compliant media MSC express TGF $\beta$ 1, a factor promoting smooth muscle regeneration, HGF, key factor for satellite cell activation, and bFGF and IGF2, factors facilitating differentiation of myoblasts. Moreover neuroregenerative factors BDNF and GDNF, and vascularization promoting VEGF were found. MSC underwent smooth muscle differentiation but did not generate striated muscle cells with high efficacy upon stimulation by cytokines *in vitro*.

We conclude that upon injection in the sphincter i) differentiation of MSC to smooth muscle cells may support its regeneration and ii) expression of paracrine growth factors may facilitate the regeneration of both, smooth and striated muscles of the sphincter complex, as well as neuroregeneration. This is currently explored in pre-clinical studies.

### Hydrophilic Crosslinking Strategies of Gelatin Scaffolds for Tissue Engineering Application

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Gelatin, thermally denatured collagen, is a promising material to develop scaffolds for bone and cartilage tissue engineering. Cross-linking of the gelatin is crucial to maintain the structural integrity of the material after implantation as it rapidly degrades in aqueous environment. The present study tends to investigate the feasibility of developing 3D gelatin (porcine skin) scaffolds through stabilization with various cross-linkers. The results highlighted the best-in-class cross-linking agent in maintaining adequate structural and physico-chemical properties of the gelatin scaffolds. The cross-linking process was optimized, in respect to type and amount of cross-linking agent as well as to duration of cross-linking process. In particular the investigated cross-linking agents were effective in covalently binding to the gelatin matrix, thus providing improved mechanical properties under dynamic mechanical loading at physiological conditions (37 °C, pH=7.4 in PBS). The scaffold porosity was uniformly distributed and well interconnected with pore size suitable for cell penetration. The scaffolds showed resistance to degradation and maintenance of the porous structure up to 21 days of soaking in PBS, also showing good swelling ability. Biocompatibility and bioactivity were investigated using human osteoblast-like cells and human chondrocytes cells and the study showed good cell adhesion, cell proliferation and scaffold colonization. In this study we verified that a suitable choice of the cross-linking agent and process enables the development of biocompatible and mechanically stable biomaterials for tissue engineering applications.

### An Rotating Integration System of Bone Tissue Engineering Grafts Based on Demineralized Bone Matrix Microcarrier

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Different types of bone defects and fractures caused by age and accidents usually require different therapeutic scenarios. An appropriate bone graft to temporarily fill the gap is needed by most treatments. Tissue engineering bone grafts (TEBG) have the advantages including well osteoconductivity, low immune rejection and robust osteogenesis ability, which is considered as an more promising choice than other grafts like titanium. Nevertheless, traditional TEBG's limitation based on the mesenchymal stem cells (MSC) is that huge amounts of MSCs are needed in order to ensure therapy effects. Microcarrier can be one of the useful solution due to its high cell expansion efficiency. However, current microcarriers are lack of osteoconductivity, and could not be transplanted to bone defect directly. Meanwhile, repeating trypsin digestion jeopardize the extracellular matrix (ECM) of MSCs, which results in quick apoptosis of MSCs when they are implanted *in vivo*. Therefore, we designed an integrin preparation system based on biodegradable demineralized bone matrix (DBM) microcarriers, that not only avoids the redundant digestion in cell expansion and osteogenic differentiation, protects the ECM environment, but also simplifies the operation, avoids potential contamination, and can be injected to defect directly as a therapy. The cell expansion ability of DBM carriers approximate the current commercial carriers, cytodex 3. BMSCs grew on DBM microcarriers maintained osteogenic differentiation potential. Orthotopic bone formation mode and ectopic bone formation mode help to investigate the possibility of DBM microcarriers' usage in injectable tissue engineering. Consequently, this integrin DBM microcarrier system could be a promising choice for bone regeneration and reconstruction.

### Pre-clinical Efficacy and Safety Evaluation of hAFSCs

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**Purpose:** Stem cell-based therapies represent new promises for the treatment of urinary incontinence. This study was performed to assess optimized cell passage number, cell dose, therapeutic efficacy, feasibility, toxicity, and cell trafficking for the first step of the pre-clinical evaluation of human amniotic fluid stem cell (hAFSC) therapy in a urinary incontinence animal model.

**Materials and Methods:** The proper cell passage number was analyzed with hAFSCs at passages 4, 6, and 8 at week 2. The cell dose optimization included  $1 \times 10^4$ ,  $1 \times 10^5$ , and  $1 \times 10^6$  cells at week 2. The *in vivo* cell toxicity was performed with  $0.25 \times 10^6$ ,  $0.5 \times 10^6$ , and  $1 \times 10^6$  cells at weeks 2 and 4. Cell tracking was performed with  $1 \times 10^6$  cells at weeks 2 and 4.

**Results:** The selected optimal cell passage number was smaller than 6 and the optimal cell dose was  $1 \times 10^6$  for the mouse model. In our pre-clinical study, hAFSC-injected animals showed normal values for several parameters. Moreover, the injected cells were found to be non-toxic and non-tumorigenic. Furthermore, the injected hAFSCs were rarely identified by *in vivo* cell trafficking in the target organs at week 2.

**Conclusion:** This study demonstrates for the first time the pre-clinical efficacy and safety of hAFSC injection in the urinary incontinence animal model and provides a basis for future clinical applications. (HI14C1642).

### Chondrocyte Sheet Directs *in Vivo* Ectopic Chondrogenesis of BMSCs by Emulating Chondrogenic Niche

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Although bone marrow-derived stem cell (BMSC) is proposed as an alternate cell source for cartilage regeneration, in subcutaneous environment, BMSC engineered constructs tend to ossify due to lack of chondrogenic niche. Therefore, it is important to know whether chondrocyte sheet can promote stable chondrogenesis of BMSCs by emulating chondrogenic niche in ectopic non-chondrogenic site. To address this issue, BMSC-PGA constructs were wrapped with chondrocyte sheet (Exp group), small intestinal submucosa (SIS, SIS group) or nothing (Blank group) respectively before subcutaneous implantation into nude mice for 4, 12 or 24 weeks. The results showed that all constructs in chondrocyte sheet group displayed typical cartilaginous features with ivory white appearance, abundant lacuna, and cartilage specific matrix deposition. These samples became more mature during *in vivo* implantation, and no sign of ossification was observed at all three time points. Moreover, cell labeling results demonstrated that the BMSCs had directly participated in cartilage formation. Samples in SIS or Blank group, however, showed partial ossification at 4 weeks and total ossification at 12 and 24 weeks *in vivo*. These results suggested that chondrocyte sheet could promote stable chondrogenic differentiation of BMSCs by providing chondrogenic niche in ectopic *in vivo* environment. This model may provide a clue for engineering ectopic cartilage (such as auricle or trachea) using BMSCs.

### Drug-eluting Bioresorbable Ureteral Stent by Co2 Impregnation

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Ureteral stents are indispensable tools in urologic practice. The main complications associated with ureteral stents are dislocation,

infection, pain and encrustation<sup>1</sup>. Bioresorbable ureteral stents are one of the most attractive designs with the potential to eliminate several complications associated with the stenting procedure. In these work we hypothesize the impregnation of Ketoprofen, by CO<sub>2</sub>-impregnation in a patented bioresorbable ureteral stent previous developed in our group and validated in a pig model<sup>2</sup>. It was evaluated of the *in vitro* elution profile in artificial urine solution(AUS) during degradation of a bioresorbable ureteral stent loaded with ketoprofen. The bioresorbable ureteral stents was developed according Barros *et al*<sup>2</sup>. The bioresorbable ureteral stents with each formulation: alginate-based, gellan gum-based(10 mg) were placed in high-pressure vessel with 10 mg ketoprofen. The impregnation conditions used were 100 bar, 2 h and different temperatures were studied (35°C, 40°C and 50°C). The impregnation was confirmed by FTIR and DSC. The *in vitro* release studied revealed an influence of the temperature in the impregnation, with 50°C operating condition to obtain higher yield. Higher yield are obtained for gellan gum-based comparing the polymers used. According to the kinetics results (released exponent(n) was between 0.45 < n < 0.89)<sup>3</sup> this systems are very promising for release ketoprofen in the first 72 h in AUS. The non-cytotoxicity characteristic of the developed ketoprofen-eluting bioresorbable ureteral stents was evaluated in L929cell-line by MTS assay and demonstrated the feasibility of this product as a medical device. This study demonstrated the possibility of combined eluting-drugs with a bioresorbable ureteral stents for different urological targets (eg. anticancer-drugs).

### Promoting Osteoblastogenesis using Lansoprazole

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The major transcription factor, Runx2, plays a pivotal role in regulating osteoblastic differentiation of the mesenchymal stem cells and bone formation. The p38 MAPK and its upstream activator of TGF- $\beta$  activated kinase-1 (TAK1) are crucial for the activation and, to a letter extent, the expression of Runx2 in osteoblastogenesis. TGF- $\beta$ /BMP signaling physiologically transmits information through MAP kinase pathways by leveraging an adaptor molecule, TNF receptor associated factor 6 (TRAF6), as well as canonical effector molecules, Smads. TRAF6, which possesses ubiquitin E3 ligase activity, catalyzes Lys63-linked polyubiquitination for intracellular signal transduction. We found by the drug repositioning strategy, in which a drug currently used for a specific disease is applied to another disease, that a proton pump inhibitor, lansoprazole, facilitated the expression and nuclear accumulation of Runx2 in osteoblast lineage cells. Lansoprazole enhanced terminal osteoblast differentiation of human mesenchymal stromal cells. Systemic administration of lansoprazole to a rat femoral fracture model accelerated calcified bone formation at the fracture sites and osteoid formation at the metaphyses. Dissection of signaling pathways revealed that lansoprazole activated a noncanonical BMP-TAK1-p38 MAPK pathway. We found by *in vivo* ubiquitination studies that lansoprazole enhanced polyubiquitination of TRAF6 and by *in vitro* ubiquitination studies that polyubiquitination of TRAF6 was attributed to the blocking of a deubiquitination enzyme, CYLD. Structural modeling and site-directed mutagenesis of CYLD demonstrated that lansoprazole tightly fits in a pocket of CYLD where the C-terminal tail of ubiquitin lies. Lansoprazole is a potential therapeutic agent for enhancing osteoblastic differentiation.

### Silk Based Microfluidic Device for the Imaging of Platelet Production

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The study of the bone marrow environment and blood cell production *in-vivo* is hindered by its inaccessibility, limiting the study of pathogenesis of diseases such as inherited thrombocytopenias. Thus far, visualization of blood cell production is limited to complex techniques and animal models. We recently demonstrated functional platelet production *in-vitro* using a silk-based bioreactor.<sup>1</sup> Here we present an *in-vitro* system that recapitulates the bone marrow vascular niche, enabling direct visualization of human thrombopoiesis. The aim is to elucidate the processes that lead to physiological platelet production, allowing further understanding of how these processes are disrupted in pathological conditions.

We developed a microfluidic device consisting of two channels, separated by a porous silk film. Composite silk films are advantageous due to their tunable mechanics, optical transparency, and ease of functionalization with ECM proteins and growth factors. Silk-fibronectin films were seeded with human megakaryocytes on the topside and endothelial cells on the bottom side to reproduce the architecture of the vascular niche where the cells are separated by a basement membrane.

To model physiological shear stress, the endothelial side was perfused and the production of functional human platelets was investigated. Furthermore, platelet formation under flow was directly visualized through confocal microscopy. On-going work aims to understand the mechanisms responsible for blunted platelet production in inherited thrombocytopenias using patient-derived megakaryocytes. The results of this study can open new roads for the study of the mechanisms of platelet production in both physiologic and pathologic scenarios.

(1) Di Buduo *et al.* Blood 2015.

### Possibilities of Various Cell Types Cultivation in New Designed Hyaluronan based Hydrogels

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Hyaluronan (HA) is a unique linear glycosaminoglycan, which is one of the chief components of the extracellular matrix. One of promising application of this natural occurring macromolecule is its usage for materials for the application in biomedicine or tissue engineering. The effective cultivation of cells incorporated into these materials is very important factor for their intended application as cell scaffolds.

The aim of this study is to present the possibility of cultivation of various cell types which can be incorporated and effectively cultured in new developed hydrogels based on materials prepared from tyramine derivatives of hyaluronic acid (HA).

So far obtained results demonstrate that these new developed HA-based hydrogels provide suitable environment for an effective cultivation of various cell types, for example of mesenchymal stem cells or chondrocytes as well as monocytes, which proliferate and produce corresponding extracellular matrix required for intended application.

This is important factor for damaged tissue healing and all these results suggest that these materials are promising candidates as cell scaffolds for tissue engineering.

### Biovasc-term®: A Platform Technology to Engineer of Vascularised Human Tissues

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Vascularization is a major challenge in creating tissues *ex vivo*. Complex tissue engineered constructs exceeding a thickness of 100–200 µm need a vascular system in order to supply the cells with oxygen and remove waste products. This restricts generation of tissues with an appropriate size for clinical application.

We developed 3D vascularized tissues based on decellularized porcine small bowel segments and preserved tubular structures of the capillary network within the collagen matrix functional associated with one small vein and artery (biological vascularized scaffold - BioVaSc®). This vascularized matrix enables the generation of a functional artificial vascular network and vascularized tissues as trachea<sup>1</sup>, bone<sup>2</sup>, skin<sup>3</sup>, fatty tissue<sup>4</sup>, intestine<sup>5</sup>, and tumour<sup>6</sup>.

Vascularized tissues are applied as tissue models in preliminary stage to animal experiments for investigations of functional parameters like penetration, distribution and metabolism of substances in different tissue layers. With the help of suitable markers, issues concerning proliferation, differentiation, cell death, but also, the initiation and graduation of tumors of the applied cell types can be examined. Furthermore, first clinical application will be shown during the presentation.

Non-invasive monitoring of such tissues during their *in vitro* maturation or post implantation is relevant for graft evaluation. However, traditional methods to analyses cell and matrix components in tissue engineered constructs such as histology, immunohistochemistry or biochemistry requires invasive tissue processing, resulting in scarification of these constructs.

At the end of the presentation an overview of non-destructive methods as impedance<sup>7</sup> and Raman<sup>8</sup> spectroscopy to characterize the complex implants will be given.

### Allopatric Renal Tissue Fabrication by 3-D Tissue Engineering

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**Background:** Transplantable renal tissues from these cells are considered to be a simple solution of kidney donor shortage. Therefore, the aim of this study is an establishment of fabrication methodology for allopatric transplantable renal tissue from renal progenitor cells.

**Method:** Allopatric microcirculation was obtained by creating a vascular bed on a femoral muscle with ligature arteriovenous. Three-dimensional tissue was fabricated by spheroid formation with primary cultured fetal rat renal cells. Renal spheroids fabricated by 48 hours cultivation were transplanted onto vascular bed without any additional extracellular matrix and suture.

**Results:** Newly vascularization originated from the host was observed at one week after transplantation. Two weeks after transplantation, we obtained clearly glomeruli constructs within transplanted tissues. Most vessels within transplanted tissue were supplied from the host.

**Conclusion:** Allopatric renal tissues containing glomeruli could be obtained by transplantation of 3-D spheroids onto the allopatric microcirculation. These results suggest that allopatric functional renal tissues will be fabricated from several kinds of renal progenitors.

**Acknowledgments:** This study was supported by Creation of innovation centers for advanced interdisciplinary research areas Program in the Project for Developing Innovation Systems “Cell Sheet Tissue Engineering Center (CSTEC)” from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

**Disclosure:** Shimizu Tatsuya is a stakeholder of CellSeed Inc. Teruo Okano is a founder and a member of the board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women’s Medical University. Tokyo Women’s Medical University is receiving research funding from CellSeed Inc.

### 3D Printed Alginate Hydrogel Scaffolds in Regenerative Medicine Techniques

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Vascular disease is the leading cause of death in the United States. The purpose of this research was to evaluate the use of modified alginate scaffolds in the production of functional vascular networks. For 3D printing a hydrogel must have a viscosity that allows it to be printed without relaxing too quickly so that it can be cross-linked to maintain the desired shape. Before printing was attempted, the relationship between alginate preparation and the resulting hydrogel viscosity was analyzed under various conditions including preparation temperature and concentration of sodium alginate. An increase in both conditions led to greater viscosity. A 6–8% sodium alginate hydrogel prepared at a temperature of 80°C exhibited the necessary characteristics for 3D printing. Additional modifications that were tested included sulfonation, methacrylation, and the addition of fibronectin. Sulfonation of alginate improves binding of heparin-binding proteins. Methacrylation of alginate enables the gel to photocrosslink while the addition of fibronectin increases cell adhesion. By combining these modifications it is hypothesized that a better biocompatible gel can be created.

Current research has indicated that gelatin “bio-paper” could be used in a method of constructing beating cardiac tissue and blood vessels (Jakab). By using modified sodium alginate as bio-paper to print a scaffold mimicking the structure of complex vascular networks, functioning blood vessels could be biomanufactured for use in transplants. Additionally, the vascular networks may also be used as temporary scaffolds to support other cell types for tissue engineering of whole organs.

Jakab, K. *et al.* Tissue Engineering Part A 2008.

### Myogenic Potential of Human Tonsil-derived Stem Cell for Skeletal Muscle Regeneration

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Stem cells are gradually regarded as the cell therapy for the skeletal muscle regeneration in damaged muscle that due to shortcomings in the organization such as congenital diseases or acquired trauma, tumor removal. In particular, mesenchymal stem cells (MSCs) that less invasive harvesting of those cells represent a valuable source for stem cell therapy. Here we show that the myogenic potential of the human Tonsil-derived mesenchymal stem cells (T-MSCs) for skeletal muscle regeneration. In this study, the T-MSCs that has characteristics of myosatellite cell were differentiated to myogenic cell *in vitro* and confirmed capable of muscle regeneration by transplant to mouse myectomy model *in vivo*. For myogenic differentiation, the spheroids were put into myoblast induction medium (DMEM/F-12: DMEM/Nutrient Mixture F-12) containing 1 ng/ml Transforming growth factor- $\beta$  (TGF- $\beta$ ) for 4 days followed by exposure to a skeletal muscle differentiation medium (low glucose-DMEM containing 2% FBS and 10 ng/ml Insulin-like growth factor 1: IGF1) for 14 days. T-MSCs were differentiated toward the myoblast and skeletal muscle cell sequentially, as evidenced by increase expression of skeletal myogenic-related markers (including MyoD,  $\alpha$ -actinin, Troponin I type 1:TNNI-1, and myogenin) and myotube formation *in vitro*. Intramuscular transplantation of T-MSCs into myectomy mice enhanced behavior function identified by gait test without the formation of teratomas. Taken together, our finding showed that T-MSCs can be differentiated to myogenic cell and are effective for muscle regeneration. Thus, these results demonstrate the therapeutic potential of T-MSC in damaged muscle.

### Skeletal Myogenic Differentiation of Urine-Derived Stem Cells using Hydrogel Loaded with Growth Factors for Potential Treatment of Urinary Incontinence

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**Introduction:** The goal of this study was to determine whether skeletal myogenic, angiogenic, and neurogenic growth factors released from hydrogel can induce human urine-derived stem cells (USCs) to give rise to a skeletal myogenic lineage, improve revascularization and innervation, and recruit resident cells to participate in sphincter tissue repair for urinary incontinence.

**Methods:** USCs were obtained from eight healthy adult donors. USCs were mixed with a hyaluronic acid hydrogel containing an optimized growth factor cocktail (including VEGF, IGF-1, FGF-1, PDGF-BB, HGF, and NGF) plus heparin. USCs mixed with the hydrogel were then subcutaneously injected into 24 athymic mice, divided into 4 groups (G) of 6 animals/each: **G1**, hydrogel alone; **G2**, growth factors within gel; **G3**, USCs with gel; and **G4**, growth factors plus USCs within gel.

**Results:** Mice in G4 had the greatest numbers of grafted cells with significant expression of human nuclei, and more cells that expressed myogenic (MyoD, myf-5, Myosin) and endothelial cell (CD31 and vWF) transcripts and protein markers, compared to the other groups. In addition, vessel formation and innervation were significantly greater in G4 mice versus all other 3 groups.

**Conclusions:** Growth factors released in a controlled manner from hydrogel efficiently improve cell survival in a synergistic manner, guide USCs to myogenic differentiation, enhance angiogenesis and innervation, and recruit resident cells to participate in tissue regeneration. These qualities suggest their potential as an alternative for cell therapy in treatment of urinary incontinence.

### Generation of Tavg using Ramsc Derived Rsmc Seeded on a Biomimetic Nano Fibrous Scaffold

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A clinical necessity in blood vessel replacement surgery with increased prevalence of cardiovascular diseases is the need of an ideal vascular substitute. Tissue engineering approach towards the development of small diameter vascular graft (<6 mm) has been the “holy grail” of vascular tissue engineering for past many decades 1. The architectural complexity and functional diversity of blood vessel poses a difficult task for drawing a line of integrity in vascular tissue engineering 2. However, generation of contractile smooth muscle cells on a scaffold equivalent to native extracellular matrix remains as a challenge in creation of a tissue engineered blood vessel. In this study, we investigated the feasibility of dual electrospun gelatin-vinyl acetate copolymer blended with poly- $\epsilon$ -caprolactone (blend ratio, GVPCL7: 3) as a potential scaffold for the differentiation of rabbit adipose derived mesenchymal stem cells (RAMSC) into smooth muscle cells (RSMC), *in vitro*. The scanning electron micrographs of tubular scaffold (ID: 2 mm) showed smooth fibres with adequate porosity and fibre diameter of 0.9  $\mu$ m. FTIR and confocal Raman spectroscopy confirmed the blend nature of scaffold. Apoptosis assay by FACS further proved scaffold induced no cell death. RAMSC isolated were differentiated to RSMC on scaffold with induction by growth factor combination for 14 days. The contractile phenotype of RSMC was confirmed by confocal microscopy and gene expression. Our results demonstrated that RAMSCs served as a reliable cell source for SMCs in blood vessel engineering, and a small-diameter medial vessel wall could be constructed using GVPCL scaffold. The TEVG of our studies hence opens the possibility of a non-immunogenic construct for potential autologous therapeutic use.

### Development of Tissue Engineered Small Diameter Vascular Grafts

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**Background:** There is an increasing need for small diameter vascular grafts for surgical revascularization procedures. However, smaller vascular grafts made from synthetic biomaterials are associated with a high incidence of thrombosis. We compared characteristics of small diameter grafts made of Polycaprolactone (PCL), a biodegradable polymer, alone or integrated with either vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (B-FGF).

**Methods and Results:** 2 mm PCL grafts were evaluated in a rat abdominal aorta replacement model for 6 weeks (n=5). All animals survived and all grafts were patent. Histological analysis showed reactive endothelial coverage, robust neointima formation and transmural cellular ingrowth. We compared PCL grafts integrated with either VEGF or B-FGF to non-modified PCL grafts via dynamic mechanical analysis (DMA) and scanning electron microscopy (SEM). We found increased tensile strength in the VEGF (3.96 MPa) and B-FGF (4.3 MPa) modified PCL grafts as compared to non-modified PCL grafts (1.52 MPa) and decreased pore size in the cytokine modified PCL grafts from micro dimensions to nano. VEGF and B-FGF integrated PCL grafts were also compared to control PCL grafts *in vivo* via subcutaneous implantation for 3 weeks in rats (n=5 per group). The cytokine modified PCL grafts demonstrated considerably more extracellular matrix than control grafts, less conspicuous macrophage infiltrate, more retained polymer fibrils and better giant cell profile.

**Conclusions:** Cytokine modified PCL grafts demonstrate strong potential for small diameter vascular replacements because of their increased tensile strength, endothelialization and extracellular matrix formation.

### Session: Poster Session 3

**Date and Time: Wednesday, September 9, 2015, 7:00 PM - 9:00 PM**

#### Synthetic Hydrogel Substrates for Xeno-Free, ROCK Inhibitor-Independent Human Pluripotent Stem Cell Culture

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Recent studies suggest substrates that facilitate increased intracellular cytoskeletal tension also promote human pluripotent stem cell (hPSC) self-renewal. These insights have been used to develop chemically-defined substrates for cell culture; however, current chemically-defined substrates are only suitable for hPSC culture in media containing animal proteins and Rho- associated protein kinase (ROCK) inhibitor. Reliance on animal proteins limits clinical translatability and studies suggest that routine dependence on ROCK inhibitor can limit differentiation potential. Here, we developed synthetic substrates capable of promoting hPSC attachment, proliferation, and pluripotency maintenance in fully-defined, xeno-free, ROCK inhibitor-independent media. We utilized a hydrogel array-based platform to systematically and independently control substrate stiffness, peptide identity, and peptide density to examine the combinatorial and synergistic effects of these properties on hPSC behavior. With the enhanced throughput capabilities of the array platform, we also examined the influence of cell seeding parameters (seeding density and colony vs. single-cell seeding) and seeding and maintenance media (with or without ROCK inhibitor) to identify the first chemically-defined synthetic hydrogel substrates that promote hPSC expansion in xeno-free, ROCK inhibitor-independent media. Results suggest that substrates presenting peptides with higher integrin-binding affinity increased total number of cells attached and substrates with increased peptide density facilitated faster initial cell attachment and enhanced survival. Additionally, substrate stiffness and peptide density synergistically promoted increased intracellular cytoskeletal tension necessary for stable cell adhesion and pluripotency maintenance over the course of 10 days.

1. Fan Y, *et al.* Stem Cell Reviews. 11, 96, 2015.

#### Electrical Stimulation for Cardiac Differentiation and Maturation of Cardiomyocytes Derived from Human Induced Pluripotent Stem Cells

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Cardiac diseases are the major cause of deaths worldwide. The ability of human induced pluripotent stem cells (iPSCs) to differentiate into bona fide cardiomyocytes provides a platform for disease modelling and drug testing to improve treatment options. However, cardiomyocytes derived from iPSCs resemble foetal cardiomyocytes and are immature. Considering the heart as an electrically active organ, we hypothesise that electrical stimulation, mimicking the endogenous electric field, can promote cardiac differentiation of human iPSCs and maturation of cardiomyocytes derived from human iPSCs.

**Methods and results:** Acute electrical stimulation (alternating current, charge-balanced biphasic pulse, 1 ms pulse width and 1 Hz frequency) at 200 mV/mm for 5 min increased the percentage of beating embryoid bodies (EBs, 11.2±2.2% vs 4.1±1.5% in no electrical stimulated, p<0.05, n=11-15) and gene expression of cardiac  $\alpha$ -actin (6.5±0.5 vs. 3.3±1.6, p<0.01, n=3-4). Beating EBs were cycling calcium and were responsive to the chronotropic agents, isoproterenol and carbamylcholine. Chronic electrical stimulation at 200 mV/mm for 7 days significantly increased the cell size (8093±2382  $\mu$ m<sup>2</sup> vs 3224±633  $\mu$ m<sup>2</sup>, p<0.05, n=18-21) and decreased the circularity index (0.64±0.05 vs 0.79±0.09, p<0.01, n=18-21) of cardiomyocytes derived from human iPSCs, indicating a more rod-like structure.

**Conclusion:** These data suggest that acute electrical stimulation can enhance cardiac differentiation of human iPSCs and chronic electrical stimulation might promote maturation of cardiomyocytes derived from human iPSCs

#### Production and Characterisation of Engineered Neural Tissue Made using CTX Human Neural Stem Cells

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The combination of cellular self-alignment in tethered collagen gels, with stabilisation through the removal of some interstitial fluid, results in the formation of sheets of robust aligned engineered neural tissue (EngNT) [1]. Previous work has demonstrated the ability of EngNT to be used as an artificial endoneurium to support peripheral nerve regeneration, using Schwann cells and more recently using stem cells that have been differentiated to resemble Schwann cells [1-3]. In order to progress this technology towards clinical translation, the use of human neural stem cells (ReNeuron, UK), as a potential allogeneic source of therapeutic cells is under investigation, along with the development of optimised production processes for manufacturing EngNT.

The aim of the current study was to investigate the feasibility of using EngNT made with human neural stem cells as an off-the-shelf therapy to treat peripheral nerve injury. Differentiated CTX human neural stem cells were seeded within type I collagen gels, and cellular self-alignment followed by stabilisation using RAFT™ (TAP Biosystems, UK) resulted in the formation of sheets of EngNT. New equipment was developed and processes optimised to facilitate production at scale. The phenotype of differentiated CTX cells was characterised in EngNT and the aligned cellular material was assembled to form a guidance substrate that supported neuronal regeneration within a repair conduit, tested using a rat model of peripheral nerve injury.



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## An Injectable Hydrogel for Stem Cell Therapeutic Treatment of Irradiated Colon

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Radiotherapy is used to treat malignant tumours but can damage the surrounding tissues. Pelvic irradiation leads to gastrointestinal complications due to intestinal/colonic damage. Stem cell injection helps restore tissue function, although multiple injections are required due to cell death at site 1. This may be overcome through using an injectable biomaterial for cell delivery. Our aim was to develop an injectable biomaterial for stem cell therapeutic treatment of irradiated colon.

**Materials and Methods:** Si-HPMC was synthesized and hydrogel formulated as described. Rheological measurements (viscosity, elastic modulus ( $G'$ ), gel-point) were performed at a range of Si-HPMC concentrations (0.6 – 2%) that could be injected through a specific catheter into the colon lumen. Adipose stem cells (ASCs) isolated from Sprague Dawley (SD) rats were seeded into the hydrogel (1.5% Si-HPMC +  $1 \times 10^6$  cells/ml) and viability assessed using Live/Dead assay. Media was collected and analysed using Luminex technology. *In vivo* assessment was performed on irradiated colorectal SD rats with described construction injected; apoptosis and proliferation assays were performed.

**Results:** Viscosity and  $G'$  increased and gelation time decreased with increasing Si-HPMC concentration. Following 21 days culture, ASCs were 70–80% viable within the hydrogel. *In vivo* analysis confirmed that cells were not apoptotic. Media assessment showed the presence of angiogenic and immunomodulatory molecules (e.g. FGF-2, VEGF, PGE2).

**Conclusion:** ASCs encapsulated in an injectable hydrogel provides a novel therapeutic approach for post-irradiation treatment with secretomic factors inducing repair.

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## The Alternating Air-medium Exposure in Rotating Bioreactors Optimizes Cell Metabolic Activities in 3d Tubular Scaffolds

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*In-vitro* dynamic culture conditions play a pivotal role in developing engineered tissue-grafts, where the supply of oxygen and nutrients, and waste removal must be guaranteed within the construct thickness. For tubular scaffolds, mass transfer was enhanced by introducing a convective flow through rotating bioreactors with positive effects on cell proliferation, scaffold colonization, and Extracellular Matrix (ECM) deposition. Thus, we investigated the influence of dynamic conditioning on cell activity under two culture configurations: (1) single-phase (medium) rotation and (2) double-phase exposure (medium-air) rotation; single-phase static culture was carried out as control. Cell line murine fibroblasts were externally seeded on tubular porous scaffolds (polyurethane foam and PGA/PLLA), and cultured under these conditions. Viability assay, DNA quantification, SEM, and histologic analyses were performed at each time point (1, 7, 14, and 21 days). Our results showed that, in comparison to the control, constructs cultured under dynamic conditions contain a significantly higher cell number

and enhanced viability. The single-phase exposure resulted similar to double-phase, but the cell scaffold penetration was limited and a partial luminal colonization reached. On the other hand, a more homogenous cell distribution was observed in double-phase rotation: cells uniformly covered not only the external surface, but also the lumen thanks to an improved wall thickness colonization. Finally, a higher ECM deposition was detected. Consequently, the alternating air-medium exposure coupled with the longitudinal rotation provided the biochemical and mechanical conditioning with the desired nutrient supply, independently from the considered polymers, thus representing the optimal condition for cell proliferation and colonization.

## Effective ECM and Elasticity for iPS Differentiation into Cardiac Marker Positive Cells or into Self Beating Cardiomyocytes

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Stem cell-based myocardial regeneration therapies have been emerged as an alternative strategies of heart transplantation. Although cardiomyocyte transplantation is promising, an effective niche for cardiac differentiation of stem cells has not been clarified. The purpose of this study is to clarify the effect of culture substrates on cardiac differentiation and maturation by separately evaluating a cardiac differentiation step, a beating-induction step, and a beating duration step *in vitro*. To this end, iPS cells and neonatal cardiomyocytes (NCMs) were cultured on synthetic hydrogels with the elastic modulus of 9, 20, 180 kPa and on tissue culture polystyrene dishes with that of 2 GPa immobilized with collagen (Col-I), gelatin (Gel), fibronectin (FN).

It was found that the cardiac differentiation assessed by cardiac marker gene expression (GATA4, MEF2D) in the iPS cells was greatly enhanced on Col-I-, Gel-, FN- immobilized culture dishes and Col-I-immobilized-20 kPa-gel. In contrast, the beating-induction assessed by contractile protein gene ( $\alpha$ -MHC, TnC1, and TnT2) expression of NCMs preferred Col-I-immobilized-9, 180 kPa-gel and FN-immobilized culture dishes. Finally, the beating duration step preferred Col-I-immobilized-9, 20 kPa-gels and Gel-immobilized-20 kPa-gels. These results indicate that the maturation of cardiac differentiation of iPS cells must be controlled stepwise on different culture substrates. This research was supported by The Ministry of Education, Culture, Sports, Science and Technology (MEXT).

## Long-term Controlled Delivery of rhBMP-2 from Collagen-Hydroxyapatite Scaffolds for Superior Bone Tissue Regeneration

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The clinical utilization of recombinant human bone morphogenetic protein 2 (rhBMP-2) for bone regeneration is associated with severe side effects, due to the non-controlled delivery which necessitates supra-physiological doses to be efficacious. However, rhBMP-2 presents outstanding regenerative properties and thus there is an unmet need for a biocompatible, fully resorbable system for targeting BMP-2 release. As such, the purpose of this work was to develop a delivery system to release rhBMP-2 in low doses from a collagen-hydroxyapatite (CHA) scaffold, previously been optimized for bone regeneration. To enhance the potential for clinical translation by minimizing complexity of the device, a microparticle/chemical functionalization-free approach was chosen to fulfil this aim. RhBMP-2 was combined with a CHA slurry using a lyophilisation process to produce a highly porous CHA scaffold which supported the controlled release of the growth factor over 21 days, as measured by ELISA, whilst maintaining *in vitro* bioactivity as demonstrated by enhanced alkaline phosphatase activity and calcium

production by osteoblasts cultured on the scaffold (7–14 days). When implanted *in vivo*, in critically size rat calvarial defects, these scaffolds demonstrated significantly increased levels of healing, evidenced by microCT and histological analysis, after 8 weeks implantation compared to an empty defect and drug-free CHA scaffolds, without eliciting bone anomalies or adjacent bone resorption. These results were obtained using 30 times less rhBMP-2 than INFUSE<sup>®</sup>, the current clinical gold standard. This work thus represents the first step of the development of a rhBMP-2 eluting material with immense clinical potential.

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### Chondromimetic Peptide- and Growth Factor-Modified Polyglycidol-Based Hydrogels for MSC Chondrogenesis

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The guided regeneration of injured joint cartilage still represents a major clinical challenge. Our overall goal, therefore, is the design of a novel platform of reactive polyglycidol [PG]-based hydrogels for controlled chondrogenesis of bone marrow-derived mesenchymal stem cells [BMSC].

Here, we present hydrogels made from thiolated hyaluronic acid [HA] cross-linked with PG derivatives either using Michael addition or thiolene click chemistry. Alternatively, pure PG gels were generated by UV-mediated cross-linking of thiolated PG. Functionalization by coupling of chondromimetic peptides yielded distinctly improved chondrogenesis of cultured BMSCs. Incorporating N-cadherin-mimetic peptides, such as HAVDI, into pure PG gels led to clearly increased GAG content and alteration of collagen type ratio, as compared to scrambled peptides or unmodified gels (biochemical quantification, histological and immunohistochemical staining). Covalent binding of TGF- $\beta$ 1 (100 nM) within HA-PG hydrogels led to strong chondrogenesis, with distinctly increased GAG and collagen type II content after 10 and 21 days, as compared to constructs receiving equal amounts of TGF- $\beta$ 1 mixed within the gels, and similar contents of ECM components as constructs receiving TGF- $\beta$ 1 (10 ng/ml) with each medium change three times per week. In conclusion, the developed hydrogels show strong potential for cartilage engineering and for analysis of peptide and growth factor effects on chondrogenesis. The convincing chondrogenic effects of bound TGF- $\beta$ 1 render the gels especially attractive. In ongoing work, combinations of coupled biomimetic peptides and growth factors are explored.

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### Modulation of Culture Conditions During Production of Psoriatic Skin Substitutes

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**Background:** Tissue-engineered psoriatic skin substitutes produced following a self-assembly method correctly represent the psoriatic phenotype. However, an increased permeability of their skin barrier, which may be caused by an essential fatty acid deficiency, was observed compared to human skin. Objective: The aim of this study was to improve the barrier function of the substitutes by restoring their lipid profile. To that end, essential fatty acid supplementation of the culture medium was performed and their implication in the pathology validated.

**Methods:** Healthy and psoriatic skin substitutes were reconstructed using culture medium supplemented with different concentrations of

linoleic acid,  $\alpha$ -linolenic acid and palmitic acid and compared with their respective counterparts, which were free of supplements. Macroscopic, histologic, immunochemistry, gas chromatography, infrared spectroscopy and percutaneous absorption analyses were performed to investigate whether the medium supplementation could have positive impacts on the barrier function of the substitutes or not.

**Results:** The macroscopic, histologic and immunochemistry assays have demonstrated that fatty acids have positive impacts on skin substitute appearance. Moreover, the characterisation by gas chromatography of the different fatty acids present in the phospholipid fractions of the epidermis showed that the lipid profile was restored with a concentration of 5  $\mu$ M fatty acid supplementation. Confirmed by infrared spectroscopy and percutaneous absorption, the lipid organisation was also modified when the medium was enriched with lipids.

**Conclusion:** These results show that implementing with fatty acids during reconstruction of psoriatic skin substitutes could restore their lipid profile in order to improve the mimicking of normal human skin.

### Osteogenic Differentiation of Adipose Stem Cells by Endothelial Cells Co-culture within Liquefied Capsules

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Inspired by the native co-existence of multiple cell types and from the concept of deconstructing the “stem cell niche”, we propose a co-culture strategy within liquefied capsules. The present team has already proven the application of liquefied capsules as bioencapsulation systems 1. Here, we intend to use the optimized system towards osteogenic differentiation. Capsules containing adipose stem cells alone (MONO-capsules) or in co-culture with endothelial cells (CO capsules) were maintained in endothelial medium with or without osteogenic differentiation factors. The suitability of the capsules for living stem and endothelial cells encapsulation was demonstrated by MTS and DNA assays. The osteogenic differentiation was assessed by quantifying the deposition of calcium and the activity of ALP up to 21 days. CO capsules had an enhanced osteogenic differentiation, even when cultured in the absence of osteogenic factors. An enhanced osteogenic differentiation in the CO capsules was confirmed by the upregulation of osteogenic markers (BMP-2, RUNX2, BSP) while angiogenic markers expression (VEGF, vWF, CD31) revealed the presence of endothelial cells. Osteopontin and CD31 could be detected, which respectively confirmed the osteogenic differentiation and the maintenance of endothelial cells phenotype. The proposed capsules can also act as a growth factor release system, as showed by VEGF and BMP-2 release profiles. These findings demonstrate that the co-encapsulation of stem and endothelial cells within liquefied injectable capsules provides a promising strategy for bone tissue engineering.

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### Synthetic Substrates for Serum free Culture of Human Stem Cell Derived Cardiomyocytes with Improved Maturity and Toxicological Sensitivity Identified using Combinatorial Materials Microarrays

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Cardiomyocytes from human pluripotent stem cells (hPSC-CM) have the potential to provide models for heart disease and toxicity screening. Whilst there have been significant advances in the generation of large numbers of hPSC-CM, they remain functionally immature. Soluble components of the culture system such as growth factors within serum and insoluble components such as the substrate on which cells adhere to are important variables controlling the biological activity of cells. Using a combinatorial biomaterial approach we develop a synthetic, chemically defined cellular niche for the support of functional cardiomyocytes in a serum-free fully defined culture system. Almost 700 polymers were synthesized and evaluated for their utility as growth substrates. From this group, 20 polymers were identified that supported cardiomyocyte adhesion and spreading. The most promising polymers were scaled up for extended culture of hPSC-CM for 15 days and assessed for functional and structural cell maturity using patch clamp electrophysiology and myofibril analysis. We found that hPSC-CM cultured on selected methacrylate co-polymers exhibited a six-fold faster upstroke velocity in their electrophysiology and significantly longer sarcomeres relative to gelatin control. The utility of increased structural maturity was demonstrated in an *in vitro* toxicity assay that found a ten-fold increase in detection sensitivity of myofibril disruption by the anti-cancer drug doxorubicin. We believe that the chemical moieties identified in this large-scale screen provide improved conditions for the use and manipulation of hPSC-CM, as well as a framework for the rational design of superior materials for their culture.

#### Healing of Rat Segmental Defects by Adipose Derived-Mesenchymal Stem Cells is BMP-2 Dose Dependent

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Transplantation of stem cells engineered to express Bone Morphogenetic Protein-2 (BMP-2) remains an attractive approach for bone repair as it provides both BMP and osteoprogenitor cells. In this study, we examined the ability of human adipose-derived mesenchymal stem cells (hADSCs) overexpressing BMP-2 to direct bone healing in rat segmental defects, focusing on the influence of BMP-2 secretion levels on tissue repair. hADSCs were genetically modified to overexpress BMP-2 using an adenoviral vector at 200, 1000 and 5000 viral particles per cell, then encapsulated into fibrin scaffolds and implanted into a stabilized 5-mm sized femoral defect within immune-compromised rats. Extra cell-loaded scaffolds were used to quantify BMP-2 secretion and bone-related marker transcript expression *in vitro* over a 3-week culture as a function of virus dose. Tissue repair was evaluated by X-ray, bone densitometry and micro-computed tomography measurements and histology. While increased BMP-2 secretion generally led to increased bone formation, high doses of secreted BMP-2 (~450 ng BMP-2/48 h/10<sup>6</sup> cells *in vitro*) were detrimental to repair tissue remodeling, as indicated by 8 week histology. qPCR analysis showed that transcript expression levels depended on BMP-2 dose: ALP, Osteopontin, Bone Sialoprotein, Chordin and PPAR $\delta$  transcripts were strongly upregulated by high secretion levels, while Osterix and FGF-2 transcripts were strongly upregulated by moderate levels (~250 ng BMP-2/48 h/10<sup>6</sup> cells). While the mechanisms by which high doses of BMP-2 compromise hADSC regenerative potential still need to be elucidated, these results demonstrate the need to optimize growth factor expression levels to stimulate effective bone repair.

#### The Vascular Microtissue Array: Parallelised Human Pluripotent Stem Cell-Derived Vascular Constructs for Pre-Clinical Screening of Drug-Induced Vascular Injury

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Human pluripotent stem cells (hPSCs) provide the opportunity to assemble human tissue constructs *in vitro*. These may be used in pre-clinical drug screening to both obviate the need for large numbers of pre-clinical research animals, and reduce late stage discovery of adverse effects when testing on human subjects.

In this work we derive vascular smooth muscle cells (SMCs; >95% TAGLN+CNN1+) and endothelial cells (ECs; >95% CD31+) from hPSCs. These populations are functional: SMCs display intracellular calcium fluxes and cause tissue construct contraction in response to vasoconstrictive stimuli, whereas ECs self-assemble into tubular networks and form tubular tissue constructs more readily than HUVECs. Combining these cell populations with a source of stromal cells (human bone marrow mesenchymal stem cells; hMSCs), a tissue hydrogel mixture and an array of micro-fabricated geometrical constraints, highly parallelised arrays of vascular tissue constructs were assembled. We demonstrate the utility of this platform (The Vascular Microtissue Array; VMA) as a pre-clinical screen of drug-induced vascular injury (DIVI), an infrequent but nevertheless significant cause of drug attrition and adverse events. So far, DIVI is only identified through histological methods. Using dual dye- or nanoparticle-release assays, the VMA can differentiate cytotoxicity between the EC and SMC compartments. The VMA should be useful as a pre-clinical, human biologic screen of DIVI, as well as for developing additional biomarkers for clinical adoption.

#### Hydrophilicity of Copolymer Materials as Scaffolds in Bone Tissue Engineering is a Critical determinant

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Porous poly (L-lactide-co- $\epsilon$ -caprolactone) scaffolds were fabricated using solvent-casting particulate-leaching method described before. The relative low hydrophilicity and the lack of natural recognition sites on the surface of degradable poly(LLA-co-CL) have greatly limited their further application in the tissue engineering field. Optimizing the wettability of the porous poly(LLA-co-CL) scaffolds is essential to promote the adhesion of cell adhesion and nutrient diffusion into the scaffold. A range of 0.5–3% (w/w) surfactant Tween 80 was used as an additive to improve the wettability and enhance cellular responses. In addition, the influence of modifying poly(LLA-co-CL) scaffolds by Tween 80 on bone regeneration was assessed. Bone marrow stem cells (BMSCs) were seeded and cultured onto pristine and modified within a dynamic environment scaffolds using biaxial rotating bioreactor (BXR) (TisXell Regeneration System). Adding 3% Tween80 to the polymer scaffolds reduced the water contact angle significantly and promoted cellular proliferation ( $p < 0.05$ ). *In vivo* evaluation demonstrated that the modified scaffolds had higher expression of runt related gene 2 (Runx2), alkaline phosphatase (ALP), osteocalcin (OC) and platelet endothelial cell adhesion molecule (PECAM-1). Furthermore, a significant bone formation was demonstrated by  $\mu$ CT after 8 weeks with 3% Tween80 scaffolds. The results indicate that blending the scaffolds with 3% of Tween80 increases the hydrophilicity of the copolymer scaffolds and significantly influences BMSCs proliferation and bone formation. Tuning hydrophilicity of copolymer scaffolds might have a significant impact on bone regeneration.

### Microarray-based Gene Expression Profiling of Psoriatic Human Skin Model Enriched with Cytokines

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The pathogenesis of plaque psoriasis involves genetic, immunological and environmental factors. Despite conspicuous progress in terms of genetic data, we are still far from a comprehensive understanding of the immune regulation of the skin in psoriasis. To overcome the temporary grunt addition of immune cells, we aim to characterize a novel *in vitro* psoriatic human skin model produced by tissue-engineering and supplemented with a cocktail of four cytokines (TNF- $\alpha$ , IL-1 $\alpha$ , IL-6, IL-17). DNA microarrays analyses were performed to determine the level of mRNA expression in psoriatic skin substitutes supplemented or not with cytokines. Cyanine 3-CTP labeled cRNA targets were prepared and incubated on a G4851A SurePrint G3 Human GE 8 $\times$ 60 K array slide. Slides were then hybridized, washed, and scanned on Agilent SureScan Scanner. The results showed that cytokines supplementation acts on the structure and the organization of epidermis, which was thicker and more irregular. A large number of genes was dysregulated in psoriatic substitutes supplemented with cytokines: upregulated genes (DEFB4A, S100A12, KYUN, IL8, CX3CL1) and downregulated genes (CCL27, ACSBG1, SERPINA12). The set of upregulated genes is mainly involved in immune response pathways or cell chemotaxis, whereas downregulated genes seem to play a crucial role in skin differentiation. These data suggest that the supplementation with cytokines could enhance the psoriatic phenotype in cultured tissues leading to a closer mimicking of the pathology. This model could be used as a new relevant tool in dermatopharmaceutical research.

### Injectable, Covalently Adaptable Hydrogels with Secondary Thermo-responsive Reinforcement for Cartilage Engineering

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While injectable hydrogels are able to be surgically implanted in a minimally invasive way, they typically rely either on external stimuli to induce gelation *in situ*, which is difficult to control in a surgical setting, or are designed to be shear-thinning, which results in mechanically weak gels with fast erosion rates. To address these limitations, an injectable hydrogel was designed with a novel combination of dynamic covalent crosslinking and physical crosslinking. *Ex situ*, the hydrogel undergoes chemical crosslinking through the formation of dynamic covalent hydrazone bonds by simply mixing two components together: hydrazine-modified elastin-like polypeptide (ELP) and aldehyde-modified hyaluronic acid (HA). This injectable, shear-thinning network provides encapsulated chondrocytes with mechanical protection during injection and significantly decreases membrane damage during syringe needle flow compared to cell delivery in saline. *In situ* at physiological temperature, secondary physical crosslinking occurs via thermo-responsive aggregation of ELP to reinforce the network, resulting in a hydrogel with viscoelastic, stress-relaxation behavior. This novel, double-network hydrogel combines the advantages of traditional physical and covalent crosslinking and has a wide tuning range of storage moduli (~50 to 5,000 Pa below 5 wt% polymer). Chondrocytes encapsulated in ELP-HA hydrogels remain highly viable after injection and through 3 weeks of *in vitro* culture. The 3D cultures demonstrate positive staining for cartilage tissue markers (type II collagen and sGAG). These dynamically adaptable ELP-HA hydrogels will be useful in fundamental studies of cell mechanotransduction in response to viscoelastic, stress-relaxation properties and for injectable, minimally invasive, regenerative medicine applications.

### The Ultrasound Elastic Imaging Monitoring of an Injectable Cs/nhac Scaffold and Comparisons with Micro-ct, Dxa and Histological Changes

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Novel evaluation methods to assess the osteogenetic properties of injectable bone biomaterials *in situ* without the necessity for specimen analysis derived from a sacrificed animal are quite imperative. To achieve this goal, ultrasound elastic imaging (UEI), an emerging non-invasive evaluation method, was employed to measure the capacity of injectable bone biomaterials *in vivo*. A new type of thermosensitive injectable biomaterial based on chitosan and nano-hydroxyapatite, namely chitosan/nano-hydroxyapatite/collagen composites (CS/nHAC), was evaluated by UEI subcutaneously on the back of rats. Pure chitosan (CS) was used as the control. After being subcutaneously injected into the back of Wistar rats, the scaffolds were evaluated by a UEI scan at time points of 24 h, 1 week, 2 weeks, 4 weeks, 6 weeks and 8 weeks, and the specimens were harvested at weeks 4 and 8 for dual energy X-ray absorptiometry (DXA), micro-CT, histological and immunohistochemical analyses. The stiffness variation of the CS/nHAC and CS scaffolds was well measured by UEI. Moreover, the dynamic changes in the stiffness, volume and blood flow of injectable biomaterials revealed by UEI can be expectedly and correspondingly explained by the results from DXA, micro-CT, histological and immunohistochemical analyses. All data showed that the injectable CS/nHAC scaffold was superior to the CS scaffold in osteogenesis. UEI could well monitor and evaluate the mechanical behavior of ossificational and vascularizational changes of injectable biomaterials *in vivo*, thereby providing an effective, practical and non-invasive assessment method for further research of injectable bone biomaterials.

### Developing an *in vitro* Model to Measure Smooth Muscle Contraction

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Smooth muscle (SM) tissue is found in many parts of the body, primarily in sheets or bundles surrounding hollow organs. Its main function being the regulation of organ tone via its contractile state. Dysfunction of SM in diseases such as asthma and atherosclerosis affect millions worldwide.

Current methods for studying SM primarily rely on *ex vivo* animal tissues or 2D *in vitro* models. These 2D models are cultured on stiff surfaces lacking the elastic properties and 3D morphology found in natural extracellular matrix *in vivo*. Therefore it is desirable to develop both an *in vitro* model of SM that possesses the ability to contract and a method in which this contraction can be measured.

In order to achieve this, primary rat aortic SM cells were cultured in collagen hydrogels and cultured under tension in order to generate aligned SM collagen constructs. When stimulated with contractile agonists, these tissues contract in a uniaxial fashion. The design of the constructs allows them to be attached to a force transducer allowing the physical force of contraction to be measured. Reproducible force measurements have been made across multiple gels.

Electrospun scaffolds provide another method of aligning cells and have the advantage of forming a confluent layer of cells in which gap junctions can be formed, allowing the cells to behave as a syncytium. By adapting the previously described hydrogel model to use aligned electrospun gelatin based scaffolds, a reliable, reproducible method for measuring the contractile force generated by SM *in vitro* has been developed.

### Response of Cardiac Repair Cells Exposed to Macrophage-derived Bmp Proteins in 2d and 3d *In Vitro* Models

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**Objective:** Following cardiac injury, there is invasion of pro-inflammatory (M1s) and pro-healing (M2s) macrophages. Any engineered cardiac tissue will inevitably interact with this inflammatory environment. We recently elucidated some important roles of BMP molecules during macrophage recruitment and CM-macrophage interactions. However, these mechanisms are still poorly understood. Our goal is to study the effects of the inflammatory environment, in 2D and 3D systems, to improve cardiac patches.

**Methodology:** M1, M2A and M2C macrophages were differentiated from human peripheral blood [1]. Human pluripotent stem cells were differentiated into cardiomyocytes (CMs) [2]. Bioartificial cardiac tissues (BCTs) [3] composed of hydrogels, CMs, and pro-survival molecules were exposed to macrophage-conditioned media and analyzed via gene expression and immunohistochemistry.

**Results:** M1 macrophage-conditioned medium affected CM proliferation and differentiation via a mechanism mediated in part by M1-derived BMPs. When CMs were allowed to form BCTs, M1 conditioned-medium still affected the gene expression of specific markers related to CM differentiation and maturation. Additionally, when functionalized with recombinant BMP2 or BMP4, the maturation and functionality of the cardiac repair cells were modulated.

**Conclusions:** We propose a new role of macrophage-derived BMP proteins in the maturation and function of CMs in 2D and 3D systems. This platform represents a step towards validating a functional cardiac patch while harnessing the inflammatory microenvironment-derived BMP proteins.

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## Matrix-stiffness Driven Osteogenic Differentiation of Human Adipose Derived Stem Cells

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The phenotypic signature and function of stem cells are governed by their ability to integrate local micro-environmental signals, among matrix-mediated triggers, such as matrix biomechanics, were shown to influence the lineage specification of stem cells [1]. The interplay between cells and matrix becomes relevant when designing tunable extracellular matrix (ECM)-like platforms to mimic the regenerative cellular niche. Herein, we introduce a unique class of ECM-like substitutes that resemble the stress-stiffening behavior of biological networks [2]. These matrices are based on a highly versatile, thermoresponsive, synthetic polyisocyanopeptide (PIC) coupled with cell-adhesive molecules (RGD). We investigated the putative effect of PIC-RGD-based matrix-stiffness over stem cell behavior and differentiation potential towards the osteogenic lineage.

PIC-RGD hydrogels with low (20–30 Pa=soft) and high (200–300 Pa=hard) storage moduli ( $G'$ ) were loaded with human adipose derived stem cells (hASCs) and cultured in either expansion or osteogenic differentiation conditions. The surface marker profile of hASCs, as well as their morphology, proliferation rate and protein deposition, were evaluated prior (day 0) and after encapsulation (day 7). The osteogenic commitment was confirmed by calcium quantification (day 28).

We show that soft PIC-RGD sustained the immediate proliferation and arrangement of hASCs in 3D-like networks and reduced spontaneous differentiation compared to hard PIC-RGD where cellular spouting was delayed up to 7 days. Under such conditions, cells were privileged towards a strong osteogenic phenotype, characterized by a collagen-enriched matrix and subsequent strong mineralization.

Our results recognize the role of matrix-stiffness in lineage assignment and introduce PIC-RGD-based 3D platforms with tunable mechanical properties for tissue engineering applications.

## Injectable Hydrogel for the Delivery of Bone Anabolic Factors in Osteoporotic Bone

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Decreased bone strength and healing capacity make treatment of osteoporotic bone challenging. We aim to use a thermoreversible injectable hydrogel for the delivery of osteogenic drugs to stimulate bone healing locally and to increase implant stability in osteoporotic bone. Thermoreversible semi-synthetic hyaluronan-poly(*N*-isopropyl acrylamide) (HA-PNIPAM) with 15% w/v composition was designed to be a flowing liquid at room temperature and a stable physical gel when exposed to body temperature. The viscoelastic properties, gelling temperature and % of PNIPAM grafting onto HA dimers were characterized by rheology and <sup>1</sup>H NMR spectroscopy. The *in vitro* release studies of osteogenic factors, bone morphogenetic protein-2 (BMP-2), strontium ranelate and icariin showed a high retention of the drugs in the gel, with a release of 10–35% within 14 days. Cell culture studies with human mesenchymal stem cells (hMSCs) were conducted to define the effective concentrations of BMP-2, strontium ranelate and icariin to achieve osteogenic differentiation. Cell culture studies also confirmed the bioactivity of BMP-2 released from the hydrogel. Animal studies using the HA-PNIPAM hydrogel around a screw in osteoporotic rat tibia showed no adverse reaction to bone turnover as assessed by a longitudinal *in vivo* microCT. Further animal studies have been planned to test HA-PNIPAM gel for drug delivery in the same model. To conclude, HA-PNIPAM gel is a versatile release system for the study of osteogenic factors *in vitro* and presents no adverse effects on bone turnover *in vivo*, and is therefore a promising drug carrier to study influence of osteogenic factors in osteoporotic model.

## Tailoring Surface Nano-roughness of Electrospun Scaffold for Skeletal Tissue Engineering

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Electrospun nanofibrous scaffolds provide an alternative approach for skeletal tissue engineering. It is demonstrated that the creation of nanoscale surface features on polymer surfaces, which mimic the natural roughness of native tissues, can affect cell behavior such as cell morphology, proliferation and differentiation. In this research, electrospun polyethylene-oxide-terephthalate/polybutylene-terephthalate (PEOT/PBT) fibers with a similar fiber dimension but varied surface roughness (porous and grooved) were fabricated without any post-electrospinning treatment. Microscopy analysis and protein adsorption assay results showed that the higher the humidity applied during electrospinning the rougher the surface of fiber obtained, followed by higher protein adsorption properties. Human mesenchymal stromal cells (hMSCs) were seeded on scaffolds and cultured in osteogenic and chondrogenic differentiation media up to 21 days. Cell shapes and differentiation were investigated by biochemical and molecular assays showing a differential influence of surface topography on cell chondrogenic and osteogenic potential. This work shed light on the potential of controlling surface nano-roughness of electrospun scaffold for skeletal tissue engineering.

## The use of Expired Platelet Concentrates as a Cell Culture Supplement

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Bone-forming stem cells, such as mesenchymal stromal cells and human embryonic derived progenitor cells, possess characteristics that might aid in the development of novel medical treatments. Growing interest in these cells exists, making safe and efficient culture procedures necessary. It is important to eliminate animal derived products such as fetal bovine serum (FBS) from the culture process. In the Blood bank, we have been developing an alternative supplement from expired platelet concentrates that can be used instead of fetal bovine serum for expansion and differentiation of bone-forming cells. Bone marrow derived Mesenchymal stem cells (BM-MSC) and human embryonic derived progenitor (hES-MP) cells were expanded in culture media supplemented with either fetal bovine serum or platelet lysate from expired platelet concentrates obtained from the Blood bank, Reykjavik, Iceland. After expansion, the expression of surface markers and osteogenic, adipogenic and chondrogenic differentiation was evaluated as well as their ability to suppress mononuclear cell proliferation. Platelet lysates from expired platelet concentrates fully supported BM-MSC and hES-MP during expansion. Compared to cells grown in cultures supplemented with fetal bovine serum, the cells supplemented with platelet lysates maintained mesenchymal characteristics and exhibited equivalent growth and differentiation features. Furthermore, osteogenic differentiation of BM-MSC in osteogenic media supplemented with platelet lysates was superior to that of MSC osteogenesis when supplemented with fetal bovine serum. No significant difference was observed for hES-MP cells. Platelet lysates from expired platelet concentrates obtained directly from the Blood bank can replace fetal bovine serum for MSC and hES-MP cell culture.

#### Thermoresponsive, Photocrosslinkable and Tissue-Specific Extracellular Matrix Hydrogels

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The complex network of extracellular matrix (ECM) molecules plays specific multiple functional roles within each different tissue. In addition to providing structural support, the ECM regulates cellular processes that influence cell metabolic activity, proliferation, and differentiation. A main goal in developing ECM-based tissue engineering scaffolds is that they support and enhance specific tissue formation as well as offering technical processability [1]. We report here the development and characterization of a thermoresponsive and photocrosslinkable hydrogel comprised of ECM from different musculoskeletal tissues that is able to preserve the native tissue specific effect. Hyaline cartilage, meniscus fibrocartilage and patella tendon were decellularized and urea-extracted to prepare a soluble ECM fraction. Compared to commonly used proteolytic extraction, urea extraction preserved non-collagenous, low molecular weight components of the ECM. The urea-insoluble fraction, mostly collagen, was then solubilized and underwent a methacrylation reaction to functionalize amine groups to produce a photocrosslinkable biomaterial. Human bone marrow mesenchymal stem cell *in vitro* differentiation tests revealed that treatment with the urea extracts resulted in tissue specific effects. The collagenous methacrylated hydrogel, first gelled at 37°C and then UV crosslinked, provided a 3D mechanically stable hydrogel that overcame the shrinkage effect of embedded cells. The ECM-methacrylated material may be used as a promising bioink for 3D printing of cartilage that possess suitable physical hydrogel properties, while preserving native tissue specific effects for the stem cell microenvironment.

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#### Development of Janus Hydrogel Microcapsules as Novel Biomaterials-Stem Cells Construct for 3D Co-culture Applications

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Co-cultures of two or more cell types and biodegradable biomaterials of natural origin have been successfully combined to recreate tissue microenvironments. Segregated co-cultures are preferred over conventional mixed ones in order to better control the degree of homotypic and heterotypic interactions. Hydrogel-based systems in particular, have gained much attention to mimic tissue-specific microenvironments and they can be microengineered by innovative bottom-up approaches such as microfluidics. In this study, we developed bi-compartmentalized (Janus) hydrogel microcapsules of methacrylated hyaluronic acid (MeHA)/methacrylated-chitosan (MeCh) blended with marine-origin collagen by droplet-based microfluidics co-flow. Human adipose stem cells (hASCs) and microvascular endothelial cells (hMVECs) were co-encapsulated to create platforms of study relevant for vascularized bone tissue engineering. A specially designed Janus-droplet generator chip was used to fabricate the microcapsules (<250 µm units) and Janus-gradient co-cultures of hASCs: hMVECs were generated in various ratios (90:10; 75:25; 50:50; 25:75; 10:90), through an automated microfluidic flow controller (Elveflow microfluidics system). Such monodisperse 3D co-culture systems were optimized regarding cell number and culture media specific for concomitant maintenance of both phenotypes to establish effective cell-cell (homotypic and heterotypic) and cell-materials interactions. Cellular parameters such as viability, matrix deposition, mineralization and hMVECs re-organization in tube-like structures, were enhanced by blending MeHA/MeCh with marine-origin collagen and increasing hASCs: hMVECs co-culture gradient had significant impact on it. Such Janus hybrid hydrogel microcapsules can be used as a platform to investigate biomaterials interactions with distinct combined cell populations.

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#### Nanotopography Modulates Cell Collective Migration

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Topological cues play important roles in cell migration. Structures at nanometer level demonstrated promising effect to induce individual fibroblasts and mesenchymal cells to migration along its orientation. Here, we report that nanotopography can promote epithelial cell collective migration in a different manner. The migration of epithelial cell sheets on nanogratings was promoted when cell sheet migrated across the nano grating structures. On the other hand, cell sheet migration was impaired when cell sheet migrated along the grating structures. We found this epithelial collective migration is dominated by the lamellipodia protrusion at cell leading edge and the actin bundle recycling at cell rear end. We found this nanotopography promoted cell sheet migration is both Rac1-GTPase activity dependent and cell adhesion dependent. In sum, we suggest the use of nanostructured biomaterials in promoting cell collective migration and corresponding nanostructures could be used in different applications, such as the wound-healing process, and the tissue regeneration.

#### Development of Injectable and Resorbable Calcium Phosphate Cements Incorporating Carbohydrate Porogens for Designer Degradability

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Calcium phosphate cements (CPCs) have been extensively used to fabricate injectable scaffolds for craniofacial bone regeneration. In order to enhance the rate of CPC degradation and simultaneously favor bone tissue ingrowth, the addition of several porogens has been investigated. However, most porogens interfere with cement setting, degrade over months, and hence the porosity of the resulting composite is limited.

In this work, we investigated the use of carbohydrate microparticles (CMs) as fast-degrading water-soluble porogens for injectable CPCs and characterized their effect on cement setting, formation of a porous network following porogen leaching, and physicochemical properties of the resulting composite.

Composites were tested on setting time, cohesiveness, mechanical strength, thermogravimetric analyses (CM loading efficiency), microcomputed tomography (open and closed porosity, and pore interconnectivity) as well as leached in phosphate buffered saline (PBS) at 37°C.

CMs were produced either with sucrose or glucose, resulting in an average particle diameter of  $134 \pm 9 \mu\text{m}$  and  $149 \pm 8 \mu\text{m}$ , respectively. CM/CPCs (20/80 and 40/60 weight ratio) were prepared and showed porogen loading efficiency close to 100%. Furthermore, micro-computed tomography and thermogravimetric analyses proved that CMs dissolve very rapidly (<5 days) even when incorporated within CPCs, resulting in the formation of a CM/CPC composites with macroporosity after cement setting and porogen leaching.

In conclusion, this study demonstrated the ability to fabricate CM/CPC composites with tunable physicochemical properties while producing porous CPCs with designer porosity and degradability. Future studies will investigate CM/CPCs biocompatibility both *in vitro* and *in vivo*.

#### Potential use of miR-146b-5p to Enhance Articular Cartilage Regeneration

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Articular cartilage injury results in loss of chondrocytes and diminishment of accompanying specialised extracellular matrix and can result in an osteoarthritic phenotype. Articular cartilage is not sufficiently replaced by endogenous regeneration, stem cells have been considered as a propitious option for articular cartilage regeneration. MicroRNAs are a class of small non-coding RNA which regulate gene expression post transcriptionally and shown to be important in the proliferation and differentiation of stem cells. Critically, identification of microRNAs which influence stem cell fate offers new approaches for *in vitro* and *in vivo* application of microRNAs to regenerate cartilage and may have significant implications in disorders such as osteoarthritis. Using human skeletal stem cells from six independent patient samples cultured in an *in vitro* micromass set-up using TGF- $\beta$ 3 across 21 days we have identified, for the first time, using TaqMan microRNA qRT-PCR, microRNA-146b-5p as being significantly down-regulated during the process of chondrogenesis with a target gene, transcription factor; SOX5. With western blotting we have shown a ~50% reduction in SOX5 protein expression upon overexpression of miR-146b-5p through transient transfection. Additionally we have identified a ~200 fold increase in expression of miR-146b-5p in human articular chondrocytes from osteoarthritic donors validated by up-regulated expression of metalloprotease MMP13; thus having involvement in the pathogenesis of osteoarthritis. Ultimately application of miR-146b-5p with other identified microRNAs involved in chondrogenesis in combination with stem cell therapy could help the enhancement of regeneration of cartilaginous tissue. Additionally miR-146b-5p could serve as a potential therapeutic target in the treatment of osteoarthritis.

#### New Skin Equivalent Models to Study The Effect of Environmental Changes and Aging on Skin

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Severe skin damages such as burns can be a fatal risk because skin protects our body from external harmful stimuli and maintains physiological homeostasis. For that reason, 3D skin models have been developed and they are widely used for the basic physiological research and clinical applications. By using two types of reconstructed skin models, we tried to find out the physiological changes of skin according to external environment and internal intrinsic aging process.

First, we developed a full-thickness skin equivalent (SE) model consisted of dermis and epidermis to understand the effect of humidity and temperature to skin. Then, through dermal and epidermal modification, we developed full-thickness pigmented skin equivalent (PSE) models - neonatal and aged model - to study the relationship between aging and skin pigmentation. As a result, abnormalities in gene expression related to the epidermal barrier function and epidermal differentiation were observed under low humidity and temperature condition. These changes also led to the reduction of epidermal thickness and abnormal stratum corneum structure. Moreover, skin pigmentation was increased according to the cellular aging. The darkness (L-value), epidermal melanin content, and melanin synthesis related gene expression were increased in aged model. The expression profile of inflammatory cytokines showed significant difference between neonatal and aged models.

These results showed that skin barrier function can be affected by external environment and skin aging can induce epidermal hyperpigmentation. These results provide a new insight that can protect skin from environmental changes and improve the skin pigmentation disorders according to aging.

#### Curved Microneedle Patches by 3D Printing for Transdermal Drug Delivery

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Microneedle arrays (MNAs), as a minimally invasive approach, allows for enhanced transdermal drug delivery by forming aqueous channels through the stratum corneum to allow drugs to diffuse to the dermal microcirculation. Current methods of fabricating microneedles such as micromoulding or lithography have only been able to produce needles on flat planar surfaces. To further its potential applications, it is imperative that MNAs be curved to fit the contours of various anatomical parts of the human body in order to allow for better contact of the microneedles with the skin surface and further enhance efficacy of drug delivery. 3D Printing, with its flexibility in allowing for various materials (e.g. metals and polymers) and precise control of geometries can be applied in achieving the required curvatures on MNAs. In our study, we developed a simple approach of designing contoured microneedle arrays that are personalized to individual body parts and fabricated a device meant to be worn on the head through the use of 3D printing. Feasibility of this approach was explored by first fabricating macro-sized needles on curved surfaces using Fused Deposition Modeling, then followed by scale-down of the method to fabricate microneedles using Digital Light Processing, which has a higher print resolution. The fabricated microneedles were found to possess suitable mechanical strengths for skin penetration. In addition, its safety on skin applications has been demonstrated by using cytotoxicity assays.

#### Different Solvent use in Relation to Biomechanical Characteristics of Plga Scaffolds

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Electrospinning (ES) is a technique used for creating scaffolds for tissue engineering. However, the solvent for producing the fibers can interfere in their morphology.

This study has aimed to analyze mesenchymal stem cell (MSC) growth on poly(lactic-co-glycolic acid) (PLGA) electrospun fibers produced with different solvents.

Two combinations of solvents were used to produce PLGA 14% scaffolds: tetrahydrofuran:dimethylformamide 3:1 (TD) and dichloromethane:ethanol 8:2 (DE). MSCs cultivated on a well plate were used as control. The fibers were fabricated at 25°C, using 12 V, a flow rate of 1.14 mL/h and 15 cm distance between the needle and collector plate. Fiber morphology was analyzed by scanning electron microscopy. 106 MSCs were seeded on the scaffolds. After two days, cell viability and adhesion were measured by MTT and DAPI assays, respectively. Cytotoxicity tests are in progress. The DE scaffolds showed a mean thickness of 93 µm and fiber diameter of 543 ± 177 nm, while the TD scaffolds were shown to be thicker, with 106 µm ( $p < 0.001$ ) with a smaller fiber diameter, 301 ± 113 nm ( $p < 0.001$ ). Only the DE scaffolds showed beads. Cell viability was equal in both scaffold types ( $p = 0.73$ ) but lower than the control ( $p < 0.01$ ). DAPI assay showed that the DE scaffolds presented more attached cells than the TD scaffolds.

In the DE scaffolds, with smaller thickness and larger nanofiber diameter, more cells were attached. These findings could be explained by the larger pore size in these scaffolds, which may facilitate oxygenation for the attached cells.

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#### Preliminary Tests with Stem Cell Therapies for the Treatment of Skin Lesions in an Animal Model

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During the last few years, we have evaluated two different approaches applying epidermal stem cells (ESC) and adipose-derived stem cells (ADSC) on preliminary tests in rats to evaluate the regeneration on a full depth skin model of cutaneous ulcer in the interscapular area. To test these therapies, stem cells were isolated from skin or adipose tissue from adult Sprague-Dawley rats and grown *in vitro*. Cell identification was performed by immunodetection with CD71, CD49 and cytokeratins 10, 14 and 15 for ESCs and CD29 for ADSCs. To assess skin regeneration with ESC, a plasma-based matrix (PBM) with fibroblasts and keratinocytes, a PBM with only fibroblasts or a PBM without cells was applied. A control group without treatment was used. To evaluate the regeneration induced by ADSC, the injury was treated with cells injected directly into the wound. Neobol<sup>®</sup> was used as a positive control and a saline solution was used as a negative control. In both cases, the treatment progression was assessed by measuring the wound's size at least four times during two weeks of treatment. Macroscopic evaluation and histological analysis of areas where either cellular treatment was applied showed faster improvement compared to controls. In the specific case of the ESC experiment, the PBM with fibroblasts and keratinocytes showed a better regeneration, overall. Interestingly, areas treated with ADSCs evidenced presence of vascularization which was not seen in the control samples. These experiments constitute the first steps in Costa Rica towards evidencing the potential of both kinds of cellular therapies.

#### Development of a Three-dimensional Cell Culture Model that Mimics Female Genital Mucosae

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**Background:** Vaginal mucosae are often exposed to chemicals, microorganisms and viruses that induce inflammation and enhance women's susceptibility to infections. Most studies on female genital mucosae responses were performed on available animal models or *ex vivo* models. Using a unique self-assembly technique, we report here the production of a fully autologous 3D model that is specific for studying vaginal mucosae responses.

**Objectives:** 1- To validate that our human 3D model mimics female genital mucosae, 2- To better understand susceptibility to pre-exposure prophylaxis (PrEP) agents.

**Methodology:** Vaginal stromal and epithelial cells were isolated from vaginal biopsies of the same HIV-1-negative donor. Stromal cells were cultured and allowed to form their own extracellular matrix, and vaginal epithelium was seeded on top of the stromal layer and differentiated *in vitro* using growth factors and various estrogen concentrations.

**Results:** Mucus secretion and the presence of glycogen were validated using PAS staining. Immunofluorescence using antibodies against cytokeratin, Lamin and collagen identified the three key structures of the equivalent (epithelium, basement membrane and stromal component). Primary human monocyte-derived macrophages (MDM) infected with HIV-1 or free HIV-1 viral load were co-cultured on the apical surface of our equivalents to measure virus transcytosis 24, 48, 72 h post-infection in the multilayer vaginal model.

**Conclusion:** Our experimental model indicates that the 3D vaginal equivalent engineered in our laboratory is similar to native vaginal tissue, and can be used to study intravaginal delivery of PrEP, microbicides, contraceptives and/or anti-HIV agents.

#### Engineering 2D Cardiac Tissues using Biomimetic Protein Micropatterns Based on the ECM in the Embryonic Heart

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A primary goal of cardiac tissue engineering is the formation of aligned cardiomyocytes capable of synchronous contractions and maximal force production. Previous work engineering cardiac tissues on 2D surfaces have primarily used fibronectin micropatterned onto the substrate in 20 µm wide lines of alternating high and low protein density (20x20) to induce cell alignment in cardiomyocyte monolayers. We hypothesized that micropattern dimensions closer to the diameter of fibronectin fibrils in embryonic heart tissue, when heart muscle forms *in vivo*, would improve cardiomyocyte alignment. To test this, two micropatterns were designed; (i) 2 µm wide lines with 2 µm spacing (2x2) and (ii) a biomimetic pattern derived from 3D images of fibronectin fibrils in the 5-day-old embryonic chick heart. Results showed that the 2x2 pattern produced higher cardiomyocyte alignment compared to 20x20 controls. Further, we observed that unlike other patterns, the biomimetic pattern's ability to align cardiomyocytes was dependent on cell density, producing poorly aligned cells in low-density cultures and well aligned cells in high-density confluent monolayers. Additionally, inhibition of cell-cell interactions using anti-N-cadherin blocking antibodies decreased cardiomyocyte alignment at intermediate cell densities, suggesting that cell-cell interactions can play an important role in the formation of aligned myocardium. In the future we will use human induced pluripotent stem cell-derived cardiomyocytes to engineer more clinically-relevant human heart muscle and analyze physiological characteristics of the tissues including contractile force and action potential propagation.

#### Bioprinting the Heart: Applications in Tissue Fabrication and Organs-on-a-chip

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Heart is one of the most vital organs in human body, functioning as a biological pump that circulates blood via the cardiovascular system throughout all other organs to maintain their homeostasis. Therefore, in order to repair injured heart and understand its responses towards pharmaceutical compounds, it is of paramount importance to engineer cardiac tissues possessing not only strong biological relevance but also high degree of functionality. Here we have developed a novel method based on 3D bioprinting to build a porous scaffold with multilayer interlacing hydrogel microfibers made from custom-designed bioink. By tuning the aspect ratio of the unit cells of the scaffolds we could induce better alignment of the cardiomyocytes seeded on the surface of the microfibers in the interstitial space of the scaffold. Importantly, the endothelial cells printed inside the microfibrous frames formed the lumen structure resembling the vasculature over the course of culture. We further designed a microfluidic cardiac bioreactor embedding a pair of Pt electrodes to promote the maturation of the bioprinted cardiac construct via continuously applied flow and electrical stimulation. Computational simulations were performed to identify the most suitable stimulation conditions. The cardiac tissues primed in the bioreactor exhibited improved maturation, stronger expression of sarcomeric  $\alpha$ -actinin, and enhanced spontaneous beating. We believe our heart-on-a-chip platform in conjunction with bioprinting and induced pluripotent stem cells would provide an enabling technology for the construction of next-generation functional, vascularized cardiac tissues for applications in regenerative medicine and personalized drug screening.

#### Injectable Peptide Hydrogel for the Treatment of Barrett's Oesophagus

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Barrett's is a precancerous condition whereby a change in the epithelium is observed caused by gastro-oesophageal reflux disease. Current surgical treatments elicit rapid inflammatory responses resulting in subsequent strictures. Though this can be suppressed by direct injection of steroids, an intervention is required to improve treatment outcome. In this study, we propose the use of synthetic peptide hydrogels, which are injectable, mimic nano-architectural structure of extracellular matrix, are able to provide a protective barrier to the treated tissue area and encourage rapid re-epithelialisation.

A variety of synthetic peptides were dissolved at 30 mg/mL in HPLC grade water and neutralized to allow hydrogel formation. Following buffering in culture media overnight, primary rat oesophageal fibroblasts were incorporated into the peptide hydrogels, whereas epithelial cells were seeded on top mimicking the *in vivo* arrangement of these cell types. Cell-gel constructs were cultured for 3 days after which, cell viability and metabolic activity was monitored. Mechanical/degradation properties of hydrogels were also assessed in acellular and cellular conditions.

The inherent mechanical properties and peptide sequence of the hydrogels influenced cell viability (stromal fibroblasts and epithelial cells). Stromal fibroblasts were homogeneously incorporated into hydrogels and demonstrated good cell viability and typical morphology. Epithelial cells proliferated well and formed a sheet on top of the hydrogels, whilst retaining morphology and cell-cell tight junctions.

Assessment of these peptides provides a platform to further develop a composite hydrogel system with incorporated anti-inflammatory properties, which will be tested further under simulated gastric conditions.

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#### Tissue Engineered of Normal Immortalized Oral Keratinocytes (OKF 6) Co-Cultured at an Air-Liquid Interface with Normal Human Primary Oral Fibroblasts (pHOF): Potential Model for Osteonecrosis of the Jaw Studies

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Osteonecrosis of the jaw (ONJ) has been associated with bisphosphonate (BP) treatment of Paget's disease of bone, bone metastases, as well as postmenopausal and steroid-induced osteoporosis. The inhibition of bone resorption has been effectively employed in bone-disease treatment, but bisphosphonate-specific toxicity on oral mucosa causes impaired soft tissue healing followed by the onset of inflammation. The modern generation of BPs that contain a nitrogen group (N-BPs) have been intravenously administered to improve the management and prevention of complications associated with bone cancer metastasis, but they are potent and accumulate in the bone. This study presents results of the construction of a three-dimensional model comprised of an air-liquid interface co-culture of normal immortalized oral keratinocytes (OKF 6) and normal human primary oral fibroblasts (pHOF). Co-culture OKF 6 and pHOF in tissue construct form stratified epithelia and expressed differentiation-related protein expression involucrin mimicking histological appearance of normal oral mucosa. The long-term goal of the project is to investigate the effect of a commonly prescribed N-BP, zoledronate, on the tissue model's viability, inflammatory cytokine release, and senescence. When each of the OKF6 and pHOF monolayer cultures were exposed to zoledronate at increasing dosage (10, 30, 50, and 70 microM), viability was reduced proportionally as measured by the PrestoBlue<sup>®</sup> assay. Further analysis will be conducted to characterize the model's response to zoledronate with the hope of testing topical rescue approaches.

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#### Spermidine Cross-Linked Gellan Gum Hydrogels with a Broad Range of Moduli for Studying the Influence of Matrix Stiffness on Adipose-Derived Stromal Cell Fate

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As well as soluble factors, the physical and biochemical properties of the extracellular matrix (ECM) are heavily implicated in directing cellular behaviours, including focal adhesion formation, cytoskeleton assembly, proliferation and differentiation<sup>1</sup>. Numerous studies have demonstrated that the elastic modulus of ECM has a significant impact on mesenchymal stem cell fate, with stiff matrices ( $\geq 30$  kPa) directing osteo- or chondrogenic differentiation, and compliant matrices ( $\sim 5$  kPa) tending to influence adipo- or neurogenesis. This correlates with the broad range of moduli of human tissues ( $>30$  orders of magnitude:  $\sim 167$  Pa, breast;  $\sim 5.4$  GPa, cortical bone). Adipose-derived stromal cells (hASC) have great potential in tissue engineering and regenerative medicine due to their high accessibility and yield compared to bone marrow-derived stromal cells<sup>2</sup>. By harnessing cell-ECM interactions, it may be possible to gain control over hASC fate by designing scaffolds with appropriate physical properties, without need for costly differentiation media. Gellan gum is a biocompatible, degradable, anionic polysaccharide, widely used in food products. In the present study, gellan gum hydrogels containing spermidine cross-linker<sup>3</sup> and high sucrose levels were investigated for their effects on matrix stiffness. Hydrogels containing 0.25/0.50/0.75% gellan gum, 10/50% sucrose and 0.01/0.05% spermidine (w/v) were successfully synthesised. Well-gelated hydrogels with a broad range of moduli ( $\sim 20$ –114 kPa) and failure stresses ( $\sim 3$ –26 kPa) and a low degradation rate were achieved, with increasing concentrations of each component resulting in increased mechanical properties. Early results

indicate high cytocompatibility of encapsulated hASC. On-going gene expression and histoimmunohistochemical analyses are assessing the differentiation of hASC in response to matrix stiffness.

#### Effects of Dexamethasone on Tissue Engineered Skeletal Muscle Units

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Volumetric muscle loss (VML), muscular trauma that overwhelms the native repair mechanism, necessitates surgical intervention. Limitations to current VML treatments present a need for exogenous Sylgard substrate sources. To date, engineered muscle produces forces substantially lower than native muscle, limiting its potential for repair. In this study, we examined the trophic effects of dexamethasone (DEX), a glucocorticoid that stimulates myoblast differentiation and fusion into myotubes, on our tissue engineered skeletal muscle units (SMUs). Using our established SMU fabrication protocol, muscle isolates were exposed to four DEX concentrations (0, 5, 10, and 25 nM). Following seeding onto a laminin-coated Sylgard substrate, DEX addition was initiated on days 0 or 6 in growth medium or on day 9 after the switch to differentiation medium and was sustained until SMU formation. During fabrication, proliferation was measured with a BrdU assay, and myogenesis was observed through immunostaining for MyoD, desmin, and  $\alpha$ -actinin. After SMU formation, isometric tetanic force production was recorded to quantify functional improvement. The addition of 10 nM DEX beginning on either day 0 or 6 yielded optimal SMUs. These SMUs demonstrated advanced sarcomeric structure, a 42% increase in myotube diameter, and a 108% increase in myotube fusion index, compared to untreated controls. Additionally, these SMUs exhibited a 6-fold rise in force production. Thus, we have demonstrated that DEX improves myogenesis and increases force production in our SMUs. Future research will focus on understanding the mechanism behind these effects and evaluating the regenerative potential of these SMUs *in vivo*.

#### Constructing Complex *In Vitro* Microenvironments using Optical Micromanipulation

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The accurate study of cellular microenvironments is limited by the lack of technologies that can manipulate cells in 3D at a sufficiently small length scale. We have previously demonstrated the ability of a non-destructive optical micromanipulation technique to simultaneously position multiple cells into 3D patterns at high resolution. We have now applied this technique to construct and analyze embryonic and adult stem cell *in vitro* micro-environmental analogues. We demonstrate how the architectural organization of small numbers of embryonic stem cells (mES) determines downstream proliferative and early differentiation activity. Polymer microparticles were also positioned within these structures generating temporally controlled chemical microenvironments inducing additional changes in aggregate activity. Adult tissue analogues were generated by positioning polymeric and extracellular matrix based materials with multiple cell types forming sophisticated multicellular models. We have also developed higher throughput functionality into this technology expanding the potential applications to areas such as drug screening as well as higher replicate capacity. The continued application of this technology will enable novel insights into biological microenvironments by allowing researchers to form complex architectures with sub-micron precision of cells, matrices and molecules.

#### The Effect of ECM and Mechanobiology on VIC Nodule Formation

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Aortic valve stenosis caused by calcific nodule formation is the leading cause of aortic valve replacement surgery [1]. To reduce the need for surgery and to better understand calcific aortic valve disease (CAVD), we have created *in vitro* models to mimic disease-state changes such as an increase in tissue stiffness, collagen content, and proteoglycan content. In our previous work, on 2D polyacrylamide gels, we demonstrated the effect of substrate stiffness and ECM composition on nodule growth. Initial nodule development is hindered when valve interstitial cells (VICs) are grown on hyaluronic acid (HA) as compared to collagen; however, nodule growth is seen to drastically increase at later time points on HA gels. Novel imaging techniques allowed for nodule staining in live cell culture and the assessment of nodule development over time. A greater distribution of nodule size is seen on HA and periostin gels as compared to collagen, but, after three weeks in culture, HA gels have a greater average nodule size. Current and ongoing work involves the use of a 3D, silk-based, hydrogel system designed to mimic the mechanics of healthy valve (5 kPa) and diseased valve (35 kPa) tissue. Initial results indicate an increase in cell activation on stiffer substrates when grown with collagen. Ultimately, we seek to assess the role of compositional changes to the ECM proteins in promoting VIC calcification with the end goal of potentially identifying a new therapeutic target for CAVD.

[1] Lloyd-Jones *et al.* Circulation. 2010; 121:e46-e215.

#### Continuous Electrojet Writing as a Robust Technique for Bio-Interface Fabrication

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With growing demands for tailored cellular microenvironments in bioengineering, there is tremendous potential in combining nanotechnology and new biomaterial fabrication techniques to construct the defined biochemical and physical inputs of an extracellular matrix (ECM). Here we introduce a new direct writing technique, continuous electrojet writing (cEJW), to directly "print out" designed biomimetic fibril structures. In particular, the fibril resolution can be tuned crossing three orders of magnitude, from 100 nm to hundreds of microns. This technique can be applied to a wide range of materials, including proteins such as gelatine and collagen type-I, biodegradable polyesters and various synthetic polymers. Furthermore, cEJW can be performed on a variety of material substrates, ranging from conductive and insulating materials to pre-fabricated structures such as PDMS and hydrogels pre-casted with microchannels. Comparing to conventional electrospinning methods with operation voltages exceeding 1 kV, cEJW enables low operation voltages of typically sub-100V which permits direct printing of electric-sensitive materials and bio-elements. cEJW opens up the potential of incorporating well-defined ECM-like structures within microfluidic devices to reconstitute a tissue-to-tissue interface *in vitro*, further extending to applications in tissue scaffolds and organ-on-chips.

#### Patterning of Neurons on Diamond-like Carbon by Pulsed Laser Ablation

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Development of electrodes for direct interface with the brain has the potential to greatly improve prostheses for victims of spinal

injury. However, development of such electrodes relies upon careful engineering of the biological-electronic interface with control of scarring, cell phenotype and tissue organisation. Diamond-like-carbon (DLC) may be an ideal material for coating electrodes as it has flexible electrical properties, it is extremely stable *in vivo* and is already used as a coating for implants. Chemical modification is essential, however, to control and promote adhesion, differentiation and organisation of neurons and glia.

A combination of atom transfer radical polymerisation (ATRP) and pulsed laser ablation (PLA) was used to modify the surface properties of DLC with a high degree of spatial control. DLC was rendered non-fouling through functionalisation with polyethylene glycol (PEG) brushes. The top layer of DLC was ablated to reveal patterns onto which polyornithine and laminin were adsorbed. NG108 cells and neural stem cells (NSCs) adhered onto ablated patterns and adopted oriented morphologies. Upon differentiation the NG108s expressed  $\beta$ 3-tubulin and remained confined to the ablated patterns. In contrast, over time the NSCs differentiated but broke free of the patterns and populated the entire ablated region. However, extremely long neurites were observed following topographical cues from the ablated tracks over several hundred micrometres. Spatial control over neuron cell adhesion and differentiation was achieved on DLC using a combination of ATRP and pulsed laser ablation, demonstrating the potential for preparing *in vitro* neural networks on DLC with a great degree of control.

#### Bilayered Collagen-Hyaluronate Scaffolds: A Novel Platform for 3D *In Vitro* Respiratory Tissue Modelling

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Improved, physiologically-relevant three-dimensional (3D) airway models are required to provide better tools for the development of novel inhalable therapeutics. Accordingly, this study applied tissue engineering strategies to achieve three objectives: (i) the development and characterisation of a bilayered collagen-hyaluronate (CHyA-B) scaffold for 3D airway cell culture recapitulating the proximal airway; (ii) the assessment of the growth of a functional airway epithelium in 3D; and (iii) the creation of an epithelial-fibroblast co-culture model. CHyA-B scaffolds were fabricated by a specialised freeze-drying process to create a bilayered film on top of a porous scaffold. The film layer was seeded with the Calu-3 bronchial epithelial cell line and the porous sub-layer with Wi38 fibroblasts for culture at an air-liquid interface. SEM imaging confirmed a bilayered scaffold structure with a dense top-layer of 20–50  $\mu$ m thickness and a porous sub-layer with a mean pore diameter of 100  $\mu$ m. Mechanical testing demonstrated adhesive strength between the two layers. Calu-3 cells proliferated on the scaffolds, with histological analysis revealing a tight epithelial monolayer formation. Additionally, significantly increased MUC5AC gene expression was observed, compared to conventional culture. Furthermore, immunohistochemical staining detected mucus secretion, indicating cellular functionalization. Co-cultured Wi38 fibroblasts were observed in the porous sub-layer at 14 days, reflecting *in vivo* bronchial tissue structure, and cell-cell crosstalk is predicted to increase epithelial barrier strength. Overall, CHyA-B scaffolds are a platform technology that incorporates an extracellular matrix analogue with multiple co-cultured airway cell types to provide an improved 3D *in vitro* model of the bronchial region of the respiratory tract.

#### Using a 3d Co-culture Model to Simulate Hypoxic-ischemic Cns Injury

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Hypoxic-ischemic brain injury in preterm infants is a significant cause of childhood disability, associated at the cellular level with a

selective loss of oligodendrocyte precursor cells (OPCs) and activation of astrocytes and microglia. Progress in developing therapeutic interventions is hampered by the lack of available experimental models in which treatments can be tested in a controlled and reliable manner. 3D culture models recreate key cellular features of the CNS tissue environment, allowing neuronal and glial cells *in vitro* to behave similarly to their *in vivo* counterparts [1,2,3]. Mixed glial cells obtained from cerebral cortices of neonatal rats were maintained in collagen hydrogels and subjected to a low oxygen and low glucose environment for 24 h to simulate hypoxic-ischaemic injury. Live/dead analyses were combined with immunofluorescence staining to identify specific cell types within the co-culture population and to enable the quantification of markers of glial responses. Conditions were optimised in order to recreate the selective OPC death and reactive gliosis associated with the disease condition. The ability of potential therapeutic compounds to reduce OPC death or alter the timing and extent of reactive gliosis was tested. This approach provides a simple, consistent 3D co-culture tissue model that can be used as an experimental platform for neurobiological research, mimicking cellular aspects of hypoxic-ischaemic brain injury in babies.

[1] East *et al.* (2009) Journal of Tissue Engineering and Regenerative Medicine 3(8), 634–646.

[2] East *et al.* (2013) Regenerative Medicine 8(6), 739–746.

[3] Haw *et al.* (2014) Journal of Neuroinflammation 11, article no. 134.

#### Biomaterials Substrate Topography Enhances Neuronal Differentiation of Pluripotent Stem Cells and Neural Progenitor Cells

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Biomaterials substrates can be modified to provide suitable mechanical cues for efficient manipulation of stem cell responses in the *in vitro* stem cell niche. We are interested in studying the response of human pluripotent stem cells (hPSCs) and murine neural progenitor cells (mNPCs) to substrate topography in the presence of neural differentiation media. A Multi ARCHitectural (MARC) chip containing fields of various geometries and size was developed to investigate the influence of topography spatial arrangement on differentiation. Human ESCs and mNPC grown on anisotropic patterns such as micro-gratings expressed significantly higher percentage of b-III-tubulin and microtubule-associated protein 2 (MAP2) positive neuronal cells and lower percentage of glial fibrillary acidic protein (GFAP) positive glial cells, thereby supporting neuronal differentiation. The percentage of GFAP positive cells were significantly higher than the neuronal cells when hPSCs or mNPCs were grown on the isotropic patterns such as wells, thus supporting glial differentiation. In the presence of FGF-8 and SHH, highest percentage of neuronal (b-III-tubulin positive) and dopaminergic (tyrosinehydroxylase positive) populations were generated on the micro-gratings.

The temporal presentation of topography may also play a significant role in the differentiation. The rate of neuronal differentiation of hPSCs on gratings was examined. The topography-contact during differentiation induced a higher level of neuronal maturation

compared to culture with only topography-priming. The results suggested that the topography contact during the differentiation period is necessary and significant for topography induced differentiation, and the effect of topography-priming and topography-contact will be additive in enhancing the differentiation.

### Investigating Cell Fate Decisions through Microenvironmental Control and High-Content Biophysical Analysis

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There is a growing appreciation that microenvironmental factors play a major role during cell-fate decisions, but the cellular micro-environment is notoriously multivariate, with complex signaling occurring among cells, soluble factors, and the surrounding proteinaceous matrix. Progress in directing cell fate in regenerative medicine has been hindered by an inability to uncouple and rationally control distinct signaling and biophysical cues.

We have developed a biomaterials based platform for high-content, single-cell analysis of cellular biophysics and decision-making by adapting microcontact printing ( $\mu$ CP) technology into the format of standard 6- to 96-well plates ( $\mu$ CP Well Plates). As a chemically defined platform,  $\mu$ CP Well Plates allow for rational and independent control of cell geometry, substrate composition, and soluble factors and enable straightforward analysis of thousands of uniform single-cells per well-plate. Combining this platform with live-cell fluorescent imaging, we perform high-content biophysical analysis of cell bodies, nuclei, and chromatin. We find that this multi-level biophysical analysis can be used to discriminate cells in discrete epigenetic states during human somatic cell reprogramming. Further, single cell patterning reveals that core nuclear properties can be controlled by altering cell geometry alone.

Understanding how cells integrate biophysical and biochemical cues during cell-fate decisions is critically important to a wide variety of regenerative medicine approaches. Our data offer insights into the interplay of these cues during decision-making processes and present biomaterial-based systems for their rational control.

### Core Shell Dual Drug Delivery Scaffolds for Bone Tissue Engineering

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Scaffolds that stimulate osteogenesis as well as angiogenesis have been shown to be a promising approach for bone tissue engineering. For this purpose, a core shell structured hydrogel scaffold composed of alginate and calcium phosphate was designed, allowing the encapsulation of ions/growth factors to be delivered through a diffusion dependent mechanism. The fiber like hydrogel was crosslinked when placed in contact with a calcium and cobalt chloride solution. Bone morphogenetic protein 2 was loaded into the core structure to stimulate osteogenesis, whereas the outer shell of the structure (as well as the core) was cross-linked with different concentrations of cobalt to promote angiogenesis. BMP2 release was shown to be sustained, only releasing 20% of the protein after 21 days, whereas cobalt had a rather fast initial release. Cobalt stimulated angiogenesis, showing increased expressions of angiogenic proteins and genes. The release of cobalt showed a synergistic effect with BMP2 in promoting osteogenesis both *in vitro* and *in vivo*. The results confirmed the importance of using cobalt as an angiogenic element, which when combined with osteogenic factor like BMP2, can stimulate synergistically bone tissue regeneration.

### 3D Microfabrication and Cell Polarity Formation in Multicellular Spheroids

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Highly- and randomly-packed hepatocytes in multicellular spheroids (MCSs) are prevented from not only gas and nutrients exchange but also cell polarity formation. The objective of this study is evaluation of the effect of sinusoid-like and hepatic cord-like microstructures on cell polarity establishment in the MCSs. We decided to use mouse E14.5 fetal hepatocytes in this study because they are sensitive to cell polarity. Alginate hydrogel beads (20  $\mu$ m in diameter) were made by an inkjet system. The MCSs were formed by injecting 1  $\mu$ l fresh medium containing 1000 fetal hepatocytes and same number of hydrogel beads into the 3% methylcellulose medium including hepatic differentiation factors, like oncostatin M [1]. After 7 days culture, we observed microstructures and cell polarity in the MCSs with hematoxylin-eosin (HE) staining and immunostaining using anti-E-cadherin, anti-multi-drug resistance protein 2, anti-ezrin and anti-CD13 antibodies. Albumin secretion rate was determined by enzyme-linked immunosorbent assay method. The results of HE staining showed MCSs formed without hydrogel beads showed extensive cell death. This was thought to be caused by abnormal cell polarity formation. The MCSs containing hydrogel beads exhibited sinusoid-like spaces and mono to bi-layered cord-like structures, and cell death was obviously suppressed. Immunostaining indicated that cell polarity was not fully but partially established. Improvement of cell viability was also supported by the albumin secretion data. Based on these data, we concluded that fabrication of sinusoid-like and hepatic cord-like microstructures helped cell polarity acquisition in the MCSs.

1. Kojima N, Takeuchi S, Sakai Y, Actuat. B-Chem. 198, 249, 2014.

### Multi-Scale Vascular Network Model Created by 3D Bioprinting and Its Applications in Tissue Engineering

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Integrating vascular hierarchical structures spanning arteries down to capillaries can provide better vascular perfusion and more physiological tissue architectures. To create multi-scale vascular network, we developed a 3D bioprinting method to first construct larger (lumen size of 0.5–1 mm) vascular channels and then, to create adjacent capillary network through a natural maturation process, thus providing a feasible solution to connect capillary to the large vascular channels. In the model, micro-vascular bed was formed in between two large perfused vessels, and then connected to the vessels by angiogenic sprouting from the large channel edge. The large fluidic vascular channels had a tight, confluent endothelium lining, presenting selective barrier functions under the physiological flow culture condition. The model demonstrated faster and broader diffusion pattern through a thick tissue filled with high density of cells, presenting the functional significance of this model on perfusion and supporting tissue viability. Our bioprinting technology has a great potential in engineering vascularized thick tissues and vascular niches, as the vascular channels are simultaneously created while cells and matrices are printed around the channels in desired 3D patterns. To demonstrate this potential, we developed an advanced bioprinting method to create a vascularized skin tissue with epidermis, dermis, and a multi-scale vascular network. We also developed a 3D glioma-vascular niche model to investigate cell-cell interactions between vascular cells and glioblastoma multiforme cells. The 3D vascular niche platform can be adapted to other biological systems and will be used as a valuable tool to model cell-cell interactions and to control microenvironment.

### Serum Albumin Coated Allograft Augments Bone Formation and Reduces Donor Site Pain after Harvesting Bone-tendon-bone Autografts

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Donor site pain affects about 30% of the patients after bone-tendon-bone (BTB) harvesting during anterior cruciate ligament surgery. We compared the functional and morphological differences between donor sites filled with blood clot, autograft chips, or serum albumin coated bone allograft in a double-blind human study of 40 patients undergoing ACL surgery. The patella site was either filled with albumin coated allograft or with blood clot. Knee function was evaluated by the IKDC, Lysholm and VISA scores as well as a visual analog scale of pain during standing, kneeling or crouching at 6 weeks and at 6 months post-op. Both the patients and the evaluators were blinded towards the treatment group. At 6 weeks the patients were still under recovery from the operation itself and the overall knee function was compromised, however, the IKDC, VISA and pain scores were significantly better in the treatment group. At six months, the knee functional scores were close to normal, with a slight but significant improvement in the IKDC score in the treatment group. More importantly, kneeling pain was still prominent in the controls but was significantly lower in the treatment group. Computed tomography showed significantly smaller bone defects and higher density new bone in the albumin treated groups. Results from the current study showed that donor site pain, a most disturbing long-term side effect of BTB surgery is significantly less if the bone buildup in the patella and the tibia is augmented by serum albumin coated bone allografts.

#### **Pulsed Focused Ultrasound Enhances Stem Cell Homing to Skeletal Muscle in a Murine Model of Muscular Dystrophy and Homing is Suppressed by Ibuprofen**

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Homing of iv-infused stem cells to diseased tissues is an obstacle to successful cell therapy. Mechanotransduction from pulsed focused ultrasound (pFUS) in skeletal muscle locally increases chemoattractants (cytokines, chemokines, cell adhesion molecules) that induce mesenchymal stromal cell (MSC) homing. Stem cell therapies for muscular dystrophy (MD) are promising, but are hampered by poor cell homing and the need for direct injections that are impractical clinically. Furthermore, drugs for MD could potentially interfere with molecular homing cues and cell tropism to undermine cell therapies. MDX mice (9 weeks) received pFUS (1 MHz, 5 MPa, 10 ms pulse, 5% duty cycle, VIFU 2000) to hamstrings with and without ibuprofen (30 mg/kg po) 15 min pre-pFUS. Mice received 10<sup>6</sup> human MSC 3 hr post-pFUS. pFUS stimulates complex molecular biological responses in dystrophic muscle consisting of pro-inflammatory cytokines and growth factors. Minimal cell homing was observed to skeletal muscle in MDX mice, but pFUS treatment enhanced MSC homing by ~15 fold (p<0.001). Numerous cells were observed in interstitial spaces between myofibers. pFUS failed to increase cell homing when mice were pretreated with ibuprofen. pFUS-enhanced homing of stem cells to dystrophic skeletal muscle may represent an ideal platform to deliver cells to tissues that are perpetually in a sub-acute or chronic inflammatory states that not otherwise conducive to active homing of cellular therapies. Additionally, anti-inflammatory drugs are a clinical standard-of-care to treat MD. Ibuprofen suppression of pFUS-enhanced cell homing in MD reveals negative drug/host interactions that are uncontrolled for in clinical trials developing MD cell therapies.

#### **A Two-Step Method to Improve the Interface Strength of Engineered Bone-to-Bone Ligaments**

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Tissue engineered bone-to-bone ligaments are a promising graft source for anterior cruciate ligament (ACL) reconstruction. However, progress is hampered by the inability to recreate the enthesis, the interface between ligament and bone, *in vitro*. We hypothesized that engineering ligaments with additional tissue at the enthesis would improve interface strength between the brushite and soft tissue. "Single anchor constructs" were first made by embedding ACL cells in fibrin cast around single brushite anchors. After 5 days, ACL cells were encapsulated in a 1 ml fibrin gel cast around two single anchor constructs to form "2 step" constructs. Control constructs were engineered using unmodified brushite anchors. An additional group was formed similarly but with additional cells to control for the increased cell number in 2 step constructs (400 K). After 14 days in culture, the 2 step constructs demonstrated significantly increased interface strength (+58%), UTS, and collagen content. The 400 K group did not show significant differences from controls. Embedding two 5 day old single anchors construct in fibrin revealed preferential cell migration along the line of tension between the two anchors. Histological examination also revealed a region dense in cells and collagen adjacent to the brushite-soft tissue interface present in 2 step constructs but not control constructs. In summary, we have demonstrated a method to improve interface strength and collagen content of engineered ligaments which our data suggests may be due to cell migration-driven remodeling of the extracellular matrix.

#### **Development of a Tissue-Specific Extracellular Matrix Based Hydrogel for the Enhancement of Peripheral Nerve Repair**

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Surgical reconstruction of damaged nerves is possible, but return to function is slow and often incomplete. A solution that improves return to function would significantly increase quality of life for affected patients. A nerve-specific extracellular matrix hydrogel (NS-ECM) was investigated for its ability to improve peripheral nerve reconstruction.

Porcine sciatic nerve was decellularized and processed into a hydrogel. The presence of nerve-specific structural and functional components within the hydrogel was analyzed using SEM, immunolabeling, and ELISA. Bioactivity of these components was tested *in vitro* through neurite outgrowth and Schwann cell migration assays. A rodent sciatic nerve injury model was used to assess NS-ECM as luminal filler for critical length nerve gap repair. A 15 mm gap was repaired on one side using a silicone conduit and filled with NS-ECM hydrogel, or sterile saline. At days 7, 14, 28, and 90, implants were excised and gastrocnemius muscles were harvested and weighed. The normal, uninjured contralateral side was used as a native control.

NS-ECM maintained nerve-specific structural and functional components following decellularization and processing and was found to promote neurite outgrowth and Schwann cell migration *in vitro*. NS-ECM treated animals displayed more robust tissue formation across the gap and greater infiltration of Schwann cells from the proximal stump. Results demonstrate that the application of NS-ECM reduced muscle atrophy compared to saline control (NS-ECM 22% ± 7%, Control 17% ± 0.3%).

NS-ECM provides an ideal environment for the regenerative process which occurs following nerve injury and has the potential to promote improved functional outcomes.

#### **Ultra-thin Tissue-Tumor Organoid Microfluidic Platforms For *In Vitro* Diagnostics**

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3D tissue models are increasingly being implemented for drug and toxicology testing. However, creation of tissue-engineered constructs for this purpose often rely on complex biofabrication techniques that are time consuming, expensive, and difficult to scale up. Here, we describe a strategy for realizing multiple tissue constructs in a parallel microfluidic platform using an approach that is simple and can be easily scaled for high-throughput formats. First, we fabricated parallel channel microfluidic devices, in which liver cells mixed with a UV-crosslinkable hydrogel solution were introduced to and photo-patterned through photomasks to produce stable tissue constructs *in situ*. Any remaining uncrosslinked material was washed away, leaving the structures in place. These constructs recapitulated some liver function, including albumin and urea production over a 7-day culture time course under recirculating media flow. As a proof of principle for toxicology analysis, we exposed the constructs to ethyl alcohol (0–500 mM) and showed that cell viability and secretion of urea and albumin decrease with increasing alcohol exposure while markers for cell damage increase. We have since implemented an alternative device that uses a low-cost fabrication approach to realize ultra-thin fluid channels and chambers. Within these devices, we have employed sequential photo-patterning to create miniature multi-zoned organoid and tissue-tumor organoid structures for chemotherapeutic screening studies. Miniaturization and parallelization of biofabrication and tissue culture techniques compatible with screening studies will be instrumental in efficiently adapting engineered 3D tissues to widespread drug and toxicology testing.

#### Injectable Hyaluronate-based Hydrogels For Cell Delivery

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Natural polymers have gained much attention to date for fabrication of injectable hydrogels useful in many biomedical applications, as they have excellent biocompatibility and biodegradability compared with synthetic polymers. Sodium hyaluronate is one of the most widely used natural polymers in drug delivery and tissue engineering applications. However, hyaluronate hydrogels can be typically formed by addition of excipient chemical cross-linking reagents, which may induce acute or chronic side effects. In this study, we fabricated hyaluronate hydrogels using only natural polysaccharides without additional cross-linking molecules. Hyaluronate was partially oxidized with sodium periodate to introduce aldehyde groups to the backbone. Hydrogels were prepared under physiological conditions by simply mixing solutions of oxidized hyaluronate and glycol chitosan via Schiff base formation between aldehyde groups in oxidized hyaluronate and amino groups in glycol chitosan. Various physicochemical characteristics of hyaluronate/glycol chitosan hydrogels were investigated *in vitro*. We found that the degree of oxidation, polymer concentration, as well as hyaluronate/glycol chitosan ratio were important to control the viscoelastic properties of the gels. We also found that viability of chondrocytes encapsulated in hyaluronate/glycol chitosan hydrogels was dependent on the stiffness of the gels. This approach may be broadly applicable to the development of injectable biomaterials.

#### *In Vivo* Structural and Cellular Remodeling of Engineered Bone-Ligament-Bone Constructs Used for Anterior Cruciate Ligament Reconstruction

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Anterior Cruciate Ligament (ACL) ruptures rank among the most prevalent and costly sports-related injuries. The tendon grafts cur-

rently used for ACL reconstruction may be limited by unsuitable biomechanical properties and poor integration with the host. Our laboratory has addressed this issue by engineering multiphasic bone-ligament-bone (BLB) constructs which, when used for ACL repair, develop structural and mechanical properties similar to the native ACL. The purpose of this study was to examine the acute remodeling process that occurs as the BLB grafts advance toward the adult ligament phenotype *in vivo*. To accomplish this, we implanted BLB constructs fabricated from male cells into female host sheep and allowed three days, one week, two weeks, and four weeks for recovery. At each time point, the BLB grafts (n = 4 per time point) were explanted for histological analysis. Picrosirius Red staining was used to quantify collagen alignment. Immunostaining was performed to examine the presence of elastin, collagen, vasculature, and innervation. Immunostaining for neutrophils as well as pro- and anti-inflammatory macrophages was used to characterize the host immune response, which we hypothesize may aid in the remodeling process. PCR analysis was performed to detect original male cell content in the grafts and migration into surrounding host tissue. These data revealed an acute process of macrophage and neutrophil infiltration, increasing collagen density and alignment, vascularization, and complete repopulation of the graft with host cells within two weeks. This study offers a better understanding of the processes that advance our constructs toward the adult ligament phenotype.

#### The Potential of the Synthetic Fibrin-like Gel in Wound Healing

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**Objective:** During the wound healing process, a fibrin clot forms and provides a primary matrix for cells, assisting them to migrate and permeate the wound site. With the rapid advances in synthetic biomaterials, the characteristics of natural fibrin are capable of being imitated. We propose the synthetic gel may hold the potential to accelerate skin wound healing.

**Methodology:** To synthesize fibrin-like biomolecular hydrogels, a gel network is formed by crosslinking two types of branched PEGs attached with two counter reactive substrates for factor XIIIa respectively. The incorporation of RGD and MMP cleavage sites therefore enables this synthetic hydrogel to mimic two essential biological functions of an ECM: cell adhesion and protease degradation. Human skin equivalent models, which are derived by culturing skin keratinocytes and fibroblasts at the air:liquid interface on a dermal scaffold, have been proposed to serve as useful *in vitro* models to recapitulate and further understand the complicated healing process of skin. The partial-thickness and full-thickness wounded human skin equivalent models created in this study were used to assess the potential of this synthetic fibrin-like gel in the wound healing situation.

**Results:** We have demonstrated that keratinocyte re-epithelialization in partial-thickness wound healing is improved by a synthetic fibrin-like gel. In addition, the migration and proliferation of fibroblasts and keratinocytes were increased in full-thickness wound healing when the synthetic fibrin-like gel was injected into the wound.

**Significance:** The novel treatments assessed in this project, with the ability to enhance healing, may be developed further for use in clinical applications.

#### Use of Carbohydrate Adjuvants to Improve Potency of Culture-expanded Adult Human Mesenchymal Stem Cells for Cell-based Therapies

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Despite an increase in the number of human mesenchymal stem cell (hMSC)-based therapies being trialed, there remains an inability to generate sufficient numbers of high quality hMSCs should a treatment gain market approval. Moreover, current best practice to cultivate therapeutic numbers of hMSCs can adversely affect their multipotentiality, immunomodulatory properties and clinical efficacy that is further hindering the potential of hMSC-based therapies. As such, there is now high demand for a cost-effective strategy that can generate a safe and efficacious supply of hMSCs to clinicians for immediate transplantation in quantities currently unavailable. Present strategies for hMSC expansion rely on either excessive doses of expensive, recombinant protein growth factors as culture supplements that readily degrade or immuno-selection methods that are costly and still require further post-selection expansion, often in the presence of growth factors. In this study we developed a strategy for the growth of hMSCs based on culture supplementation with a carbohydrate compound purified from commercial sources of porcine mucosal heparan sulfate (HS). Our data show that culture supplementation with this HS variant results in increased proliferation of hMSCs under serial passaging, while at the same time preserving their stem-like quality, all in the absence of costly exogenous growth factors.

#### Regulation of Human Nucleus Pulposus Cell Phenotype and Behavior by Laminin-Mimetic Peptide Coupled Substrates

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Nucleus pulposus (NP) cells of the intervertebral disc undergo a phenotypic and behavioral shift with age, changes thought to initiate disc degeneration. We have previously shown that soft substrates (~0.3 kPa) conjugated with full-length laminin (LM-111) promote matrix synthesis and expression of healthy NP molecular markers. Here, we identify LM-mimetic peptides that promote human NP cell attachment, and reveal how matrix stiffness and peptide specificity affect NP phenotype and behavior.

Peptide sequences engaging LM-specific cell receptors were screened by quantifying primary human NP cell attachment to adsorbed peptides (0.1–200 µg/mL, 1 hr attachment). Peptides with maximal cell attachment through integrin  $\alpha 3$  (TSP1:CGGFQGVLQNVRFVF and Pa3:CGGPPFLMLLKGSTR), integrin  $\alpha 6$  (AG10:CGGNRWH-SIYITRF), and syndecans (AG73:CGGRKRLQVLSIRT) were selected for further study. NP cells were seeded on peptide-coupled soft (0.3 kPa) or stiff (14 kPa) polyacrylamide hydrogels, cultured for four days, and analyzed for biosynthesis, morphology, and NP-specific markers.

Human NP cells attached to peptides TSP1, Pa3, AG10, and AG73 in a dose-dependent manner, with maximal cell attachment at 200 µg/mL peptide comparable to 65% cell attachment for LM-111 at 40 µg/mL. Substrate stiffness and ligand significantly affect sGAG production, with peptides TSP1 and AG10 at 0.3 kPa increasing sGAG from 5.7 µg/µg DNA on 14 kPa LM-111 to 17.7 and 20.8 µg/µg DNA respectively. Morphological and molecular marker expression similarly differed amongst substrates of different stiffnesses. Laminin-mimetic peptides are able to mediate human NP cell attachment, can increase NP cell biosynthesis when presented on a soft substrate, and may allow for the design of biomaterials that promote a healthy young NP phenotype for a variety of disc therapies.

#### Modulation of Fiber Organization within Electrospun Polycaprolactone/collagen Nanofiber Matrices to Facilitate the Integration of Nanofiber/cell Multilayered Constructs

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Electrospinning with the capability of producing fibers in the range of nanometers represents a mainstream to produce versatile nanofibrous matrices to recapture the key characteristics of extracellular matrix of native tissues. In recognition of the limited cell infiltration into such electrospun nanofiber matrices, it becomes highly desirable to generate large interstitial space. In this regard, the aim of this study was to differentially modulate the spatial organization of nanofibers within the mats to achieve large interfiber distances for their efficiency in forming the integrated 3D skin-like constructs. Polycaprolactone (PCL)/collagen nanofibers were collected onto various grounded conductive surfaces to obtain the nanofiber mats composed of random, aligned and meshed fibers. The fiber diameter, morphology, and pore size were characterized by Micromaster™ inverted digital microscopy and scanning electron microscopy (SEM). Rat fibroblasts or keratinocytes were seeded onto various electrospun fiber mats. Totally, 15 layers of cell-seeded nanofiber mats were assembled either with only fibroblasts or with a mixture of both cells. The assembled constructs were performed for tensile test, histological analysis, methylene blue and immunofluorescence staining. Compared to aligned and random fiber mats, meshed mats with the spinning time of 20 and 25 seconds showed sufficient mechanical strength with large pore size for cell infiltration, which resulted in significant improvement of the integration of cell/nanofiber layers. In conclusion, the meshed nanofiber mats show their advantages in promoting cell infiltration for better formation of 3D tissues.

#### Construction of Functional Liver Tissue Model by Cell Sheet Technology for Drug Test

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Liver has important functions in human body. For example, drug metabolism is one of the most important liver functions to maintain our health. Drug metabolism is a function to change the chemical structure of drugs by metabolic enzyme, and discharge drugs from the body to outside. However, the development of toxicity in the metabolic process sometimes becomes a clinical problem. Therefore, drug efficacy and drug toxicity test give an important thing in drug development. However, hepatocytes lose their biological function rapidly *in vitro* for drug screening. Therefore, the purpose of this study is to construct functional liver tissue model and drug testing device with cell sheet technology.

Firstly, we fabricate the hepatocyte cell sheet and the hepatocyte-endothelial co-cultured cell sheet. In order to mimic the liver *in vivo*, hepatocyte sheet was cultured between two co-culture cell sheets in static. As a result, while hepatocyte sheet in conventional culture lose its function in two weeks, multi-layered cell sheets sustained and improved their function of albumin secretion for 14 days.

Secondly, microfluidic device for perfusion culture was fabricated from poly-dimethylsiloxane (PDMS), and monolayer hepatocyte sheet was cultured within it for 7 days. As a result, hepatocyte sheet in perfusion culture improved the function of albumin secretion after 7 days.

By layering co-culture and perfusion culture, construction of tissue which is high-sensitive to drug toxicity had shown a promised potential for drug test.

#### Skin Substitute Model to Study the Maturation and Organization of the Elastin Network

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Elastic fibers form a complex network that contributes to the elasticity of connective tissues. Alterations in the elastic fiber network are involved in several diseases such as pseudoxanthoma elasticum, a genetic disorder characterized by a progressive mineralization of dermal connective tissues. The aim of this work was to develop an *in vitro* tissue-engineered skin substitute allowing to study maturation and organization of the elastin network. The basic model of skin reconstructed by the LOEX self-assembly approach consist to cultivate dermal fibroblasts, from newborn or adult skin biopsy, in the presence of ascorbic acid, which promotes extracellular matrix assembly and allows the formation of thick sheets of collagenous tissue. Keratinocytes with or without melanocytes were then added in order to reconstruct the epidermis. Since high ascorbic acid concentration decreases tropoelastin mRNA and prevents its attachment on the microfibrillar network, aldosterone and spironolactone were used with ascorbic acid as treatment to stimulate the elastogenesis through the IGF1 pathway. Immunohistochemical analysis of elastic fiber components in the basic model (without melanocyte) revealed that elastin and fibrillin-2 staining was practically absent in adult but strong in newborn reconstruction. Aldosterone and spironolactone combine with ascorbic acid increase the fibrillin-2 and elastin expression on the microfibrillar network. Addition of melanocytes influence the elastogenesis processes by increasing the elastin expression without treatment in newborn and adult reconstruction and was improved with aldosterone and spironolactone treatments. We concluded that our skin substitute model is a promising tool to study the maturation and the organization of the elastin network.

#### Evaluation of Delivery of Mesenchymal Stem Cells using Small-Gauge Needles: Tailoring Administration of Cell-Based Therapies for Efficient Clinical Translation

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Numerous cell therapy procedures use injection-based administration to deliver high density cell preparations. Administration of these therapies can pose major challenges, due to efficacy being dependent on cell vitality and function post-injection. Following on our previous work<sup>1</sup>, we used a comprehensive tool-set for assessment of cell delivery post-ejection, to allow clinicians to make informed judgements regarding administration requirements for clinical trials and answer critical questions regarding possible reasons for failure to deliver sufficient numbers of viable cells.

Primary human mesenchymal stem cell (hMSC) suspensions were drawn up into 100 µL Hamilton syringes with 30 and 34 gauge needles, before being ejected at controlled rates ranging from 10–300 µL/min. Effects of ejection rate and needle size were comprehensively evaluated, using various standard and multiplex assays, in terms of viability, apoptosis, senescence, and other parameters of cellular health. Ejections at the slower flow rates under investigation resulted in a significantly lower percentage of dose being delivered as viable cells among the ejection rates tested. Normalised caspase-3/7 activity measurements ejected at 10 µL/min were also significantly higher than control cells. Quantification of the differentiation of ejected hMSCs revealed that ejection rate exerts an effect upon cells' differentiation capacity, thereby possibly influencing success of cell-based therapies. This study conclusively shows that ejection rate has significant impact on the percentage of cell dose delivered and cellular health post-ejection, and therefore emphasises the importance of careful consideration of administration protocols required for successful cell delivery.

1 Amer, M., White, L. & Shakesheff, K., J Pharm Pharmacol, doi:10.1111/jphp.12362 (2015).

#### Comparative Analysis of Human Somatic Stem Cells-Derived from Different Tissues in Respect to Bone and Cartilage Tissue Engineering

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Somatic stem cells (SSCs) are characterized as undifferentiated cells with ability of long-term self-renewing and plasticity. Due to these properties SSCs represent promising tool for regenerative medicine. This study was aimed at analysis of characteristics of SSCs derived from bone marrow, adipose tissue and umbilical cord in respect to bone and cartilage tissue engineering. SSCs were isolated and cultured using standard protocols. They were maintained in D-MEM/F12 with 10% of FBS and antibiotics up to 3th passage. The proliferation was analyzed by CEDEX XS. The cell cycle and expression of selected markers was assessed by flow cytometry and morphology was analyzed by inverted microscope and TEM. Pellet cultures and chondrogenic medium containing TGF-β were used to induce chondrogenic differentiation. Osteogenic differentiation was induced by osteogenic medium (dexamethasone, L-ascorbic acid-2-phosphate and beta-glycerophosphate). Chondrogenic and osteogenic differentiation was analyzed by real-time PCR. SSCs from all sources were attached on the Petri dishes after 24 h and started to proliferate in colonies. After 4–8 days they reached 80% confluency and were subpassaged. In next 3 passages they displayed fibroblast-like morphology. They showed similar kinetics of proliferation and cell cycle course. They also shared expression of CD73, CD90 and CD105; and were negative for CD14, CD20, CD34 and CD45. TEM showed similar morphology. Cells from all sources underwent chondrogenic and osteogenic differentiation. In summary, SSCs from all analyzed sources shared biological properties. They had similar chondrogenic and osteogenic potential and therefore they are suitable for cartilage and bone regeneration.

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#### Temporospatial Control of Growth Factor Bioavailability Mediated by Biodegradable Semiconductor Hydrogels

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Semiconductor copper sulphide nanoparticles (CuSNPs) are a class of inorganic photo-absorbers that provide an alternative to gold nanoparticles (AuNPs) in converting optical energy into thermal energy. CuSNPs exhibit strong optical absorption at near infrared (NIR) wavelengths (650–1100 nm) originated from the d-d transitions of copper (Cu) ions and hence, unlike plasmonic nanomaterials, their optical properties are not influenced by nanoparticle size or shape. Moreover, CuSNPs are not dependent on the dielectric constant of the surrounding environment to absorb NIR light and can be metabolized in the body, thus overcoming the major drawbacks of AuNPs in photothermal therapy protocols. CuSNPs biodegradation, which involves a slow release of Cu ions, can account for attractive bioeffects in regenerative medicine by influencing cell proliferation, matrix remodeling or growth factor release from producing cells. In this work, CuSNPs were added as fillers within *in situ* polymerized fibrin hydrogels (CuSNPs-hydrogels). These matrices were used to encapsulate cells derived from a mesenchymal multipotent C3H/10T/1/2 cell line, which harbor a transgenic growth factor controlled by a heat-activated and rapamycin-dependent gene switch. In the presence of rapamycin, NIR irradiation of CuSNPs-hydrogels efficiently enabled photothermal regulation of transgenic growth factor bioavailability. Furthermore, biodegradation of CuSNPs embedded in fibrin matrices resulted in a sustained release of Cu ions which



promoted phenotypic changes in multipotent cells and dramatically influenced the dynamic remodeling of NIR-responsive scaffolds.

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#### **Acellular Muscular Tissue and Muscle Precursor Cells for *In Vivo* Regeneration**

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Numerous pathologies lead to considerable and invalidating loss of skeletal muscle -volumetric muscle loss (VML), which can be congenital (abdominal wall defects, diaphragmatic hernia, etc.) or acquired (large muscle resection due to tumours or traumas). Effective clinical treatments for these conditions are extremely limited to date. By preserving the complexity of environment and biofactors after tissue decellularization, natural scaffolds derived from skeletal muscles show great potential for the treatment of VML. Moreover, acellular muscle can be used as instructive environment in which seeded cells can lead to functional maturation of skeletal muscle *in vitro* and *in vivo*. We developed three different decellularization protocols to obtain acellular muscles: detergent-enzymatic treatment (DET), anti-polymerizing agent-enzymatic treatment (Lat B) and detergent alone (SDS). All the methods lead to efficient decellularization and good preservation of extracellular components and three-dimensional architecture of the tissue. To understand whether acellular scaffolds were able to support myogenesis *in vitro*, satellite cell-derived precursors were seeded and cultured into decellularised muscles in combination with other muscle resident cells. Although with different degree of outcome, all the scaffolds allowed cell engraftment, migration, proliferation and muscle differentiation. Moreover, to test the ability of unseeded or repopulated scaffolds to be transplanted *in vivo*, we also generated a mouse model of VML by surgically removing the EDL muscle, then replaced with our tissue engineered skeletal muscles. Preliminary results showed local muscle regeneration 3 months after implantation, suggesting great potential for decellularised muscles in muscle reconstruction and regenerative medicine strategies.

#### **Sortase Mediated Ligation to Covalently Tether Bioactive Proteins to 2D and 3D Hydrogel Systems**

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Synthetic extracellular matrices are widely used in regenerative medicine and as tools in building *in vitro* physiological culture models. Synthetic hydrogels display advantageous physical properties, but are challenging to modify with large peptides or proteins. Here, a facile, mild enzymatic tethering approach is presented. Sortase-mediated ligation was used to conjugate human epidermal growth factor fused to a GGG ligation motif (GGG-EGF) to preformed PEG hydrogels or PEG macromers containing the sortase LPRTG substrate. The reversibility of the sortase reaction was then exploited to cleave tethered EGF from the hydrogels for analysis. Analyses of the reaction supernatant and the post-ligation of preformed hydrogels showed that the amount of tethered EGF increases with increasing LPRTG in the hydrogel or GGG-EGF in the supernatant. Sortase-tethered EGF was biologically active, as demon-

strated by stimulation of DNA synthesis in a 2D culture of primary human hepatocytes and endometrial epithelial cells. In addition, results with the PEG macromers demonstrate that EGF integrates into 3D PEG hydrogels and that the EGF is released from crosslinked PEG-EGF macromers upon sortase reaction on the gels. Furthermore, the tethering of several EGF molecules to single PEG-stars macromers allows a local clustering effect for growth factor presentation to cells. The simplicity, specificity, and reversibility of sortase-mediated ligation and cleavage reactions make it an attractive approach for modification of hydrogel systems.

#### **The Role of Autophagy during Chondrodifferentiation of Human Mesenchymal Stem Cells**

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Cartilage lesions due to injury heal poorly, leading to early onset of osteoarthritis, chronic joint pain and disability. Mesenchymal stem cells (MSCs) in combination with polymeric carriers can be used to induce differentiation of the cells towards chondrogenesis. Autophagy is a cellular mechanism, required for differentiation of adult stem cells. Influencing autophagy during differentiation of MSC to chondrocytes may alter cell fate. Therefore, we investigated the effect of rapamycin, an autophagy activator, during differentiation of MSC in 3D microtissues to chondrocytes. Cartilaginous microtissues were prepared by mixing droplets of human MSCs in chitosan (sChi) with droplets of oxidized alginate (oxAlg). Microtissues were induced in chondrocyte differentiation media in presence and absence of rapamycin for 10, 14 and 21 days. Changes in gene and protein expression for collagen type II and X and autophagy markers Atg5 and LC3 were investigated by real-time-PCR and immunofluorescence. Upon chondroinduction, down regulation of key autophagy gene Atg5 was observed in the non-stimulated group. We observed an increased expression of collagen 2 and hypertrophic marker collagen X starting at day 10 with highest expression on day 21. Rapamycin treated group showed increased Atg5 gene expression with the highest peak at day 14. Treated microtissues exhibited better cell morphology and viability with high expression of Atg5 and punctated immunostaining pattern for LC3 confirming accumulation of autophagosomes. Our study demonstrates that autophagy plays an important role in chondrodifferentiation of MSC. Administration of rapamycin could serve as novel therapeutic approach for treating cartilage injuries for future clinical applications.

#### **Oxidative Stress in Intervertebral Disc Cells can be Inhibited by the Natural Polyphenol Epigallocatechin 3-gallate**

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Recently, cellular senescence and death has been shown to be positively correlated with intervertebral disc (IVD) degeneration and aging *in vivo*. Cells in degenerated discs often induce vascular ingrowth into an originally avascular tissue, which increases formation of reactive oxygen species and accelerates premature aging followed by cell death. We have previously described the anti-inflammatory and analgesic effect of the polyphenol epigallocatechin 3-gallate (EGCG) in IVDs. However, its effect on oxidative stress-induced senescence and cell death of primary IVD cells *in vitro* has not yet been studied, which is the aim of this project. Oxidative stress was induced by sub-lethal (25  $\mu$ M, 2 h) or lethal (50  $\mu$ M, 24 h) doses of H<sub>2</sub>O<sub>2</sub> (n=15). 10  $\mu$ M EGCG significantly increased the metabolic activity (MTT assay, p<0.05) and viability (PI/A flowcytometry, p<0.05) of stressed IVD cells *in vitro*. In order to determine the mechanism of EGCG action, the activity of EGCG in the acute stress phase (=antioxidant activity) and in the following recovery phase (=interaction with cellular components) was studied on an *in vitro*

model system of premature senescence, induced by sub-lethal oxidative stress. This model was established and validated using early (mitochondria depolarization, p53/p21 activation) and late (cell cycle arrest,  $\beta$ -galactosidase) senescence markers. Obtained results indicate that EGCG inhibits oxidative stress-induced senescence and death of IVD cells via its antioxidant activity (reduced  $\beta$ -galactosidase,  $p < 0.05$ ). In summary, an *in vitro* model system of premature senescence was successfully developed, validated and used to demonstrate the action of EGCG in oxidative stress response of primary IVD cells.

#### **Towards Modeling Limb Development: High-Throughput Microfluidic Platform for 3D Mesenchymal Stromal Cell Cultures**

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The first stages of embryonic limb development - namely cell condensation, undifferentiated proliferation and chondrogenesis - are tightly regulated by the interplay of specific signaling pathways (Wnt, FGF, TGF $\beta$ /BMP). The development of reliable *in vitro* models to screen the effect of such morpho-regulatory factors on stem/progenitor cells is crucial to elucidate and possibly recapitulate developmental processes. Towards this aim, we report an innovative microfluidic platform designed to generate and culture 3D stem/progenitor cells as perfused micromasses (PMMs), consisting of two functional elements: (i) a serial dilution generator (SDG) and (ii) a 3D culture region. Patterns of different combinations/concentrations of morphogens are generated through the SDG and delivered to downstream culture units, each comprised of 10 cubic microchambers (side 150  $\mu$ m). Human bone marrow-derived mesenchymal stromal cells (hBM-MSCs) cultured within microchambers were able to undergo spontaneous condensation within 3 hours upon seeding, generating PMMs uniform in size ( $77 \pm 15$  cells,  $\Phi = 56.2 \pm 3.9$   $\mu$ m). As compared to traditional static macropellet cultures, exposure to morphogens involved in the first stages of embryonic limb development (i.e., Wnt, FGF, TGF $\beta$ ) yielded more uniform and repeatable responses of 3D PMMs, and a 34-fold higher percentage of proliferating cells at day7. The use of a logarithmic SDG led to the identification of an unexpected concentration of TGF $\beta$ 3 (0.1 ng/ml) permissive to hBM-MSC proliferation and inductive to chondrogenesis. Allowing the investigation of developmental signals involved in the first stages of limb bud formation in a high-throughput fashion, this microfluidic platform represents a powerful tool towards the definition of 'developmental-inspired' approaches for skeletal tissue regeneration.

#### **Role of Intermediate Filaments in Mesodermal Differentiation of Pluripotent Stem Cells**

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Differentiation of pluripotent stem cells, a source for cell-based therapies, can be directed using mechanical forces. Our lab has shown that fluid shear stress, can promote pluripotent stem cell (ESC) differentiation towards the mesodermal lineage, specifically towards the endothelial phenotype 1,2. Others have demonstrated in mature endothelial cells that cytoskeletal actin microfilaments and vimentin, an intermediate filament, are remodeled or displaced by applied fluid flow. It is unknown, however, if fluid shear stress in PSCs also results in cytoskeletal remodeling of actin and vimentin. The objective of this study was to determine the effect of laminar shear stress on gene expression of smooth muscle actin and vimentin in pluripotent stem cells.

Pluripotent mouse embryonic stem cells (PSCs) were seeded onto collagen type IV-coated glass slides for 2 days and then exposed to fluid shear stress of 1.5 or 5.0 dynes/cm<sup>2</sup> for a subsequent 2 days.

Samples cultured under static conditions served as controls. At both shear stress magnitudes vimentin gene expression was significantly upregulated compared to controls however, changes in smooth muscle actin expression was not detected. Taken together with our previous findings of shear stress mediated mesodermal differentiation, this study suggests that vimentin, but not actin microfilaments, is required for mesodermal differentiation of pluripotent stem cells. Future studies with vimentin knockout or vimentin silenced ESCs would help elucidate the necessity of vimentin in differentiation toward mesodermal phenotypes. This in turn may help inform strategies to promote efficient differentiation toward specific phenotypes for tissue engineering and regenerative medicine therapies.

#### **A Tissue-engineered Microphysiological Platform for the Study of Human Organ Fibrosis**

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Organ fibrosis is a progressive, life-threatening medical condition characterized by excessive deposition of extracellular matrix (ECM) in the connective tissue, leading to impairment of normal organ architecture and function. Despite the increasing prevalence of fibrosis in various fatal diseases, our understanding of its development and progression remains rudimentary due to the failure of existing models to recapitulate complex human-relevant fibrotic responses. Here we present a microengineered modular platform that leverages three-dimensional (3D) cell culture in compartmentalized micro-devices to replicate organ-specific alterations in the micromechanics of stromal tissue during fibrosis. This system combines tissue-engineered 3D hydrogel constructs impregnated with human fibroblasts with perfusable microchannels to mimic the stromal-vascular and stromal-epithelial interface. The ability to tune fibrotic responses using this model was demonstrated by varying soluble microenvironment to form a normal stroma consisting of quiescent human lung fibroblasts (HLFs) or to induce the development of fibrotic foci comprised of proliferating HLFs and a dense ECM. Furthermore, we demonstrated the potential of this system for therapeutic screening by showing attenuated fibrotic responses via inhibition of integrin-mediated signaling known to promote organ fibrosis *in vivo*. Similar results were obtained using human hepatic stellate cells as a model of liver stroma. To better model the complexity of post-injury fibrogenesis *in vivo*, we incorporated M2-polarized macrophages and demonstrated their profibrotic influence on the stromal microenvironment. Our model serves as a robust platform for mechanistic investigation of fibrotic disease processes, as well as identification and screening of new therapeutics.

#### **A Mechanically Robust Injectable Hydrogel Scaffold for Adipose-Derived Stem Cell Delivery for the Treatment of Peripheral Arterial Disease**

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Peripheral arterial disease is a condition affecting the lower limbs that can lead to critical limb ischemia with high risks of amputation and mortality. A promising treatment strategy involves delivering adipose-derived stem cells (ASCs) to stimulate revascularization, healing, and functional recovery. However, when delivered in saline, ASC treatment has been limited by substantial cell loss following intramuscular injection.

To improve ASC retention and survival, a modular delivery scaffold was developed composed of two components: i) methacrylated glycol chitosan (MGC) functionalized with a cell adhesive peptide (GGGGRGDS), and ii) a diacrylate copolymer of poly(ethylene glycol) (PEG) and poly(trimethylene carbonate) (PTMC), in a triblock PTMC-*b*-PEG-*b*-PTMC configuration (PEG-PTMC) to provide mechanical robustness. To facilitate minimally invasive injectable delivery, a thermal initiator system of ammonium persulfate

(APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) was optimized.

Significant changes in equilibrium compressive modulus and tensile modulus of MGC were facilitated by blending with PEG-PTMC, enabling these properties to be aligned with that of native tissue (10–15 kPa). Strain at failure under compression and tension were significantly increased to 95% and 200%, respectively. During 1000 cycles of tensile loading, no change in modulus was observed. By varying APS and TEMED concentrations, crosslinking time upon heating was controlled between 1 to 10 minutes. Viability and retention of ASCs following injection, crosslinking, and encapsulation was measured over 7 days *in vitro*. These results suggest that this modular system would provide an effective, minimally invasive means to delivery ASCs in a scaffold that is suited to the loading conditions of the lower limb.

### Hybrid Collagen-alginate-hyaluronan Hydrogels for Sustained Maintenance of Chondrocytes

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Redifferentiation of chondrocytes outside their native microenvironment is a critical aspect that restricts their therapeutic application for cartilage tissue engineering. Here, we provide an engineered composite 3D hydrogel matrix that combines collagen with an alginate based hydrogel, in order to provide encapsulated chondrocytes similar microenvironments to those found in native tissue. Results showed that the incorporation of collagen mimicked to a higher extent the mechanical properties of native cartilage. Costochondral chondrocytes were encapsulated inside the hydrogels, showing increased proliferation as well as chondrocyte spreading in the collagen containing hydrogels. Furthermore, higher levels of chondrogenic associated molecules were found compared to the pristine hydrogels after 21 days. Chondrogenic gene expression was also enhanced at 7 and 21 days, although it was considerably down-regulated at 21 days. Therefore, the study shows that hybrid matrices mimicking native microenvironments of native tissue are promising platforms for chondrocytes culture and therapeutic delivery for cartilage tissue engineering.

### Combining Mesenchymal Stromal Cell Infusions with Pulsed Focused Ultrasound to Kidneys Increases MSC Production of Interleukin 10 to Improve Acute Kidney Injury

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Pulsed focused ultrasound (pFUS) to murine kidneys before *iv* infusion of mesenchymal stromal cells (MSC) enhances MSC homing to kidneys during cisplatin (CIS)-induced acute kidney injury (AKI), improves prevention and AKI, and better rescues established AKI compared to MSC alone. Combination pFUS+MSC reduces inflammation and alters immune cell profiles to anti-inflammatory phenotypes. However, molecular mechanisms behind combination therapy remain unknown. C3H mice were given AKI by cisplatin (15 mg/kg). Kidneys were treated with pFUS (1 MHz, 5% dc, 100 pulses) followed by *iv* injection of 10<sup>6</sup> human MSC. pFUS alone increased mouse interferon gamma (INFg) in kidneys and pFUS+MSC-treated kidneys had significantly more human interleukin (IL)-10 than with MSC alone ( $p < 0.001$ ). To test the hypothesis that INFg elevations in pFUS-treated kidneys stimulates subsequently-infused MSC to produce IL10, INFg-KO mice with AKI were treated with MSC with or without pFUS. pFUS+MSC did not increase IL10 from MSC or improve AKI compared to MSC alone. However, incubating MSC cultures with mouse INFg before

infusion restored human IL10 production in the INFg-KO mouse and improved AKI outcomes. To examine the link between INFg and IL10, IL10 was knocked down in MSC given to wild-type mice with and without pFUS. Even in with pFUS-elevated INFg levels, pFUS+MSC (with IL10 knockdown) failed to improve AKI outcomes compared to MSC without pFUS. These data suggest that improved AKI outcomes through combination MSC/pFUS therapy is the result of pFUS increasing INFg levels in the kidney to stimulate production of anti-inflammatory IL10 by MSC that home to the kidney.

### Differentiation of Mouse Embryonic Stem Cells in Simulated Microgravity

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This work tests the hypothesis that the proliferation and differentiation of mouse embryonic stem cells (mESCs) towards a definitive endoderm phenotype, a precursor of alveolar epithelium, will be significantly enhanced when cells, encapsulated or as suspension cultures, are maintained in rotating wall vessel (RWV) bioreactors. When maintained as suspension cultures in the RWV, mESCs formed embryoid-body like structures. Encapsulation in either alginate or collagen type-I spherical hydrogel beads did not affect mESC viability. Following encapsulation, the cells were cultured under static conditions or in a RWV bioreactor for 6 to 11 days. After six days of spontaneous differentiation (in the absence of LIF), non-encapsulated cells showed reduced expression of the endodermal marker *Foxa2* in RWV, as compared to static culture. Similarly, in encapsulated cells (collagen and alginate) in RWV, ectodermal (*Zic-1*) and mesodermal (*Brachyury T*) markers were upregulated in comparison to endodermal marker (*Foxa2*). Collagen beads showed increased mass transport properties, resulting in 150% more viable cells than in alginate. To induce endoderm-specific differentiation, cells were treated with A549 cell-derived conditioned media. After six days, the expression of a definitive endoderm marker (*Cxcr4*) in collagen was 2 folds greater in the RWV than under static conditions. By day 11 the cells exhibited significantly higher expression of a panel of definitive endoderm markers (*Foxa2*, *Sox17*, *Cxcr4*) in alginate beads than in collagen beads. We suggest that 3D culture in RWV bioreactors is an important tool for enhancing the efficacy of endodermal differentiation of embryonic stem cells. (\*Guimaraes & Stabler: equal contribution).

### Human Chondrocytes and Dental Pulp Stem Cells Enhance their Chondrogenic Differentiation Potential via a Two-Step Fusion Culture Technique

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Cartilage tissue engineering based on isolated and culture-expanded chondrocytes or mesenchymal stem cells has been studied in various *in vitro* models, but the quality differs with respect to the morphology and the physiology of the synthesized tissues. The aim of our study was to promote *in vitro* chondrogenesis of human articular chondrocytes using a novel scaffold-free three-dimensional (3-D) cultivation system in combination with chondrogenic differentiation factors. Moreover, this innovative culture system was applied on human dental pulp stem cells (DPSCs).

Chondrocytes and third molar derived DPSCs were expanded in monolayer culture and then transferred to a scaffold-free 3D-culture in the presence, or absence, of TGF- $\beta$  and BMP-2. Cartilage-like spheroids were formed and cultured as individual aggregates or as bigger microtissues engineered via a special fusion technique based on gravity.

Conducting these tissue engineering processes with chondrocytes, the fused tissues were morphologically similar to native hyaline cartilage and showed a higher differentiation degree compared to individual spheroids. These highly differentiated fused microtissues contained S100-positive chondrocytes embedded in a cartilaginous matrix (proteoglycans and collagen type II). However, in case of DPSCs after 21 days in fusion culture, *in vitro* chondrogenesis was just initiated, indicated by the expression of the early differentiation transcription factor Sox9 and the production of cartilage-specific proteoglycans.

Our fusion culture system enhanced *in vitro* chondrogenesis of human articular chondrocytes and DPSCs after expansion in a monolayer culture. These 3-D hyaline cartilage-like microtissues may be useful for *in vitro* tissue engineering or even for clinical application in cell-based therapies.

#### **Micropatterned Nerve Guidance Conduit Incorporate with Neurotrophic Gradient and Schwann cells to Facilitate Peripheral Nerve Regeneration**

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The current gold standard treatment in clinic for peripheral nerve injury is autologous nerve graft. Although there is no immune response after graft and complete extracellular matrix and Schwann cells are preserved in the nerve tissue, limited amount of available nerve and possible neuroma formation accompanied with permanent functional loss at donor site are the main concerns. In these years, the nerve guidance conduit has been developed as an alternative way to repair peripheral nerve. However, without incorporating proper stimulating factors, the prognosis is still not very satisfied. In this study, a biodegradable polymer (poly (glycerol-co-sebacic acid), PGS) based novel nerve guidance conduit has been developed and characterized. By incorporating three stimulating factors: 1) micro-patterned surface, that can directionally guide the regenerated axon as physical cue; 2) neurotrophic gradient membrane, that can continually attract regenerated axon outgrowth from the proximal to distal stump as chemical cue; 3) Schwann cells, that can support the growth of regenerated neurite and form myelin sheath around regenerated axon as biological cue, we expect that this scaffold can be used as a promising nerve guidance conduit for peripheral nerve regeneration. The results showed that the micro-patterned surface with specific dimensions of channels and chambers can be fabricated with good uniformity, the neurotrophic gradient distribution on gelatin membrane was successfully achieved, and Schwann cells can be successfully inoculated in designed micro-patterned area. The promoting effect for neurite outgrowth and extension by combining these three stimulating factors will be studied in this research work.

#### **Gradients of Decellularized Cartilage and Demineralized Bone Matrix in Microsphere-Based Scaffolds for Regeneration of the Osteochondral Interface in Rabbits**

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Extracellular matrices (ECM) such as decellularized cartilage (DCC) and demineralized bone matrix (DBM) have been gaining

interest as scaffolds in the field of osteochondral regeneration due to their ability to provide instructive cues for guiding cellular function. However, these ECM scaffolds lack sufficient mechanical properties to support joint function during tissue regeneration. Alternately, scaffolds made from synthetic polymers are robust but lack signals for directing cell differentiation. Therefore, we hypothesized that the incorporation of opposing gradients of DCC and DBM in polymeric microsphere-based scaffolds would provide both biological and structural signals to the surrounding cells thus, leading to regionalized tissue formation when implanted *in vivo*. We fabricated poly(D,L-lactic-co-glycolic acid) (PLGA) microsphere-based scaffolds with encapsulated DCC and DBM in opposing gradients, and implanted them into 3.5 x 2 mm (Diameter x Height) osteochondral defects in rabbit knees for 12 weeks, with analyses including gross morphology, mechanical properties and histology. All animals exhibited normal movement during the 12-week period. Gross signs of inflammation or infection were not evident upon visual inspection of the joint surface at the time of tissue retrieval. The synovial fluid had a normal color in all the rabbits except for one rabbit that had yellowish fluid in one of the knees. No differences were observed between the PLGA-only and DCC/DBM gradient groups in terms of gross morphology. Mechanical and histological analyses are in progress to further test our hypothesis, and will be included in the presentation.

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#### **Pre-vascularized Tissue Model with Complex Microarchitecture via 3D Bio-printing**

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Vascularization has been the bottleneck for tissue engineering of viable and functional tissues, especially for long-term *in vitro* or *in vivo* investigations. I will present an advanced 3D bioprinting system-microscale continuous optical printing ( $\mu$ COP) and its application in the fabrication of prevascularized tissue models with complex microarchitectures. The  $\mu$ COP platform allows rapid and scalable fabrication of highly-specified biomimetic structures. We utilize naturally derived biomaterials (hyaluronic acid methacrylate, gelatin methacrylate, etc.) with appropriate mechanical, biological and chemical properties to create the cell-friendly microenvironment to guide cell proliferation and promote tissue function. With our technology, we are able to precisely pattern multiple cell types in a heterogeneous 3D distribution without post-fabrication perfusion or seeding. Our results demonstrate that this prevascularization approach by our  $\mu$ COP platform can support vasculature-like endothelial network both *in vivo* and *in vitro*. For *in vivo* study, anastomosis between pre-formed vasculature and the host circulation can be induced. We also demonstrate that prevascularization can facilitate the cell survival and function, especially for tissues with active metabolism, such as cardiac tissues.

#### **Novel 3d Printed Bioceramic Scaffolds as *In Vitro* Models for Bone Tissue Regeneration**

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The use of porous three-dimensional bioceramic scaffolds to support bone tissue growth and regeneration is a longstanding area of interest. Nevertheless there are still some limitations in their use, particularly related to the mechanical loads that these materials can

withstand. Together with the development of novel bioceramic formulations, the aim of this research was the fabrication and characterisation of customised ceramic scaffolds for bone tissue engineering applications using 3D powder printing technology.

Two silicate-based glasses (NCL2 and NCL7) containing different elemental combinations: Al and Fe (to improve the mechanical properties), Cu and Zn (important for maintaining the bone matrix and density), and also Ca, Na, K, Mg and Mn (essential elements for all living organisms) were produced along with apatite wollastonite (AW) used as comparison material. Each formulation was processed to "green" parts using a ZPrinter® 310 Plus 3D printer. Subsequently the green bodies were sintered to obtain consolidated 3D porous structures. The scaffolds exhibited architecture and porosity (~35%) values comparable to those of natural bone as demonstrated by SEM and micro CT investigations. Interestingly, the *in vitro* biocompatibility assessed using MC3T3 cell line, indicated a biological positive response with a cell viability of ~70% after 7 days similarly to AW. Evidences of cell-material interaction were evaluated using confocal microscopy. The 3D printed silicate-based scaffolds exhibited suitable architecture and good mechanical and biological properties indicating their potential for bone reconstruction applications.

### The Paracrine Relationships that Mesenchymal Stem Cells Establish with Osteoblasts and Endothelial Cells are Influenced by Biomaterials Features

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Bone tissue engineering approaches involve culturing human mesenchymal stem cells (hMSC) on support structures. Upon implantation, transplanted hMSC establish paracrine relationships with neighboring host osteoblasts and endothelial cells. It remains largely unknown whether these cross-talks are modulated by the features of the substrates that harbor hMSC. In this study, hMSC from bone marrow were seeded on porous polystyrene or flat polyester substrates and then co-cultured with human osteoblasts (hOB) or endothelial cells (HUVEC) using a transwell system that allows humoral interactions of both cell types without direct cell contact. Co-cultures of hOB and hMSC seeded on porous substrates contained higher levels of procollagen type I than co-cultures harboring hMSC seeded on flat surfaces. Viability and alkaline phosphatase activity of hOB co-cultured with hMSC seeded on porous polystyrene were higher than in hOB co-cultured with hMSC seeded on flat substrates while the levels of TGF- $\beta$  and the degree of cell layer mineralization were lower. The OPG/sRANKL ratio in co-culture media was substantially lower when hMSC were seeded on polystyrene scaffolds and M-CSF levels increased. Survival and migration ability of HUVEC were higher in co-cultures of hOB and hMSC seeded on porous substrates. Tubule formation was enhanced when HUVEC were incubated with conditioned media from hMSC seeded in polystyrene scaffolds. Altogether, these data indicate that the structural features of substrates influence the paracrine interactions that hMSC establish with osteoblasts and endothelial cells.

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### Discovery of a Novel Polymer for Human Pluripotent Stem Cell Expansion and Multi-Lineage Differentiation

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Human pluripotent stem cells (hPSCs) are proving valuable for various biomedical applications due to their ability to self-renew and be differentiated into numerous lineages representative of the three embryonic germ layers<sup>1</sup>. We apply a high throughput materials discovery approach to identify a new polymer for hPSC culture using microarray screening of an unprecedented chemical space (141 monomers, polymerized alone and mixed pairwise to form 909 unique polymers, tested in 4356 individual assays). This identified the first synthetic polymeric substrate that achieves both pluripotent hPSC expansion (in the commercially available culture media, StemPro and mTeSR1) and subsequent multi-lineage differentiation into representatives of the three germ layers, namely cardiomyocytes, hepatocyte-like cells and neural progenitors. This polymer can be applied to common cultureware and used off-the-shelf after long-term storage providing a scalable and cost-effective polymer growth substrate enabling the clinical potential of hPSC-derived cells to be realized.

1. Celiz, A. D. *et al.* Materials for stem cell factories of the future. Nature Materials 13, 570–579 (2014).

### Recapitulation of Mesenchymal Cell Condensation using Human Mesenchymal Stem Cell-Collagen Microspheres

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Mesenchymal cell condensation is a critical transitional stage that precedes cartilage or bone formation. A microencapsulation technique was previously established to entrap mesenchymal stem cells (MSC) in collagen microspheres. Here, we hypothesize that the MSC-induced gel contraction mimics the mesenchymal cell condensation and thereby regulates MSC differentiation. The effect of cell density and timing for chondrogenesis induction was investigated. In general, gene expression of SOX9 was upregulated at early time points (day 0–2) while RUNX2 was gradually increased. COL2A1 was constantly expressed in intermediate cell density and was increased at later time points in high cell density but decreased in low cell density. COL1A1 was downregulated except the intermediate cell density with constant expression. COL10A1 was constantly expressed except the low cell density with a decreasing trend. Osterix was gradually upregulated and peak at day 7. However, aggrecan and ALP expression was lower than the monolayer throughout the culturing period. Nuclear localization of Sox9 was observed at day 0 and day 1 whereas nuclear localization of Runx2 was observed from day 0 to day 7 in low and intermediate cell densities but only at day 0 in high cell density. Deposition of collagen II and collagen X were increased with time and cell density. Upon chondrogenesis, gene expression of SOX9, COL2A1, and aggrecan were higher in MSC-collagen microspheres when chondrogenesis was induced at day 3 than that at day 0 and day 1. This work suggests the importance of timing of induction in developing protocols for stem cell chondrogenesis.

### Bioengineering Ligament Tissue Construct by Combining Cell Sheet Technology and Electrospinning

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In this work, we developed a tissue engineered ligament construct (TELC) combining a low fibre density solution electrospun scaffold

and cell sheet technology. The polycaprolactone (PCL) membrane was seeded with ovine Mesenchymal Stem Cells (oMSCs) and cultured for four weeks with ascorbic acid (100 µg/mL) conditioned medium. After 4 weeks of culture, a mature cell sheet was formed and the scaffold was rolled into a ligament-like bundle and subsequently braided with 2 other bundles to create the TELC. Cell viability was assessed by Live/Dead staining showing that most cells were viable in the TELC after rolling and braiding and no significant cell death compared to the control scaffold was observed. The TELC was also analysed for *in vitro* ligament tissue formation by Hematoxylin and eosin staining (H & E). The stained images showed the formation of ligament tissue *in vitro*, which was confirmed by Van Gieson's staining for collagenous fibres. *In vivo* assessment of the TELC was performed in immunocompromised rats by subcutaneous implantation for 4 weeks. The H & E stained *in vivo* tissue images showed the regeneration of ligament tissue with adequate collagen fibres. The collagen fibre formation was further confirmed with Van Gieson's staining with the TELC showing improved alignment and collagen fibre formation compared to the control scaffold. These findings demonstrate the potential application of stem cell seeded PCL scaffolds for ligament regeneration.

#### Fabrication of Scaffold-free Tissue Engineered Blood Vessel using Nanopatterned Human Mesenchymal Stem Cell Sheet

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An integrated microphysiological system of human tissue engineered constructs for *in vitro* drug screening and toxicity study purposes is a promising alternative to *in vivo* animal models due to their better simulation of the human physiology. Tissue engineered blood vessel (TEBV) is an essential component of such microphysiological system, not only for transport purposes but also for vascular therapeutics. In this study, we have fabricated a scaffold-free TEBV (1 mm i.d.) made up of nanopatterned human mesenchymal stem cell (hMSC) sheets as the wall and human endothelial progenitor cell (hEPC) coating as the lumen. The burst pressure of the scaffold-free TEBV was measured to be higher than that of human physiological vascular microenvironments (normally < 200 mmHg) after three weeks of sequential culture in a rotating wall bioreactor and perfusion at 6.8 dynes/cm<sup>2</sup>. The scanning electron microscopy images of various TEBV sections indicated 3D spiral and interwoven organization of the cell layers, and immunofluorescence staining showed extensive extracellular matrix (ECM) formation of the scaffold-free hMSC sheet based TEBV in resemblance to that of native blood vessels. The TEBV exhibited flow-mediated vasodilation, vasoconstriction after exposure to 1 µM phenylephrine and released nitric oxide in a manner similar to that of porcine femoral vein. HL-60 cells attached to the TEBV lumen after TNF-α activation to suggest a functional endothelium. Overall, the nanopatterned hMSC cell sheet-based TEBV fabricated in this study holds promise as a more efficient drug screening process with respect to drug-induced vascular injury.

#### Bovine Bone Matrix in the Osteoinduction and Differentiation of Human Mesenchymal Stem Cells

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The Nukbone (NKB), is a bovine bone matrix used as a biomaterial for treating bone lesions. However, the information on its capacity about osteoinduction is limited.

The objective of this work is demonstrate the osteoinductive capacity of NKB in primary cultures of human mesenchymal stem cells (hMSCs), through the estimation of aspects such as proliferation, adhesion and cellular differentiation toward the osteoblastic lineage.

The results indicate that the NKB has osteoinductive properties in hMSCs cultures, because the activation of key genes in the osteoblastic differentiation as CBFA-1 and Osteocalcin is induced, without the addition of soluble inducers. In addition, NKB promotes cell adhesion and proliferation similarly to the positive control, which is a culture of hMSC without NKB but with osteoblastic inducers as ascorbic acid, B-glycerophosphate and dexamethasone. This osteoinductive property of NKB, might favour its best use in regenerative medicine.

#### Effect of Mechanical Stimulation on Osteogenic Differentiation of Self-Assembled Collagen-Cell Seeded Hydrogels

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Mechanical forces and 3D topological environment can control MSCs fate 1, 2. It is hypothesised that dynamic compressive strains influence cell morphology and expression of hMSCs osteogenic cell marker. This study investigates the effect of mechanical stimulations on soft cellular microspheres when subjected to dynamic fluid compression in microfluidic chamber, compression bioreactor and orbital shaker.

5 µl microspheres were produced through gelation of 2 mg/ml bovine collagen I (2500–3200 cells/droplet). Three loading conditions were studied: (1) by passing the microspheres through a 0.7 mm microfluidic chamber at 200 µl/min for 3 cycles/day for 5 days; (2) applying 10% compression strain with Bose bioreactor for 15 and 40 min/day for 5 and 10 days; (3) fluid shear stress at 50 rpm for 40 min/day for 10 days. Cell viability, ALP activity, mineralization, gene expression and histological cross sections of samples were compared with controls.

Low concentration (2 mg/ml) of collagen can be an efficient cellular microenvironment which supports long term cell viability. ALP activity increased steadily from day 6 post-encapsulation up to day 28. All samples showed an ALP peak at day 28 post-encapsulation with free floating and 5 days controls manifested the highest activity (0.0014) and (0.0013). Microfluidics condition manifested the lowest ALP level at day 21 among other experiment groups (> 0.0003). Mineralisation was confirmed in all samples from day 14 post-encapsulation onward by alizarin red staining with highest ARS level in cyclic compression group. Calcium level increased from > 119 µg/l to > 400 µg/l over 28 days. Dynamic Compression presents a model to study hMSCs osteogenic differentiation.

#### The Infrapatellar Fat Pad from Diseased Joints Inhibits Chondrogenesis of Mesenchymal Stem Cells

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Microfracture is used to stimulate mesenchymal stem cells (MSCs) to repair cartilage defects. However, instead of hyaline cartilage, fibrocartilage is formed. The goal of this study was to investigate whether factors produced by IPFP influence chondrogenesis of bone marrow-derived MSCs, whether this could be influenced by joint pathology or obesity, and whether macrophages which reside in the IPFP play a role.

IPFP obtained during total knee replacement (TKR), anterior cruciate ligament reconstruction (ACL), and subcutaneous adipose tissue (SAT) was used to make conditioned medium (CM). Additionally, CM was made of monocyte-derived macrophages of 3 donors. The effect of CM on MSC chondrogenesis was evaluated.

CM from both IPFP sources and pro-inflammatory macrophages significantly decreased gene expression of collagen type 2 (COL2A1;  $p < 0.01$ ) and aggrecan (ACAN;  $p < 0.05$ ), the COL2A1/COL1A1 ( $p < 0.01$ ), and ACAN/versican ratio ( $p < 0.05$ ). CM from SAT significantly decreased COL2A1 ( $p = 0.01$ ) and not ACAN. The effect of CM from lean (BMI < 25) or obese (BMI > 30) IPFP donors or from TKR and ACL IPFP donors was not different. GAG deposition was not influenced by IPFP CM. Collagen type 2 staining (determined with immunohistochemistry) was decreased by IPFP CM, whereas collagen type 1 was not influenced.

Factors secreted by the IPFP from diseased joints inhibited chondrogenesis of MSCs and shifted production of hyaline cartilage to fibrocartilage. Type of joint pathology and obesity do not influence this effect. The IPFP and its macrophages could be a target for future therapies to improve the results of the microfracture procedure in the knee.

### Using Laser Ablation to Fabricate Fully Biodegradable Microfluidic Devices for the Regeneration of Vasculature

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Polymeric microfluidic systems are widely used in vasculature regeneration for the purpose of tissue engineering. Biocompatible and biodegradable polymeric materials with high mechanical strength, flexibility, optical transparency, optimal degradation properties and biocompatibility are critical to the success of device fabrication. Micro- and nano-patterning of scaffolds used in tissue engineering shows possibility in signaling cell morphology via contact guidance *in vitro*, as well as higher mechanical tissue adherence properties. With the advances in MEMS over the past decade, micro- and nano-patterning techniques for polymeric materials have become more common. However, high cost and low flexibility of MEMS remains an expensive and time consuming molding creation process. By applying laser ablation onto biodegradable scaffolds, micro- and nano-pattern is created and microfluidic devices are constructed in this work on polydimethylsiloxane (PDMS) and two novel classes of biodegradable polymer, poly(glycerol sebacate) (PGS) and poly(1,3-diamino-2-hydroxypropane-co-polyol sebacate)s (APS). The results of seeding epithelial MDCK cells and HUVEC endothelial cells on grooved biodegradable polymer and microfluidic channels under constant flow are presented. Cell adhesion, proliferation and contact guidance responses are observed. This work presents a novel method for rapid prototyping and manufacturing of tissue engineering scaffolds for vasculature and tissue.

### Development of Three-dimensional Culture System for Bovine Nucleus Pulposus Cells

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**Introduction:** Nucleus pulposus is a central component of the intervertebral disc and rich in extracellular matrix (ECM). In the pathology of degenerative disc disease (DDD), the changes in ECM may be related to changes in nucleus pulposus cells (NPCs). To study NPCs, it is important to culture them *in vitro* in an environment similar to the native tissue. Many factors such as the interactions between NPCs and ECM, the topological cues and the mechanical properties of the constructs should be considered. If a 3D NPC-matrix system mimicking the native NP is developed, it can be used in future studies in regenerative medicine and DDD as a model platform.

**Objective:** The objective is to maintain the survival and phenotype of NPCs in a 3D culture system, by resembling the native tissue

microenvironment. Specifically, we compare two starting materials, namely collagen and collagen-GAG constructs.

**Methodology:** bNPCs were either encapsulated in collagen microspheres or seeded on collagen-GAG constructs fabricated by coprecipitation. They were cultured up to two weeks before evaluation. Cell morphology, F-actin distribution and viability were revealed by staining. Phenotype and components in the ECM were analyzed by qRT-PCR, immunohistochemistry and histology.

**Results:** bNPCs can survive, proliferate and maintain some phenotypic markers, in our 3D systems. Collagen-GAG constructs may be better than collagen alone for long term culture of bNPCs.

**Conclusion:** Collagen-GAG constructs may be a good starting material for culturing NPCs. Including other ECM components into the culture system may further help in supporting the cell phenotype.

### 3D In Vitro Liver Tissue Model of Human Liver Organogenesis: a Platform to Study Developmental Diseases

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Current *in vitro* models of liver tissue do not address the need for a system that enables the study of human liver development and developmental diseases. We have generated a 3D *in vitro* liver tissue model using bipotent human fetal liver progenitors (hFLPs) seeded on decellularized liver extracellular matrix (LECM) that mimicks human liver development. LECM discs (300  $\mu$ m thickness, 8 mm diameter) were prepared from these scaffolds and seeded with  $0.5 \times 10^6$  hFLPs. The cells were differentiated for 3 weeks in culture. The seeded discs self-assembled into 3D organoids and the cells differentiated into hepatocytes and cholangiocytes. Immunostaining analysis showed clusters of cells expressing hepatocytic markers like albumin, HNF-4 $\alpha$ ,  $\alpha$ -1 antitrypsin and CYP3A4 and ductular cells expressing bile duct specific markers namely, CK19, SOX9, Ep-CAM, ASBT and  $\beta$ -catenin. The organoids also expressed hepatocyte and cholangiocyte specific genes. Functionally, the hepatocytes within the organoids secreted albumin and urea and displayed phase I drug metabolism, while cholangiocytes displayed apicobasal polarity. Hepatocytes displayed transcriptional switch from  $\alpha$ -feto protein to albumin, while cholangiocytes exhibited biliary morphogenesis process, not a phenomenon observed during liver development. The duct morphogenesis process was interrupted by inhibiting Notch signaling, thus creating a liver developmental disease model exhibiting a phenotype of ductal malformation similar to Alagille syndrome. Our system has thus demonstrated the capability of recapitulating hepato-biliary organogenesis in a 3D *in vitro* model consisting LECM. This model provides novel approaches for studying liver development, liver bioengineering, drug discovery and toxicology, and ultimately for treatment of liver disease.

### Microfluidic Regulation of Cancer Cell Phenotype

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Cancer cells in their native microenvironment are spatiotemporally heterogeneous due to continuous cell proliferation, angiogenesis, matrix remodeling, invasion and metastasis. They are regulated by a series of chemical and physical cues in this microenvironment including cytokine and oxygen gradients, continuous signaling with over-expression, abnormal blood flow patterns and elevated interstitial fluid pressure. Particularly, cancer cells are exposed to continuous shear stresses a lot more than normal tissue as a result of the

elevated interstitial fluid flow. These shear stresses alter cancer cytophysiology and lead to the phenotypical plasticity. We develop a microfluidic platform to mimic continuous shear stress in elevated interstitial fluid flow in native tissue. We investigate the phenotypic and morphologic changes of esophageal cancer cells under a shear stress, which is developed by continuous laminar flow. We characterize the phenotypic plasticity of laminar-flow exposed cells in terms of expression of cancer stem cell markers (CD44 and CD24), ALDH activity, E-cadherin, N-cadherin and vimentin. To better understand the dynamic characteristics of the cancer microenvironment, we performed computational fluid dynamics and numerically model shear stress exposed on adherent cancer cells in the microfluidic chip channel. We defined the shear stress forces on cells and further experimentally analyzed the membrane stiffness of live cancer cells with atomic force microscopy. We also demonstrate the chemotherapeutic drug regulation with the developed cancer microfluidic platform. This study provides a new tool to investigate the role of continuous shear stress on cancer cells potentially useful for novel bioengineered microenvironments and cancer research.

#### Defined Culture Conditions for Efficient Derivation of Mesenchymal Stem Cells from Human Induced Pluripotent Stem Cells

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The invasive nature and variability efforts to isolate mesenchymal stem cells (MSCs) from adult tissues have prompted researchers to use human induced pluripotent stem cells (hiPSCs) as an alternative source. However, current approaches to derive MSCs from hiPSCs involve culturing cells in serum-containing medium on animal feeders and require prolonged culture time. We have developed an efficient method to induce direct differentiation of hiPSCs into cells with MSC-like properties under completely defined culture conditions. Briefly, hiPSCs cultured in mTeSR1<sup>TM</sup> medium in a monolayer culture reached confluency in 7 days and were then dissociated using animal component-free (ACF) dissociation reagents and seeded at  $1.5 - 7.5 \times 10^4$  cells/cm<sup>2</sup> on defined substrate. Three different ACF media formulations (MesenCult<sup>TM</sup>-ACF, and two variants thereof; ACF2 and ACF3) were tested. The proliferative potential of hiPSC-derived MSCs was measured and cell phenotype was analyzed by flow cytometry. After 1 week in culture, hiPSCs started adopting MSCs-like morphology and could be expanded for up to 10 passages in all three ACF media. Preliminary data from one experiment indicated the average of the fold expansion at each subculture (P1-P10) was lower (2.5 fold) in MesenCult<sup>TM</sup>-ACF than in ACF2 (4.7 fold) and ACF3 (5.3 fold) media. More than 90% of MSC-like cells expressed CD73, CD105, CD90 and CD146 at 21 days. The hiPSC-derived MSCs were able to differentiate into adipocytes, osteogenic cells and chondrocytes *in vitro*. These data indicate that MSC-like cells can be derived efficiently from hiPSCs using completely defined culture conditions offering the possibility for generating patient-specific MSCs.

#### Promoting Chondrogenesis Through Paste-Like Hydrogel Precursors

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Although hydrogels are promising for cartilage tissue engineering, they are prone to leaking from the defect site after implantation. To address this major drawback, we incorporated particles of decellularized cartilage (DCC) into a methacrylated hyaluronic acid (MeHA) hydrogel precursor solution to yield a paste that would 'set' in place once implanted. We hypothesized that incorporating DCC

would result in a material that facilitates cell differentiation and cartilage matrix deposition. Rat bone marrow mesenchymal stem cells were encapsulated within DCC/MeHA composites with MeHA only and native cartilage/MeHA composites used as a control. Additional controls were MeHA gels and DCC/MeHA composites supplemented with TGF- $\beta$ 3. We found that the compressive moduli of hydrogels containing 10% native cartilage particulates were 79% higher than that of hydrogels containing 10% DCC after 1 day of culture. At 6 weeks, although there was no significant change in the compressive moduli of 10% DCC hydrogels cultured with or without growth factor, there was a 2.7-fold increase in the compressive modulus of MeHA-only gels supplemented with growth factor compared to without growth factor supplementation, suggesting DCC alone may promote chondrogenesis. Analyses of the gene expression, biochemical content, rheology, and histology of these networks are currently ongoing. Ultimately, this technology may lead to improving the clinical translation of hydrogels for cartilage repair.

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#### BMP-2 Outperforms TGF- $\beta$ 3 for Chondrogenic Differentiation of Induced Pluripotent Stem Cells

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Current cellular therapies for cartilage repair have focused on the use of bone marrow mesenchymal stem cells (MSCs) with limited success. Though capable of chondrogenic differentiation, MSCs have limited proliferation and differentiation potential, especially in older populations. Induced pluripotent stem cells (iPSCs) are a novel, autologous cell source for cartilage repair without the limitations of MSCs. While chondrogenic factors have been studied widely for use with MSCs, similar work lags for iPSCs. Therefore, the objective of this study was to compare the effects of two known chondrogenic factors for MSC differentiation, BMP-2 and TGF- $\beta$ 3, on the chondrogenic differentiation of iPSCs.

Human skin fibroblasts from a 50-year old female were reprogrammed via an mRNA-based method to produce iPSCs. iPSCs were further differentiated into mesenchymal progenitors (iPSC-MPs) and evaluated in pellet culture for chondrogenesis. iPSC-MP pellets were stimulated with chondrogenic medium (CM) in the presence or absence of BMP-2 (100 ng/mL) or TGF- $\beta$ 3 (10 ng/mL) for 21 days. Upon harvest, pellets were analyzed for evidence of sulfated glycosaminoglycans (GAG) via Safranin O histological staining and dimethylmethylene blue (DMB) assay.

Pellets cultured with BMP-2 produced the largest pellets and most intense Safranin O staining. These results were supported by DMB analysis. Neither CM only or CM+TGF- $\beta$ 3 produced significant Safranin O staining or GAG content.

These results suggest that BMP-2 is an important factor for iPSC chondrogenesis whereas TGF- $\beta$ 3 is not. Furthermore, it emphasizes that the use of chondrogenic factors that have been studied for MSC chondrogenesis may not directly translate for iPSCs.

#### Selective Removal of Cells Deviated from the Undifferentiated State in Hipsc Colonies by using Botulinum Hemagglutinin Proteins

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Understanding the regulation of cell-cell and cell-substrate adhesion in cultured human induced pluripotent stem cells (hiPSCs) can facilitate the design of culture strategies for maintaining undifferentiated hiPSCs. The botulinum hemagglutinin (HA) treatment disrupted cell-cell adhesion in hiPSC colonies, and the cells deviated



from the cells at the undifferentiated state were detached from the hiPS colonies. The vacated space was filled by newly dividing the undifferentiated cells along with prolonged incubation. Because E-cadherin-mediated cell-cell adhesion was disrupted, undifferentiated cells in HA-treated colonies exhibited aberrant cytoskeletal organization featuring diminished actin stress-fiber formation and focal adhesions; however, during the culture period, these cells fully recovered their cytoskeletal structures and E-cadherin and retained the expression of pluripotency-associated transcription factors and surface markers and their potential to differentiate into cells of the 3 embryonic germ layers. However, the deviated cells completely lost their actin cytoskeleton and focal adhesions because E-cadherin binding was disrupted, and this eventually led to their apoptosis. Furthermore, in the undifferentiated cells, the cadherin/integrin-regulator Rap1 was concentrated at cell-cell adhesions, whereas it was detected in cytoplasmic regions in the deviated cells. We attribute this effect to Rap1-induced enhancement of cell-cell adhesion (through cortical F-actin reorganization) and E-cadherin recruitment in the undifferentiated cells in hiPSC colonies, indicating that HA treatment could serve as a method of maintaining undifferentiated hiPSCs. Our results suggest a robust and stable hiPSC culture-propagation strategy that involves HA-mediated selective removal of cells that deviate from the undifferentiated state.

### Nanoscale Design of Bone Scaffolds Through Biomineralization Process Modulating Cell Behavior

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Biomimetic scaffolds specially designed to support cell and tissue growth have been focused on “bulk properties” such as porosity and mechanical response [1]. Nevertheless, exceptional properties of bone rely on its complex hierarchical structure spanning from nanoscopic to macroscopic level. In fact, it has been already reported that surface modifications at the nanoscale provides different cell behavior [2]. We propose the synthesis of bioinspired scaffolds with tailored final surface features such as chemical composition, roughness as well as crystallinity and size of apatite nanocrystals (HA). To this aim, collagen like-peptide (CP) was mineralized through neutralization process [3] in the absence (CP-HA) and in the presence of magnesium (CP-MgHA). Moreover, non-mineralized scaffolds (CP) were prepared as a control system. Surface roughness and wettability of CP, CP-HA and CP-MgHA dense scaffolds were measured by Atomic Force Microscopy (AFM) and optical tensiometer, respectively. The biological response of MC3T3-E1 cell line to scaffolds was assessed, too. Cells were incubated onto dense scaffolds to evaluate the cell viability, morphology and differentiation. These results can provide a new biomineralization route to design biomaterials with improved cell-material interaction and thus promoting cell attachment and tissue growth.

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### 3D Bioprinting of Human Skin

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Annually, 11 million people worldwide suffer from burns so severe that they required medical attention. Out of these 265,000 cases are fatal, this leaves millions of surviving burn victims each year suffering from the disfigurement, disabilities and associated stigma and trauma. In many countries, burns are among the top causes of disability-adjusted life-years, possibly because young children are at an increased risk of burns. In the year 2000, \$211 million were spent on the care of children who had suffered from burns. Ultimately, by the development of 3D bioprinting technology, patient specific living tissue can be biofabricated with high accuracy using the patients' own cells. In this project, we explore the feasibility of 3D bioprinting human skin. Skin constructs are have been biofabricated by alternating one-by-one layer of supporting hydrogel bioink and human cells. Firstly, bioink based on nanocellulose and alginate has been combined with well-defined density of human fibroblasts. Then, the several layers of keratinocytes premixed with melanocytes in polysaccharide hydrogel bioink have been deposited by 3D bioprinter. Image data acquired from two photon fluorescence microscopy images of human skin is used as input for creating a computer-aided design (CAD) file for 3D bioprinting. We believe that this emerging technology offers unique opportunity for biofabrication of constructs with the ability to exactly mimic the microarchitecture and thus properties of human skin. The approach can potentially be used in clinics to increase quality of life of burn patients or applied for pharmaceutical and toxicology testing.

### An Automated Bioreactor for Screening of the Effects of Complex Biomechanical Stimuli on Osteoarthritic Chondrocytes

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Osteoarthritis (OA) is one of the most common degenerative diseases. The mechanical stimulation of chondrocytes through movement regulates cartilage homeostasis *in vivo*. Guidelines of Department of Health and Human Services recommend at least 150 minutes of moderate exercises to improve discomfort in people with OA, often interpreted as the need for at least 10,000 steps/day. However, there is no certainty that this interpretation meets the time-intensity exercise suitable for subjects affected by OA. To verify the effect of different patterns of stimulation on OA chondrocytes, we developed a novel bioreactor that provides the *in vivo* like mechanical stimuli of high hydrostatic pressure (HP) and bidirectional interstitial perfusion. The two stimulation phases are generated using a single piston operated by an electromechanical linear actuator and a two-way valve controlled by a stepper motor. A PID control uses the signal of a load cell mounted in the piston to real-time regulate the stroke of the piston in order to maintain the desired pressure. Programmable patterns are managed and constantly monitored through a PC-based software developed in NI Labview.

We mechanically validated the bioreactor applying perfusion speeds ranging from 30 to 1000  $\mu\text{m}/\text{sec}$  and HP up to 10 MPa, with cycles of 5–20 minutes at frequency from 0.3 to 1.5 Hz (max 4 hour/day). We also preliminary tested the bioreactor with primary human OA chondrocytes seeded on collagen scaffolds. This is the first pump-less bioreactor system able to serially provide both perfusion and HP to 3D constructs in an automated way.

### Fibroblast Growth Factor-2 Counteracts Osteogenic Priming during Expansion of Human Periosteum-Derived Cells in 3D Perfusion Bioreactors

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**Introduction:** Long-term culture of progenitor cells leads to loss in differentiation capacity, restricting their potential for cell therapy applications. Fibroblast growth factor-2 (FGF-2) increases proliferation potential and retains the differentiation capacity of mesenchymal stem cells (MSCs)<sup>1</sup>. However, its effect on human periosteum-derived cells (hPDCs) and bioreactor cultures is not yet explored. Bioreactors allow for (3D) cell growth in controlled culture environments. Nonetheless, osteogenic priming in perfusion bioreactors has previously been reported for dynamic cultures in growth medium<sup>2</sup>. We hypothesize that perfusion bioreactors and FGF-2 will increase hPDC proliferation rate and counteract flow induced osteogenic priming.

**Methods:** hPDCs were seeded in culture flasks (2D) and on porous Ti6Al4V scaffolds for the perfusion bioreactor (0.1 ml/min). After 14 days with FGF-2 supplemented media (0, 5 and 15 ng/ml), the cumulative cell population (2D), metabolic activity (3D), DNA content and RT-qPCR was defined.

**Results:** A 1.4–1.5 folds higher cell quantity/DNA content was detected for FGF-2 treated cells in 2D (5–15 ng/ml) and 3D (15 ng/ml), respectively. Furthermore, the addition of FGF-2 resulted in a significant down-regulation of osteogenic markers (Runx2, CollagenI) in 2D and 3D while the chondrogenic marker Sox9 was up-regulated in 2D, implying suppressed differentiation towards the osteogenic lineage.

**Conclusion:** The proliferative effect of FGF-2 on MSCs is confirmed here for hPDCs in 2D and 3D. Gene expression data suggest that FGF-2 counteracts the osteogenic priming observed in perfusion bioreactor.

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#### Mesoporous-bioglass-nanoparticles for Odontoblast Differentiation

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Mesoporous bioactive nanoparticles (MBN) has been widely used in bone and dentin regeneration as carrier of drug and nucleonic acid; however, reports on the biological effects of MBN itself on dental pulp stem cells are rarely studied. This study aims to investigate the effects of MBG on odontogenic differentiation of dental pulp stem cells. Rat dental pulp stem cells (rDPSC) were primarily cultured directly with MBN *in vitro*. The cell viability did not change up to 50 µg/mL MBG. The internalization of fluorescein-isothiocyanate (FITC) conjugated MBG was observed by confocal microscope images at 1 hr incubation condition and FITC conjugated MBN was observed in ~95% of cells at 4 h incubation condition by flow cytometry experiments. Results from decrease of uptake after 1 h pretreatment of rDPSC with inhibitor of endocytosis showed internalization of MBN was performed by ATP dependent manner and majorly macropinocytosis. The mineralization capacity and expression of odontogenic-related proteins and genes (osteocalcin, dentin sialophosphoprotein, dentin matrix protein 1 and Collagen, type I, alpha 1) of rDPSC were significantly up-regulated with MBG, which was explained by odontogenic ion release such as Ca<sup>2+</sup> and Si<sup>4+</sup> inside the cell. This study reveals that MBG induces the odontogenic differentiation of dental pulp stem cells and may serve as a potential material for dentin regeneration.

#### Development of an Injectable Cell-laden Microniche for Mesenchymal Stem Cell Therapy

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Mesenchymal stem cells (MSCs) have shown promise in treatment of diabetic wounds, critical limb ischemia, and myocardial infarction by modulating local immune responses, promotion of tissue regeneration, and angiogenesis. This is accomplished through secreted and non-secreted factors such as VEGF, PGE<sub>2</sub>, TGF-β, and IDO. Currently, delivery of MSCs is limited by poor short-term engraftment, survival, and maintenance of cell potency. In this study, we examine the effects of injectable biodegradable cell microcarriers on MSCs' trophic properties. We have developed two systems of injectable biodegradable microcarriers: one composed of poly(lactide co-glycolide) (PLGA) and the other created from cross-linked gelatin. Both microcarriers systems were synthesized using a single emulsion process. The PLGA microcarriers were surface modified with fibronectin to promote cell attachment and the gelatin microcarriers were stabilized for use in physiological conditions by cross-linking with genipin at elevated temperatures. Dozens of MSCs quickly populate each carrier, creating an injectable cell-laden microcarrier. MSCs are stable and viable on the carriers for greater than five days providing a platform on which MSCs can be grown and then directly injected. These biodegradable injectable cell-laden microcarriers have the potential to promote superior control of MSC phenotype, viability, and maintenance of cell function post injection.

#### Decellularized Thymus for *In Vitro* Culture of Functional Adult Thymic Epithelial Cells

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Thymic epithelial cells (TEC) are a critical component of the three-dimensional (3D) thymic stromal network mediating T-cell development and self-tolerance. Models supporting functionally competent adult TEC *in vitro* are currently lacking, hampering thymus engineering strategies and a deeper understanding of the development of a competent immune system. Therefore, we aimed to establish a 3D model based on decellularized thymus for *ex vivo* culture of functional TEC.

Here we report for the first time the decellularization of thymus organs. A perfusion bioreactor-based protocol was developed for devitalization and efficient DNA removal, as verified by histology and PicoGreen assay. The main components of the thymus native extracellular matrix (collagen IV, laminin and fibronectin) were preserved, as shown by immunofluorescence. The decellularized tissue was mechanically disrupted, lyophilized and cross-linked to produce 3D porous scaffolds (Thymus scaffolds, "TS"). TS supported adult TEC culture *in vitro*, with preserved TEC phenotype at 2 weeks. Fetal TEC cultured with T-cell precursors in TS supported thymocyte differentiation *in vitro*. TS were then seeded with TEC isolated from adult mice and grafted under the kidney capsule of athymic nude mice. Host derived CD4<sup>+</sup>/CD8<sup>+</sup> double positive thymocytes were detected in the graft, and host-derived CD4<sup>+</sup> or CD8<sup>+</sup> T-cells were found in lymph nodes after 7 weeks, demonstrating graft-derived thymopoiesis *in vivo*.

We have developed a 3D model based on decellularized thymus organs, which supports the culture and functionality of adult TEC, paving the way to a better understanding of TEC/thymocytes cross-talk and unprecedented approaches for treating thymic-related pathologies.

#### Construct Development for Local Endurable Cell Therapy with Mesenchymal Stem Cells

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**Introduction:** Mesenchymal stem cells (MSCs) are promising as cell therapy in complex diseases, such as Crohn's disease and osteoarthritis, due to their immunomodulatory and trophic properties by secreting anti-inflammatory cytokines and growth factors. In the diseased location these properties of MSCs should be guaranteed for several weeks to achieve improvement of the pathology. By MSC injections this cannot be achieved. We investigated whether encapsulation of MSCs in alginate will retain MSCs in the applied locations and whether this also applies for allogeneic MSCs.

**Methods:** MSC survival and immunomodulation was evaluated after encapsulation in clinical grade alginate for 14 days *in-vitro* (n=3 donors). MSC number was measured by DNA, MSC immunomodulation by IL-6 secretion and IDO activity after stimulation with TNFa/IFNg. Encapsulated syngeneic/allogeneic SPIO-labeled MSCs (n=6) were implanted subcutaneously in three immune competent rats for one month. The integrity of the constructs and the host reaction were studied by MRI and histology.

**Results:** After encapsulation in alginate approximately 50% of MSCs survived and kept their immunomodulatory properties *in vitro*. The integrity of the constructs *in vivo* remained intact over time indicated by the continued SPIO signal assessed by MRI and the construct morphology when harvested for histology. Histologically, no foreign body reaction of the host was found against either syngeneic or allogeneic cell constructs.

**Conclusions:** These results offer a possible solution for cell therapy with allogeneic MSCs and retaining the MSCs for a long period at the diseased location. This encourages further studies on the effect of the constructs.

### Combining Gene Delivery and Decellularized Cochleas to Engineer Inner Ear Sensory Epithelium

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Mechanosensory hair cells (HCs) located in the cochlea and vestibular organs facilitate hearing and balance, respectively. Once damaged, mammalian HCs do not regenerate, which can lead to hearing loss, tinnitus, or vertigo. Sensory epithelium is isolated in the inner ear, which makes evaluation of toxic and therapeutic strategies exceptionally challenging in living organisms. An open model, where sensory epithelium could be grown in real-time would be highly advantageous to investigators exploring ototoxic effects of new drugs and efficacy of new treatments. We have shown that human Wharton's Jelly Cells (hWJCs) are capable of differentiating toward an HC phenotype when ATOH1 and its negative regulators HES1 and HES5 are overexpressed and suppressed, respectively (1). Our recent studies have shown that hWJCs are responsive to extracellular cues in the extracellular matrix of native cochleas. When hWJCs were cultured on decellularized cochleas, presentation of the exclusive hair cell marker, Myosin VIIA was detected. Presentation of Myosin VIIA was further enhanced by ATOH1-transfected hWJCs cultured on decellularized cochleas. Thus, the objective of the current work is to manipulate the intracellular expression of key NOTCH pathway genes (ATOH1, HES1, and HES5) while culturing hWJCs on decellularized mouse cochleas to induce the development of sensory epithelium. Our work is intended to create an open 3D culturing system that would enable accessible screening of ototoxic and therapeutic agents on HCs, and advance the field of HC regeneration.

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### Loss of Ccbe1 Affects Cardiac-specification and Proliferation in Differentiating Mouse Embryonic Stem Cells

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In humans, mutation in CCBE1 has been associated with Hennekam syndrome, a disorder characterized by abnormal lymphatic system development where some patients present as well congenital heart defects, including hypertrophic cardiomyopathy and ventricular septal defects. This suggests that Ccbe1 may also function during heart organogenesis. In this study, we examined Ccbe1 expression in differentiating mouse ESCs and in isolated cardiac progenitor populations, and how Ccbe1 loss-of-function affects cardiac differentiation from mouse ESCs.

Here, we have identified Ccbe1 expression in differentiating ESCs, especially in second heart field (SHF) cardiac progenitors at day 6 of differentiation. Furthermore, isolation of the same populations from transgenic mice at an equivalent time point, showed a similar Ccbe1 expression profile for the different populations. Therefore, our data shows that Ccbe1 expression is related to the appearance of specific populations of cardiac progenitors both *in vivo* and during ESC differentiation. In addition, differentiation of Ccbe1 knockdown ESCs lines resulted in a clear decrease in the expression of early cardiac mesoderm markers Mesp1 and Isl1, indicating that the development of early cardiac precursors is compromised in the absence of Ccbe1. In addition, we show that also from this day onwards the size of the embryoid bodies is severely decreased and that this is caused by decreased proliferation. Furthermore, full-length recombinant CCBE1 protein was shown to partially rescue the phenotype in differentiating Ccbe1 KD ESCs. Whether gain-of-function or supplementation with Ccbe1 in differentiating ESCs would lead to increased commitment to cardiac precursors remains yet under investigation.

### Injectable Polymeric Nanosheets for Subretinal Cell Delivery

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Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in the elderly population, whose main complication is the degeneration of retinal pigment epithelial (RPE) cells. In this regard, subretinal transplantation of RPE cells to the degenerated site has attracted a great deal of attention as an innovative therapeutics for the AMD treatment. However, poor viability, distribution and integration of the transplanted cells in suspension to the subretinal space have limited this approach. Therefore, development of cell delivery system would bring significant benefits for the AMD treatment. Recently, we developed micropatterned polymeric nanosheets which can direct growth and morphogenesis of RPE cells. Here, we examined whether nanosheets can be delivered to the subretinal space of rat eyes using a syringe needle.

Micropatterned nanosheets (0.3 mm in diameter) consisting of poly(lactic-co-glycolic acid) were prepared on a poly(vinyl alcohol) (PVA) coated substrate by a stamping technique. The freestanding nanosheets were obtained by dissolving the PVA layer. After rats were anesthetized, nanosheets were injected to the subretinal space through the sclera using a 27G syringe needle. Optical coherence tomography (OCT) images of the retina showed a shadow of the sheet like structure in the subretinal space. Also, a shadow of the nanosheet was observed at the posterior segment of the isolated eye. These results indicate that the nanosheets were successfully injected and spread into the subretinal space of rat eyes. The injection of nanosheets through a syringe needle could be a minimal invasive way to transplant organized RPE cells.

### A Tissue Engineered Bone Construct as a 3D Metastasis Colonization Model

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In prostate cancer, the most prevalent organ for metastasis is bone, where cancer cells find a rich marrow, fuelling metastasis progression. While microenvironmental factors have been acknowledged to be major components of disease establishment, no current metastasis *in vitro* model has been able to represent these features. In the case of prostate cancer bone metastasis, the patients are often receiving androgen-deprivation therapy (ADT), meaning that standard culture with serum, that naturally contains androgens, is not representative of this androgen deprived condition.

In this study, a mineralised neo-tissue was engineered utilizing melt electrospun polycaprolactone microfibres, forming a 3D porous and interconnected structure, further seeded with osteoblastic cells ( $4 \times 10^7/cm^2$ ). The construct (500–1000  $\mu m$  high, 150–300  $\mu m$  pore size) was osteogenically differentiated over 8 weeks and exhibited a dense extracellular matrix (SEM, confocal microscopy), and displayed a typical mixed population of osteocytes (core) and osteoblasts (top).

Prostate cancer cells, such as LNCaP, C4-2B, PC3 were seeded ( $3 \times 10^5/cm^2$ ) on the constructs and co-cultured in FBS or charcoal stripped serum (CSS) - representative of ADT *in vivo*. The systems were fully viable for up to 3 weeks and LNCaP in CSS displayed a neuro-endocrine phenotype representative of cancer cells metastasised to the bone. The expression of bone markers was significantly altered by the presence of cancer cells (media analysis, qRT-PCR and immunohistochemistry).

This 3D model has shown to replicate bone metastasis in an ADT state for prostate cancer, representing a powerful tool for assessing current and new therapeutic approaches for treating bone metastasis.

### Human Adipose Derived Mesenchymal Stem Cells Pre-differentiated to a Discogenic Phenotype Can Withstand the Catabolic Effects of the Inflammatory Degenerate Intervertebral Disc Niche

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**Introduction:** The degenerate intervertebral disc (IVD) niche is a milieu of catabolic/pro-inflammatory cytokines, particularly IL-1, which drives catabolic processes characteristic of IVD degeneration. For regeneration strategies, mesenchymal stem cells (MSCs) will be implanted into this degenerate niche; hence their response to the milieu needs to be ascertained to ensure that catabolic events are not exacerbated.

**Aims:** To investigate how adipose-derived MSCs (AD-MSCs) differentiated to NP cells (aNPCs) respond to IL-1beta compared to mature/native degenerate NP cells.

**Methods:** Human AD-MSCs and mature NP cells (n=4) were seeded into type I collagen hydrogels. AD-MSCs were differentiated to aNPCs using 100 ng/ml GDF6. After 14 days components of the IL-1 pathway were assessed. Hydrogels were then cultured with or without IL-1beta for 7 days. QPCR analysis of classic NP matrix, catabolic and anti-catabolic markers was undertaken, along with analysis of sGAG content (DMMB).

**Results:** Both cell types expressed IL-1R. Mature NP cells treated with IL-1 showed a significant down regulation of matrix and anti-catabolic markers and upregulation of catabolic markers. aNPCs showed no significant differences. Both cell types produced similar amounts of sGAGs, although IL-1beta caused a significant reduction in sGAG in NP cells only.

**Discussion:** The cells respond to IL-1beta in a differential manner; IL-1beta had no detrimental catabolic effect on aNPCs suggesting that these cells may be able to withstand the effects of IL-1beta in the IVD niche. Thus, pre-differentiation of AD-MSCs prior to implantation may improve the success of cell-based IVD regenerative therapies.

### A Microengineered Biomimetic Hydrogel for Studying the Role of Extracellular Matrix Stiffness in Lung Fibrosis

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Fibrosis is a major cause for progressive organ dysfunction in most of chronic pulmonary diseases. There is increasing evidence showing that altered matrix stiffness in fibrotic state actively participates in disease progression. Lack of suitable *in-vitro* models partly accounts for slow progress in understanding mechanisms involved in induction and resolution of lung fibrosis. Here, we present both 2D and 3D hydrogel based culture systems with tissue relevant tuneable stiffness that can be used for studying fibrotic disorders. Briefly, we fabricate Gelatin Methacrylate (Gel-MA) based microgels of different stiffness with 10  $\mu m$  thickness by using different percentages of GelMA. These microengineered hydrogels are highly biocompatible as evidenced by high cell viability, spreading and fibronectin deposition of primary fibroblasts. We have used this system to investigate the phenotypical and functional changes in fibroblasts in responses to increasing substrate stiffness. Firstly, we observed that growth of both MRC5 and primary lung fibroblasts is largely dependent on increasing stiffness of microgel. Moreover, by applying this platform, idiopathic pulmonary fibrosis (IPF) fibroblast showed greater response to increasing stiffness compared to normal fibroblasts. Secondly, fibroblasts become more contractile as evidenced by higher expression of alpha smooth muscle actin ( $\alpha$ -SMA) when seeded on rigid matrix which was confirmed by both immunostaining and western blot analysis. We confirmed that these changes are independent of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and their molecular basis need to be further investigated. We believe this platform will provide new opportunities for better understanding of cellular responses in fibrosis.

### Intra-discal Application of BMP-7 elicits Extra-discal Bone Formation and no Regenerative Effect

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Bone morphogenetic protein 7 (BMP-7) exerted beneficial effects on proliferation and matrix production of intervertebral disc (IVD) cells *in vitro*, and *in vivo* in rabbits with induced IVD degeneration. This study was designed to establish the most effective BMP-7 dose in a canine model with spontaneous IVD degeneration, probably the only non-induced, and therefore clinically relevant, animal model for IVD-degeneration. The lumbar IVDs of beagle dogs were injected with 30  $\mu l$  sucrose buffer only, 2.5  $\mu g$ , 25  $\mu g$ , or 250  $\mu g$  BMP-7 in sucrose buffer. MRI was done prior to surgery, and 6, 12, and 24 weeks after surgery to evaluate IVD degeneration. Radiographs and CT images were obtained post mortem (24 weeks) and IVDs were evaluated by macroscopy, histopathology, biochemical analysis, and gene expression profiling. There were no significant changes in disc height indexes and Pfirrmann scores on MRI during follow up. Radiography and CT revealed minimal to extensive peridiscal bone formation in 1/7 and in 4/7 IVDs treated with 25  $\mu g$  and 250  $\mu g$  BMP-7. Macroscopic and histological evaluation confirmed early IVD degeneration and new bone formation. Statistical analysis revealed no significant differences in GAG/DNA, nor in collagen/DNA between treatments. Bcl2 gene expression levels indicated an anti-apoptotic effect of the highest dosage of BMP-7, in line with *in vitro* findings in human IVD cells. Intradiscal bolus injection of BMP-7 did not show regenerative effects in experimental dogs with spontaneous IVD degeneration. In fact, injection of 250  $\mu g$  BMP-7 resulted in extensive peridiscal bone formation.

### Single-Walled Carbon Nanohorns Modulate Tendon Biomechanics and Tenocyte Cellular Response

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Sub-failure ligament and tendon (L/T) injury remains a significant burden to global healthcare. Due to tissue composition, structure, degree of vascularization, and function, healing is slow and usually incomplete. After injury, tissue biomechanics are compromised often leaving it prone to re-injury. Current tissue engineering approaches have focused on the repair of severe damage (usually failure) via the use of implantable constructs that completely replace the damaged tissue. Here, we present the novel use of single-walled carbon nanohorns (CNH) in the repair of sub-failure injury in L/T. Biomechanical tests with explanted porcine digitorum tendons showed the ability of CNH suspensions to modulate tendon biomechanics, most notably elastic moduli immediately after treatment. Next, *in vitro* tests showed that the immediate cell response of human tenocytes may be dependent on CNH aggregate size with endocytosis occurring mostly via clathrin-mediated mechanisms. Furthermore, prolonged *in vitro* exposure of CNH to tenocytes revealed no decrease in cell activity for up to 7 days, and no significant effect on collagen production. However, gene expression studies revealed significant down-regulation of collagen types I and III mRNA at 7 days with noticeable recovery after 14 days of exposure. *In vivo* experiments demonstrated the ability of CNH to alter stretch-injured Sprague Dawley rat Achilles tendon biomechanics and persist in the damaged matrix, most prominently at 7 days after treatment. Altogether, these results show the feasibility and potential of the utility of CNH as a novel modality for sprain and strain injuries by directly affecting cellular response and damaged tissue biomechanics.

### Composite Material for Hyaline Cartilage Repair

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Regeneration of articular cartilage is a high-priority task of tissue engineering. Actually, at least 11% of overweight, more than half of elders, as well as trauma and osteoarthritis patients need restoration of hyaline cartilage (HC). Many protocols of HC repair are offered, but several aspects including adhesion of the implant with surrounding cartilage/bone, ossification, fibrosis and restoration of normal HC tissue need to be improved. Also, simplification and acceleration of the current protocols are required.

To restore HC we propose a combination of electrospun produced scaffolds (EPS) and photopolymerized gel (PhPG). EPS were prepared from nylon 6, polylactide-co-glicolide and their mixtures with gelatin in hexafluoroisopropanol. PhPG prepared from gelatin and chondroitin-4-sulfate both modified by glycidyl methacrylate, poly(ethylene glycol) dimethacrylate, Darocur 2959 and polymerized by 365 nm LED light has compressive strength 0,12 MPa and was not toxic for human chondroblasts. Fluorescent and scanning electron microscopy demonstrate that human chondroblasts isolated from HC remain viable and produce matrix components both inside PhPG and at the surface of gelatin-containing EPS, but proliferate solely at the surface of material.

Stacks of EPS (3 sheets, PhPG wetted) were implanted in knee-joint of rabbits, photopolymerized layer by layer and their biocompatibility was evaluated at different time intervals. Observed

microscopy and histological study demonstrate crush resistance, biocompatibility and good adhesion of the implant with surrounding cartilage with the trend to develop normal HC in place of biodegradable EPS.

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### Differentiation of Adipose Derived Mesenchymal Stem Cells (admsc) *In Vitro* to Cardiomyocyte Lineage using Fibrin-based Niche

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The multi lineage differentiation capacity of human adipose derived mesenchymal stem cells (ADMSC) makes it an attractive candidate for regenerating damaged heart. However, its multipotent nature necessitates *in vitro* lineage commitment into cardiomyocyte progenitor cells (CPC) to ensure desirable post transplant differentiation. This study aimed to standardize a differentiation niche/program and to demonstrate cardiomyocyte lineage commitment of ADMSC. Human adipose tissue was collected (IEC approved) from patients undergoing coronary artery bypass grafting. Standard collagenase digestion method was employed to isolate cells from tissue. Plastic adherent MSCs were culture-expanded, characterized by flow cytometry and confirmed tri-lineage differentiation potential. Third passage cells were seeded on fibrin matrix coated culture dish in RPMI medium with fetal calf serum; growth factors were added and withdrawn at specific time points of culture to induce differentiation. Cell phenotype was analyzed between 2 d to 24 d of culture. The morphological changes of cells such as multi-nucleation, shape and cell size were encouraging and suggested lineage commitment towards CPC phenotype. Real time qPCR analysis of CPC markers Nkx2.5, GATA-4, TNNT2, MYH6, and MYL2 showed up/down regulated expressions. Immuno fluorescence for cardiac markers TroponinI and Connexin43 confirmed differentiation to cardiomyocyte by 16th day. Protein expressions were quantified using flow cytometry. The results signify the possibility of *in vitro* manipulation of autologous ADMSC to commit them to CPC for potential application in cell transplantation therapy to regenerate infarct in cardiac tissue. Since CPC were developed within 8 d of culture, cells harvested around this period may be tested for transplantation success.

### Development of Osteochondral Interface Scaffold with Opposing Peptide Gradients for Directed Spatial Differentiation

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The calcified cartilage interconnecting the bone and cartilage of osteochondral tissue plays a major role in the tissue functional properties.<sup>1</sup> Osteochondral studies often develop separate chondrogenic and osteogenic scaffolds that are combined post-cellular differentiation, resulting in inadequate interfaces.<sup>1</sup> This study investigates the development of a continuous dual-gradient peptide scaffold which directs chondrogenic and osteogenic differentiation within a single scaffold, while maintaining a strong interface.

Peptide sequences designed to bind glycosaminoglycans (GAG) or nucleate mineral were conjugated to poly( $\epsilon$ -caprolactone) (PCL) and electrospun in gradients.<sup>2,3</sup> Initial studies investigated the effect of single peptide gradients to determine whether peptides induced distinct differentiation of human mesenchymal stem cells (hMSCs) without exogenous growth factors. Unmodified, GAG-binding, and mineralizing PCL scaffolds were seeded with bone marrow-derived hMSCs and cultured for 2 weeks in standard growth medium.

By 2 weeks, both peptide-gradient scaffolds had enhanced cellular penetration compared to unmodified PCL. GAG-binding scaffolds induced early chondrogenesis with significant upregulation in SOX9

expression, while mineralizing scaffolds induced early osteogenesis with significant upregulation in RUNX2 expression compared to unmodified PCL. Further, confocal microscopy demonstrated chondrogenic and osteogenic phenotypic hMSC changes by 2 weeks for respective peptide scaffolds.

This study demonstrates GAG-binding and mineral-nucleating peptide sequences are capable of inducing chondrogenesis and osteogenesis, respectively, without exogenous growth factors. Ongoing studies are investigating the effect of opposing dual gradients of the peptides in a single scaffold for the development of an intact osteochondral interface.

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#### Oxygen as a Modulator of Epimorphic Regeneration Processes

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Epimorphic regeneration, the regrowth of a digit or limb after amputation, is limited in mammals. The mouse digit model is insightful, however, as it provides a comparative model where regeneration occurs after amputation at the terminal phalangeal element (P3) but not at the next more proximal joint (P2). Environmental factors presented after amputation, including an elevated oxygen concentration, may be important regulators of regenerative processes such as proliferation. Hence, our objective was to determine if modulation of oxygen concentration affects the proliferative capacity of stromal cells isolated from regeneration-competent (P3) and regeneration-incompetent (P2) regions of mouse digits.

P2 and P3 cells were cultured under a high (21%), low (1%), or dynamic oxygen profile. Samples exposed to dynamic oxygen profiles underwent multiple switches between 1% and 21% oxygen over 4 days: 48 h/48 h (1 switch); 24 h/24 h (3 switches); and 12 h/12 h (7 switches). In general, we found that P2 cell proliferation responded to the switch in oxygen concentration while P3 cell proliferation was regulated by the level of oxygen. Furthermore, pairwise comparisons revealed that the 7-switch profile significantly increased P2 cell number compared to the 1-switch or either fixed concentration profiles.

Here, we found that the oxygen environment after amputation can affect the initiation of regeneration by altering proliferation of the endogenous cells. Thus, modulation of the microenvironment may be one approach for promoting elusive epimorphic regeneration in humans. Subsequent studies will focus on paracrine signaling to promote morphogenesis of a complex 3D structure such as a limb.

#### Non-invasive Tissue Specific Reporter System for Monitoring of Skeletal Muscle Cell Differentiation

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**Background:** Detection of skeletal muscle differentiation plays a key role for tissue engineering. In this study, we developed a reporter system to investigate the effectiveness of murine skeletal muscle differentiation. The use of a tissue specific promoter combined with a non-invasive bioluminescence assay allowed the evaluation of biological processes during myogenic differentiation in 2D and 3D culture systems as well as in bioreactors.

**Methods:** To construct the muscle creatine kinase (MCK) specific reporter vector (pE3MCK-MetLuc ± CMVe), triple tandem of MCK enhancer was positioned to its truncated basal promoter (87 bp) upstream of either a secreted luciferase or a fluorescent protein. In addition, up to three different myogenesis related miRNA seed sequences were ligated into 3'UTR of DNA, in order to develop tracking strategy by reducing background signals in non-muscle

cells. Cells were transfected with indicated constructs by using lipofection. The reporter gene signal in the supernatant was either detected using a luminometer after conversion of substrate into light units (secreted luciferase) or by fluorescence microscopy (fluorescence protein).

**Results:** The constructs exhibited strong luciferase and fluorescence expression in cells undergoing myogenic differentiation compared to non-muscle cells. Reconstruction of pE3MCK-MetLuc vector by ligating of miRNA seed sequences resulted in low background signals in non-muscle cells.

**Conclusions:** Tissue-specific promoters combined with E-boxes and miRNA seed sequences allow the amplification of tissue specific signals as well as kinetic monitoring of myogenic differentiation in a non-sample-destructive manner.

#### Cellular-Scale Surface Modification of Allograft Bone Increases the Recruitment of Necessary Bone-Forming Proteins, Expediting Bone Turnover and Ossification

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Previous studies demonstrated that biomaterial surface features improve cellular adhesion, permitting better scaffold incorporation through positive host cell response [1–3]. This approach may improve the osteoinductive potential of orthopaedic implants. The objective of this study was to evaluate the potential for micron-scale surface characteristics on allograft cortical bone to promote progenitor cell viability and proliferation. Human adipose-derived stem cells (ASCs; 5,000 cells/cm<sup>2</sup> seeding density) were cultured up to 21 days on patterned (arrays of 10 μm diameter pits) and non-patterned bone (control). Cell population and viability were measured, surface characteristics were assessed using atomic force microscopy, and osteoinductive proteins RUNX2 and SP7 were analyzed by Western blot. Patterning increased surface area by 88.7 ± 18.12%, permitting increased cellular attachment and protein production. At 72 hours, surface roughness of patterned samples was 96.6 ± 31.3% greater than the geometrical area compared to 11.0 ± 11.6% on controls. At 7 days, bone resorption on patterned samples was maximized at 737.4 ± 408.9 μm<sup>2</sup> and pit diameter increased to 12.4 ± 2.3 μm. After 21 days, patterned bone formation was 384.45 ± 226.1 μm<sup>2</sup> and pit diameter reduced to 7.4 ± 2.0 μm. While RUNX2 production was not significantly different, SP7 on patterned samples was significantly greater than controls at 7 and 10 days (p=0.05 and p=0.0006 respectively). The cumulative production of SP7 between treatments was also significant (p=0.00236), which is indicative of a more osteoconductive surface. These results demonstrate the benefits of surface modification on biological implants to accelerate bone remodeling and ossification.

#### Neural Differentiated Marrow Derived Stromal Cells Seeded on a Biological Conduit Promotes Axon Myelination during Peripheral Nerve Regeneration

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Biological tissues with tubular structures such as arteries and veins can be used as a conduit to bridge a nerve gap. For long nerve gaps, additional cues may be required. These cues could be provided by Schwann cells and basal lamina of muscle fibers. This study is initiated to evaluate the efficacy of biological conduit seeded with neural differentiated marrow stromal cells (NDMSC) for peripheral nerve repair.

A biological conduit was created by lining muscle fibers in the a vein. The conduit was used to bridge a sciatic nerve gap of 1.5 cm in the Female Sprague-Dawley rats. Motor and sensory functions of the affected foot was assessed over a period of 12 weeks via nerve conduction and pinch tests. At the end of 12 weeks, the conduit was harvested and analysed histologically.

Improved angiogenesis and remyelination of axons were evident in biological conduits that were incorporated with NDMSC compared to those without. Using Green Fluorescent Protein as the reporter gene, we have demonstrated that the incorporated NDMSC formed the Schwann cells in the myelin sheath of the regenerated axons.

The incorporation of NDMSC improved sensory but not motor function of the foot innervated by the repaired sciatic nerve. This could be attributed to the fact that axonal regeneration in the nerve conduit was confined only to a small area. Further studies are required to improve the performance of the cell-seeded biological conduit before it can be translated to the clinic.

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### Pro-remodeling Macrophage Polarization at the Tissue-Implant Interface by Local Release of IL-4 from Coated Polypropylene Meshes

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Polypropylene mesh implantation is a common option for hernia and pelvic organ prolapse repair. However, polypropylene implants are commonly associated with a foreign body reaction and scar tissue formation at the tissue-implant interface. It has been hypothesized that transient polarization of macrophages at the tissue-implant interface to an anti-inflammatory/regulatory (M2) phenotype will promote better integration into the surrounding tissue. Therefore, a degradable nanometer thickness coating containing IL-4 (an M2 polarizing cytokine) has been developed.

Polypropylene mesh was treated with radio frequency glow discharge and layer-by-layer (LbL) coating was performed using chitosan and dermatan sulfate. Loading was achieved by incubation of IL-4 with dermatan sulfate prior to coating. Uniformity of the coating was assessed using XPS, FTIR and Alcian Blue staining. ELISA assays showed IL-4 loading and release were dependent on the number of coating layers. *In-vitro* macrophage culture assays showed that meshes loaded with IL-4 promoted polarization to an M2 phenotype (assessed by higher expression of arginase-1), demonstrating maintenance of IL4 bioactivity.

A murine model showed that implantation of IL-4 loaded meshes resulted in less dense cellular infiltrates around mesh fibers compared to controls (pristine and coated [no IL4] meshes). Additionally, the percentage of M2 macrophages expressing arginase-1 was higher for IL-4 loaded meshes compared to controls. These results suggest that the local release of IL-4 from coated polypropylene meshes is able to polarize macrophages to an M2 phenotype, and may have the potential to promote better implant integration.

### Self-healing Nanostructured Colloidal Gels for Bone Tissue Regeneration

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The extraordinary self-healing ability of many human organs is the most important source of inspirations for design of synthetic materials capable of autonomously reversing the damage development. In particular, by employing individually weak but collectively strong bindings at the microscopic level, many bone tissues such as bones showed intrinsic self-healing ability and high mechanical strength.

Enlightened by micro-architecture of bone tissues, we innovatively develop a class of colloidal gels which allow for a "bottom-

up" approach for the design of mechanically strong and self-healing scaffolds by employing organic and inorganic nanoparticles as building blocks. By introducing non-covalent electrostatic interactions, such colloidal networks typically showed a intriguing shear-thinning and self-healing behavior, which can be attributed to the fast and reversible re-establishment of the electrostatic interactions between anionic and cationic nanoparticles as well as re-arrangement of jammed particles packing upon gel equilibration. The use of nanoparticles, which can also serve as delivery vehicles for therapeutic purposes, rendered the gels of capable of programmable release of multiple biomolecules by fine-tuning individual particle degradation. Further *in vivo* studies confirmed their decent biological properties of the injectable gels as well as capacity to accelerate bone regrowth even in load-bearing physiological condition.

Therefore, these novel colloidal gels that allow autonomic repair of structural and mechanical properties, and be capable of controllable release of therapeutic biomolecules to provoke the cascade of intrinsic healing process of human tissues could potentially open a promising avenue for the design of biomaterials for tissue regeneration.

### Substrate Stiffness Influences Osteogenic Differentiation of Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells

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Studies have shown that, through a mechanism of mechanotransduction, mechanical properties of the surrounding environment can influence stem cell differentiation. *In vitro*, this can be seen by the effect of substrate stiffness on the differentiation of mesenchymal stem cells (MSCs) derived from bone marrow. The effect of substrate stiffness has not been observed on the differentiation of MSCs derived from induced pluripotent stem cells (iPSC-MSCs), which could serve as a novel stem cell source for tissue engineering. By culturing iPSC-MSCs on Polydimethylsiloxane (PDMS) substrates with varying stiffness, determined by percent concentrations of the crosslinking component of the two-part elastomer, we tested the hypothesis that mechanical stimuli can promote cellular proliferation and osteogenic differentiation. Substrates of 4%, 8%, 12%, 16%, and 20% crosslinker concentrations were utilized with lower percentages resulting in softer substrates, and run against controls grown on tissue culture plastic (TCP). Gene expression at 7, 14 and 21 days shows upregulation of osteogenic markers such as Runt-related transcription factor 2 (RUNX2), Alkaline Phosphatase (ALP), and Osteocalcin in cells cultured on substrates with 12% and 16% crosslinker concentrations. Alizarin Red staining, a quantitative measure of calcium deposition by osteogenic cells, demonstrates enhanced osteogenic differentiation of cells on 12%, 16%, and 20% substrates in comparison to softer substrates and TCP. Our results demonstrate the significant influence of substrate stiffness in the control of osteogenic differentiation of iPSC-MSCs, and suggest a means for enhanced cellular expansion and differentiation control when producing cellular populations relevant for regenerative stem cell therapy.

### Corneal Endothelial Tissue Engineering: An Improved Cell Cultivation Platform with Topographic Cues

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One of the most common indications for corneal transplantation is corneal endothelium dysfunction, which can lead to corneal

blindness. Due to donor cornea shortage, alternative treatments are needed, which rely on the successful *in-vitro* culture of primary human corneal endothelial cells (HCECs). Primary HCECs are non-proliferative *in-vivo* and challenging to expand *in-vitro*. The objective of this study was to develop a cell culture platform for enhanced cell proliferation and functional markers of donor-derived primary HCECs. Micro- and nano-pillars and wells were patterned on polystyrene by using hot-embossing technique. Human donor-cornea derived primary HCECs were cultured in dual-media on patterned polystyrene. The effect of topography on cell functions was characterized in terms of proliferation rate, cell morphology, and expression of functional markers. The results demonstrated that the proliferation of primary HCECs increased close to 3-fold, and the expression and localization of Zona Occludens-1 (ZO-1) was significantly enhanced on TCPS pillars. 250 nm pillars induced an optimal hexagonal morphology of HCECs. Higher amount of ZO-1 expression was maintained even when the topographic cues were removed in the successive seeding, showing that the cells were able to maintain or “memorize”, the topography-mediated cell behaviors. The findings emphasize that the culture system for primary HCECs can be improved by incorporating surface topographic cues for the cultivation and regeneration of human corneal endothelial cell layers for tissue engineering, cell therapy and drug screening applications. The topographic memory could specifically be useful in cell-therapy based approaches to improve the *in-situ* endothelial cell monolayer formation upon cell delivery.

#### Soft Tissue Stiffness Range Influences Early Commitment of Mouse Embryonic Stem Cells Towards Endodermal Lineage

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Differentiation decisions of ESCs during either embryogenesis or in culture *in-vitro* are influenced by exposure not only to chemical signals but also to physical forces. Physicochemical cues, such as physiologically relevant substrate stiffness may have a significant effect on stem cell fate decisions. We explored the relationship between matrix stiffness and spontaneous or growth factor-induced commitment of ESCs towards endodermal-lineage. mESCs were grown on either FN-PA or FN-TCP for 6-days, in the presence or absence of endoderm-inducers, mRNA was extracted and evaluated by qRT-PCR. The stemness marker, Oct4 gene expression was slightly down-regulated and significantly higher by about 50% in its expression on soft stiffness FN-PA gels compared to FN-TCP, but independent on the matrix stiffness in a physiological range. This finding indicates that self-renewal of mESC is maintained in soft stiffness. Under conditions of spontaneous differentiation, the expression of endoderm gene markers, Foxa2, Sox17, Cxcr4 was gradually up-regulated in the range of stiffness from 0.25 to 20 kPa and was, respectively, 6, 4 and 2.5 fold higher than in cells grown on FN-TCP. When the cells were treated for 6-days with 20 ng/ml activinA the expression of endoderm marker genes was strongly up-regulated in the same-range of stiffness 0.25 to 20 kPa and were 140, 150 and 8 folds higher expression than on cells grown on FN-TCP. The biphasic trend of the dependence of endodermal marker stimulation on the substrate stiffness was similar in spontaneous and AA-induced differentiation, indicating a direct effect of physiological “soft tissue” stiffness (emulating that of endoderm-derived soft organs) on gene expression of markers of definitive-endoderm.

#### Cyclic, Uniaxial Tensile Strain Amplitude Influences the Kinetics of Alignment and Myogenic Differentiation of Adipose-Derived Stem Cells

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Adipose-Derived Stem Cells (ASCs) have shown myogenic potential (Mizuno *et al.*, 2001) with increased potential when cultured under cyclic strain (Huri, 2013). However, the impact of strain amplitude on myogenesis is still widely unknown.

This study seeks to understand the influence of strain amplitude on ASC alignment and myogenesis. Passage three ASCs were seeded on UniFlex™ Flexcell® membranes at a density of 5000 cells/cm<sup>2</sup> and were cultured for four days prior to beginning a cyclic strain regimen. One day into the four day period, half the wells received myogenic induction cues for 24 hours (10 μM 5-Azacytadine, 5% horse serum, 1% P/S, and 1% FBS in low-glucose DMEM) while the other group was maintained in control media (10% FBS and 1% P/S in low-glucose DMEM).

Subsequently, both groups were maintained in control media for three days followed by daily cyclic strain at 0.5 Hz for one hour per day for 18 days at 0, 4, 8, and 10% strain to determine the relationship between strain amplitude and cell alignment and multinucleation.

ASCs showed alignment perpendicular to the direction of strain, while cells grown in static culture showed random alignment. The strain percentage had an impact on the kinetics of alignment, with the higher strain groups showing greater alignment at earlier time points. The samples were also stained for MyoD, Desmin, and Myosin Heavy Chain showing that both myogenic induction and cyclic culture play a role in myogenic differentiation.

#### Tailor-made Coupled Helical Coils for Soft Tissue Engineering of Tubular Urological Structures

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Coupled helical coils are promising tools to engineer tubular organs and they can be easily integrated into collagen sponges and/or hydrogels to maintain the desired dimensions during (re)modeling *in-vitro* and *in-vivo*. In this study, we developed coupled helical coils and evaluated their potential for tubular genitourinary tissue engineering applications based on (bio)mechanical characteristics. Coupled helical coils were manufactured by winding commercially available, resorbable surgical suture filaments (copoly-L-lactide/ε-caprolactone (PLCL), copoly-glycolide/ε-caprolactone (PGCL) and polydioxanone (PDS)) in a clockwise- and anticlockwise helix, resulting in tubular structures with a crosswise pattern (distance cross to cross ~4 mm). Fiber crossover points were fused together to secure them. All manufactured coils were compared with the (bio)mechanical properties of native ureteral and urethral tissue. The produced tubular structures (φ: 10 mm; l: 80 mm) showed uniform crosswise patterns (mesh area ~16 mm<sup>2</sup>). Constructs had a Young's Modulus of 1.46±0.48 MPa and Ultimate Tensile Strength (UTS) of 1.07±0.21 MPa, similar to native ureteral and urethral tissue which demonstrated a Young's Modulus of 3.60±2.40 MPa and 1.37±1.22 MPa and UTS of 1.61±0.47 MPa and 1.05±0.48 MPa, respectively. Cyclic tensile tests of PLCL fabricated coils demonstrated little “shape-memory”, as they did not spring back to their original shape after extension, while other constructs showed a high “shape-memory” effect. Furthermore, degradation tests indicated that PDS fabricated coils retained their tensile strength for 21 days, while the other constructs lost their strength within two weeks. Coupled helical coils manufactured from PDS show potential to strengthen tubular constructs produced with structurally weak biomaterials for tubular genitourinary organs.

#### Heparin-Based Microparticles for Temporal Control of Chondrocytic Differentiation

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Heparin is a sulfated glycosaminoglycan that can sequester positively-charged growth factors (GFs) involved in cellular differentiation (1). Integrated into the cellular microenvironment, heparin could potentially control cell access to GFs via sequestration and thus modulate differentiation. To investigate this, heparin microparticles were incorporated into ATDC5 cell spheroids, a model system for chondrogenesis. Additionally, heparin microparticles with a degradable shell of poly(ethylene-glycol) (PEG) were developed to temporally control heparin presentation.

Heparin methacrylamide and PEG diacrylate (low protein binding control) microparticles were fabricated via water-in-oil or water-in-water emulsions. Core-shell heparin-PEG microparticles were fabricated by suspending heparin microparticles in a PEG diacrylate solution containing dithiothreitol (to achieve hydrolytic degradation). ATDC5 spheroids containing heparin, PEG, or no microparticles were formed via aggregation and cultured 18 days. Spheroids with core-shell microparticle were also formed and cultured 7 days.

In comparison to the no microparticle control, the heparin microparticle group showed reduced glycosaminoglycan and collagen II deposition and lower chondrocytic gene expression (7.4+1.8- and 16.5+6.18-fold decrease for collagen II and aggrecan, respectively, at day 6). The PEG microparticle group was similar to the no microparticle control (1.8+0.15 and 2.85+0.21-fold decrease for collagen II and aggrecan). Core-shell microparticles were successfully incorporated into ATDC5 spheroids with no decrease in cell viability.

This study illustrates the ability of heparin microparticles, possibly through GF sequestration, to delay chondrogenesis. Development of degradable core-shell microparticles provides opportunity to temporally modulate heparin presentation. This technology may allow heparin microparticle-mediated temporal modulation of cell differentiation, a novel strategy for tissue regeneration.

(1) Bishop JR. Nature, 2007.

### Bioactive Glasses at the Osteochondral Interface: Directing the Mesenchymal Stem Cell Response for Osteochondral Tissue Repair

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**Objectives:** Bioactive glasses (BG) are smart, tailorable materials that are known to impact cell behaviour. Characteristically, BG modify their local ionic microenvironment by releasing therapeutic ions when introduced in biological fluids. Here, we evaluate the application of BG as the bone substrate of multilayered scaffolds to treat osteoarthritis. By designing appropriate glass compositions, the ionic dissolution products may enhance both bone and cartilage regeneration. In particular, the influence of magnesium and strontium incorporation in different glass compositions on mesenchymal stem cell (MSC) chondrogenic differentiation was studied.

**Methods:** Human bone marrow-derived MSCs (hMSC) were cultured as pellets in control or BG-conditioned medium for up to 21 days. The impact of BG dissolution products on hMSC metabolic activity and chondrogenesis were determined.

**Results:** Metabolic activity of hMSCs increased when cultured in BG-conditioned medium after 4 days while the DNA content of hMSC pellets was comparable to control after 21 days. Accumulation of glycosaminoglycans was significantly increased compared to day 0 and at least as high as control in presence of all BG compositions indicating chondrogenic differentiation of hMSCs. Histological assessment of hMSC pellets by safranin O/fast green and toluidine blue staining suggested that incorporation of magnesium was beneficial while strontium didn't seem to have a strong effect on cartilaginous tissue formation in these culture conditions.

**Significance:** Our data suggests that BG are versatile materials to modify the local tissue/biomaterial environment and direct cell behaviour. Analysis of differentiation marker expression will provide further insight into the effects of BG-conditioned medium on hMSC chondrogenesis.

### High Throughput Generated Fibrochondrocyte Micro-aggregates for Bottom-Up Tissue Engineering: Impact of Low Oxygen Tension

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**Objective:** In meniscus tissue engineering, enhancing the matrix quality of the neomeniscal tissue is important. When the differentiated phenotype of fibrochondrocytes is lost, the quality of the matrix becomes compromised. The objective of this study was to produce uniform fibrochondrocyte micro-aggregates with desirable phenotype and tissue homogeneity in large quantities using a simple and reproducible method, keeping in mind the criteria needed for future application in 3D bioprinting. Furthermore, we investigated if hypoxia could enhance the matrix quality.

**Method:** Porcine fibrochondrocytes were expanded at 21% oxygen until passage 3 (P3) and a gene expression profile was determined. P3 fibrochondrocytes were cultivated in chondrogenic medium at 5% and 21% oxygen in high-throughput agarose microchips containing 2865 microwells with 200 µm diameter. Evaluation included live/dead staining, (immuno-)histochemistry, dimethyl-methylene blue assay and RT-qPCR of the micro-aggregates.

**Results:** Gene expression analysis showed that porcine fibrochondrocytes rapidly dedifferentiate. Uniform, stable and viable micro-aggregates could be formed within four days. Hypoxia cultivated micro-aggregates showed an enhanced redifferentiation. Sulfated glycosaminoglycan synthesis was higher, collagen II expression was upregulated and the collagen II/collagen I ratio was significantly improved in the 5% oxygen cultures.

**Conclusions:** The beneficial use of an agarose micro-well chip for fibrochondrocyte aggregation was demonstrated. Relative to the normoxic cultured micro-aggregates, hypoxic cultured micro-aggregates enhanced the redifferentiation of the dedifferentiated meniscal fibrochondrocytes and thereby matrix quality. High-throughput production of uniform micro-tissues holds promise for the generation of larger scale tissue engineering constructs, in screening applications or optimization of redifferentiation mechanisms for clinical applications.

### Enhanced Corrosion Resistance and Cyto-biocompatibility of Plasma Immersion Ion Implanted (PIII) WE43 Magnesium Scaffolds for Bone Tissue Engineering

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Magnesium scaffolds have received attractions in bone tissue engineering due to their biodegradability, excellent biocompatibility, suitable mechanical properties and compatible elastic modulus. Furthermore, magnesium ion release in small concentration can stimulate osteoblast differentiation and new bone regeneration [1]. However, rapid degradation of magnesium alloys and subsequent hydrogen gas evolution significantly limits their clinical applications. Plasma immersion ion implantation (PIII) [2] is proved to be an effective method for suppressing rapid degradation of magnesium alloys. In this paper, PIII method was employed to enhance corrosion resistance of WE43 magnesium scaffolds by titanium and oxygen dual implantation. Surface composition and morphology of PIII-treated WE43 scaffolds were characterized by XPS and AFM. The results indicated that a dense TiO<sub>2</sub> film with approximately 120 nm was formed over the surface of WE43 scaffolds. Electrochemical and immersion tests in simulated body fluid (SBF) and DMEM are performed to systematically investigate the corrosion resistance of PIII-treated alloys. Electrochemical impedance of PIII-treated WE43 alloys increased by eighty times and the corrosion density remarkably dropped after titanium and oxygen

dual implantation. No obvious damage on the PIII-treated WE43 scaffold surface was observed and the mg ion release was retarded in comparison with the untreated after immersion in SBF for 3 days. The cyto-compatibility of PIII-treated WE43 scaffold was evaluated by MTT assay. The PIII-treated samples exhibited higher cell viability than the untreated group. Furthermore, the animal study is still ongoing. In summary, it is expected that PIII-treated WE43 scaffolds may potentially apply for large bone segmental defect in the future.

### Mimicking Cartilage Tissue Zonal Organization by Engineering Hydrogels with Dual Gradient Niche Cues

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Cartilage tissue is characterized with zonal organizations, with mechanical and biochemical cues transition in a gradient manner. However, most scaffolds developed so far are homogeneous in nature. The goal of this study is to better mimic cartilage zonal organization by engineering hydrogels with biochemical and mechanical gradient cues. Specifically, we designed a facile method that allows fabrication of tissue scale (cm) hydrogels with mechanical and biochemical gradients that support homogeneous cell encapsulation with high viability, proliferation and ECM deposition in 3D over time. To control hydrogel stiffness, 8-arm PEG-NB was chosen due to its bio-inertness, which can be crosslinked by PEG diethanol. To mimic biochemical content of cartilage, methacrylated chondroitin sulfate was incorporated. Compressive mechanical testing and sGAG assay confirmed successful formation of mechanical and biochemical gradients within hydrogels. When encapsulated in 3D gradient hydrogels, both chondrocytes and mesenchymal stem cells exhibited zonal-specific responses. Increasing hydrogel stiffness and CS content led to enhanced cartilage gene expression (aggrecan and type II collagen), increased cell proliferation, as well as markedly enhanced collagen deposition. The trend of resulting ECM deposition and cell morphology mimics the superficial to deep zones of native articular cartilage. Importantly, our results showed that cells in dual gradient hydrogels demonstrate better zonal response than those in single gradient hydrogel controls, highlighting the need for dual gradient hydrogels to achieve optimal tissue zonal organization. The platform reported here offers an enabling technology for engineering tissues with zonal organizations and may be broadly applicable in regenerating different tissue types and interfaces.

### Molecular Mechanism for Endothelial Differentiation of Mesenchymal Stem Cells Driven by *In Situ* Crosslinkable Gelatin Hydrogels

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Clinical trials involving treatment of mesenchymal stem cells (MSCs) for severe vascular diseases have highlighted the need to improve cell engraftment and to promote pro-angiogenic activities. A functional material is an ideal solution to achieve these two goals as spatiotemporal and batch-to-batch variations in classical therapeutic delivery can be minimized with accelerated *in situ* tissue regeneration. Gelatin may serve as a promising biomaterial due to its excellent biocompatibility, biodegradability, and non-immuno/antigenicity. An injectable, *in situ* crosslinkable gelatin was developed by conjugating enzymatically crosslinkable hydroxyphenyl propionic acid (GHPA). In our previous study [1], MSCs cultured *in vitro* or injected *in vivo* in GHPA spontaneously differentiated into endothelial cells, as evidenced by marked increases in endothelial cell marker expressions (Flk1, Tie2, ANGPT1, vWF), and forming an extensive perfusable vascular network. Additionally, favorable host macrophage response was achieved with decreased pro-inflammatory marker iNOS and increased reparative marker MRC1. These results indicate GHPA as a promising soluble factor-free cell delivery template with advanced functions to direct

stem cell differentiation. To understand the mechanism driving MSC differentiation, we are currently investigating the expression of integrins and their downstream signaling molecules. Preliminary results indicate that integrin  $\alpha v \beta 3$ , PI3K and ERK1 that are heavily implicated in endothelial differentiation [2] are significantly up-regulated for MSCs in GHPA. The expression levels and the phosphorylation of signaling molecules will be verified through western blotting, and small molecule inhibitors will be employed to block the pathways and further elucidate the molecular mechanism involved in GHPA-driven MSC differentiation into endothelial cells.

### Cancer Extravasation Dynamics in an *In Vitro* Model

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Extravasation of cancer cells from the blood vessel, which involves the trans-endothelial migration and tissue invasion, remains a less well understood process in cancer metastasis. Understanding the mechanisms underlying the extravasation process is of fundamental importance in developing therapeutic targets for the prevention of metastasis. The aim of this research is to investigate breast cancer extravasation *in vitro*, using a microfluidic system with adjustable biophysical and biochemical factors, to mimic the 3D *in vivo* vascular microenvironment. The device is created to mimic the interface between the blood vessel wall and the surrounding extracellular matrix. Using this microfluidic system, cancer cell extravasation from the blood vessel to the artificially simulated extracellular matrix was observed. Combined with an image analysis algorithm, cancer cell morphological changes during migration were found to be microenvironment-dependent. In particular, cancer cells were observed to exhibit invadopodia-like protrusions during the invasion process.

### Novel *In Vitro* Autologous Tissue-engineered Substitutes of Vaginal Tissues With Self-assembly

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**Objectives:** Many diseases necessitate the substitution of vaginal tissues including congenital and acquired pathologies. Existing reconstructive procedures are accompanied with many complications. Recent tissue-engineered vaginas were implanted in patients with congenital vaginal abnormality. However, the use of non-autologous tissues for vaginal bioengineering may be associated with infection, immunologic reactions and fibrosis. In this study, we aimed to build neo-vaginas with the self-assembly technique using autologous epithelial and stromal cells without the use of exogenous materials.

**Methods:** Vaginal epithelial and stromal cells were isolated from different tissue biopsies. Stromal cells from 3 patients were stimulated separately to form collagen sheets. Three collagen sheets were stacked, followed by seeding vaginal epithelial cells on top to form vaginal equivalents. These equivalents were evaluated with different histological, functional and mechanical tests.

**Results:** Stromal cells formed collagen sheets that could be handled easily. Vaginal epithelial cells formed a well differentiated stratified epithelial layer. Epithelial differentiation was marked with pancytokeratins, Involucrin and Keratin 13. Tissue impermeability was suggested with E-cadherin and ZO-1 antibodies. Mucous and glycogen production were demonstrated. Vaginal stromal tissue was shown to include collagen I, III and IV, and fibronectin. Continuous basement membrane underlying epithelial cells was evident by Laminin and collagen IV. The epithelium, basement membrane and stroma characteristics were comparable to native vaginal tissues.

**Conclusions:** Using the self-assembly, *in vitro* vaginal tissues were created with many functional and biological similarities to native vaginal tissue. This model has the potential for vaginal substitution, disease modeling for infection and testing applicants and drugs.

### Human Engineered Heart Tissue - A New Tool for Preclinical Drug Safety Screening

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**Objective:** Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) have great potential for preclinical drug safety screenings. This study evaluated the usefulness of three-dimensional, strip-format, force-generating engineered heart tissues (EHTs) from hiPSC-CM for drug screening.

**Methods and results:** 24-well EHTs were generated from hiPSC-CM (1 Mio each) in a fibrin matrix and casted between flexible silicone posts. Contractile function of coherently beating EHTs was repeatedly monitored by an automated video-based system. Histological analysis revealed a high degree of sarcomeric organization and alignment of cardiomyocytes in EHTs. Contractile force increased with increasing calcium concentrations (EC50 0.9 mM), pre-load (max. force at 110% of baseline length), calcium sensitizer EMD-57033 (10 µM), Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor ouabain (100 nM) and beta-adrenergic stimulation (100 nM isoprenaline) and was decreased by addition of the muscarinic agonist carbachol (10 µM). Ion channel modulators revealed concentration-dependent effects (mean ± SEM in % of baseline mean): The Na<sup>+</sup> channel opener ATX-II, Ca<sup>2+</sup> channel agonist Bay K 8644 and hERG channel blocker E-4031 increased relaxation time to 161 ± 5% (10 nM ATX-II; n=20), 160 ± 6% (0.1 µM Bay K-8644; n=8) and 115 ± 2% (10 nM E-4031; n=20). The Na<sup>+</sup> channel blocker TTX decreased beating rate to 73 ± 5% (0.3 µM; n=16), whereas the K<sup>+</sup> channel agonist NS-1643 increased beating rate to 154 ± 3% (300 µM; n=19). The Ca<sup>2+</sup> channel blocker verapamil reduced contraction force to 51 ± 4% (0.3 µM; n=19).

**Conclusions:** Responses of hiPSC-CM-EHTs to standard interventions principally mimic that of native human heart tissue, suggesting that human EHTs are suitable for automated toxicology screens in future drug development.

### Development of a Novel Injectable Microscaffold for the Treatment of Injured Cartilage in Ptoa

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Posttraumatic Osteoarthritis (PTOA) resulting from traumatic combat injuries has limited treatment options which often fail to repair damaged cartilage and lead to a permanent cure. Early treatment is critical for complete recovery from the disease. Our goal is to develop a novel treatment for PTOA. We believe that, shortly after injury, the recruitment of autologous stem cells to activated chondrocytes can reduce tissue damage and improve cartilage regeneration. To test this hypothesis, folate-conjugated hyaluronic acid (HA) microscaffolds ranging from 200 nm - 5 µm were fabricated to target activated chondrocytes. HA microscaffolds were chosen as bioactive agent carriers from previous studies showing injection of HA particles can reduce the symptoms of mild osteoarthritis (1-2); additionally, folate has been shown to effectively target inflammatory cells in arthritic tissues (3). The HA microscaffolds showed no toxicity to cells or tissues. Folate conjugation improved HA microscaffold's targetability to inflammatory cells, enhancing their accumulation at the cartilage defect sites. To recruit stem cells, HA microscaffolds were loaded with erythropoietin (Epo). As expected, Epo-releasing HA microscaffold injection increased stem cell recruitment and reduce localized inflammatory responses at implant sites. We believe that damaged chondrocyte targeting and localized stem cell recruitment will serve as a novel and minimally-invasive therapy to combat PTOA.

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### Effects of Nanofibrous Surface Topography on Macrophage Polarization and Foreign Body Giant Cell Formation

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Nanofibrous surface topography has been noticed as an advantageous scaffolding architecture for promoting cell differentiation. Scaffolds are implanted into a living tissue, and host tissue responses are an indispensable aspect in tissue engineering. It is known that macrophage is a primary cell that recruits firstly to implantation sites and that its polarization can profoundly influence on host regenerative responses. In this study, the effects of fibrous surface topography on macrophage polarization and foreign body giant cells (FBGCs) formation were examined through *in vivo* and *in vitro* experiments. Nanofibrous (NF) surface film was prepared from poly(ε-caprolactone) by electrospinning, and the responses to NF were compared with those to solid one. The films were implanted to the subcutaneous tissue of rats for up to 4 weeks. The samples were retrieved and analyzed through Wright staining, immuno-staining against CCR7 (M1 phenotype; pro-inflammatory) and CD163 (M2 phenotype; anti-inflammatory/regenerative), and qPCRs. Macrophages (positive cells to Wright staining) were recruited to both the films, and the ratio of M2 to M1 on NF films was much higher than that on solid ones. Formation of FBGCs on NF was significantly lesser, compared to solid one. These findings were also confirmed in the cultures of RAW264.7 cells. Furthermore, the thicknesses of capsule-like fibrous tissues around the films were significantly thinner in NF groups than in solid ones. The results in this study consistently indicate that fibrous surface topography supports the polarization of macrophages to a more regenerative phenotype and the integration with host surrounding tissues.

### Modulating Interactions between Adipose-derived Stem Cells and Neonatal Chondrocytes in 3D using Combinatorial Hydrogels

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Although cartilage was envisioned to be one of the first tissues to be successfully engineered, effective therapies for articular cartilage repair remains elusive after two decades of efforts. One critical bottleneck is the lack of abundant cell sources that can regenerate articular cartilage with desirable phenotype. Neonatal chondrocytes (NChons) has high regenerative potential for cartilage, but its clinical translation is limited by donor scarcity. We have recently reported an intriguing finding that NChons can be proliferate and produce neo-cartilage far more effectively when mixed co-cultured with adipose-derived stem cells (ADSCs) in 3D hydrogels, which allows substantial reduction of NChon number needed. The goal of this study is to investigate the effects of hydrogel stiffness and ECM concentration on synergistic interactions between NChon and ADSC. ADSCs (75%) and NChons (25%) were mixed encapsulated in 39 combinatorial hydrogel compositions with independently tunable stiffness (10, 30, 90 kPa) and biochemical compositions. Three types of methacrylated ECM molecules were examined including chondroitin sulfate (CS), hyaluronic acid (HA) and heparan sulfate (HS), each with varying concentration at 0.5%, 1.25%, 2.5% and 5% (w/v). Biochemical cues, but not hydrogel stiffness, played a dominant role

in modulating interactions between ADSCs and NChons. CS led to enhanced catalyzed cartilage formation in a dose-dependent manner while HS inhibited such synergy. Immunostaining also showed larger neocartilage nodules in CS-containing hydrogels. The results of this study offer valuable guidance in optimizing hydrogel compositions that would best support the synergistic interactions between ADSCs and NChons for robust cartilage repair.

### Engineering Angiogenesis: A Materials Approach to Vascularizing Tissue

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There is a growing interest and need to harness the complex nature of viable three-dimensional tissue scaffolds of various cellular systems in an *in vitro* culture. This work seeks to develop synthetic biomaterials that will support the development of 3D microvascular networks in an *in vitro* environment for dense tissue, such that they maintain their native physiological properties. The vascularized dense tissue constructs can serve as a model for studying human physiological and pathological processes, which will provide a platform for drug testing that more closely resembles the native extracellular matrix architecture of the tissues *in vivo*. Although there has been great progress in the development of 3D culture models that are able to maintain a high degree of cell function, few models have successfully addressed the creation of a microvascular network within the tissue. We developed methods for building microvasculature using a fibrin gel assay that incorporates polymer microbeads, which are created using microfluidic devices. These polymer microbeads contain chemical functionalizations that not only serve as anchors for endothelial cell (EC) sprouting, but can also allow us to study the mechanical properties that dictate angiogenesis.

### Static Electricity Enhances Proliferation of Human Mesenchymal Stem Cells Seeded on Collagen and Fibronectin

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**Introduction:** Human Mesenchymal Stem Cells (hMSCs) are multipotent cells that have the capacity to differentiate into multiple cell lines. hMSCs have been shown to secrete fibronectin and collagen, which can function as extracellular matrix (ECM) components. Cell matrix adhesion plays a role in hMSC morphology, migration, proliferation, differentiation and survival. Administering an electric current has been shown to increase ECM synthesis of collagen, secretion of growth factors and expression of growth factor receptors. In this study, the effects of fibronectin and collagen, at varying concentrations and in combination, on hMSC proliferation were examined using a 2D proliferation assay. Furthermore, hMSCs were seeded in the presence and absence of static electricity (SE) exposure. SE was administered via a cathode ray tube television setup to generate and maintain a constant static electric field.

**Results:** It was observed that hMSCs have a greater capacity to proliferate on an ECM composed of collagen at 5 µg/mL than collagen at 10 µg/mL or fibronectin at 5 µg/mL and 10 µg/mL. Collagen and fibronectin showed enhanced proliferation compared to uncoated cultures. When hMSCs were seeded in the presence of SE, treating plates with SE enhanced hMSC proliferation in uncoated wells. In the presence of SE, an increase in cell proliferation was observed for fibronectin 5 µg/mL compared to collagen. The combination of collagen and SE caused a reduction in proliferation when compared to collagen without SE treatment. Both collagen and SE appear to have a positive effect on hMSC proliferation and future studies should explore the effects of migration and differentiation.

### Matrix Metalloproteinase-dependent Collagen Matrix Remodeling by Adipose Stromal Cells Regulates 3-D Sprouting Angiogenesis

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Adipose-derived stem cells (ASCs) can regulate both physiological and pathological angiogenesis through their ability to (i) secrete proangiogenic factors including vascular endothelial growth factor (VEGF) and (ii) stabilize blood vessels in the form of pericytes. However, ASCs also play an important role in extracellular matrix (ECM) remodeling yet the resulting effects on angiogenesis remain largely unclear. Here, we utilized a microfabricated 3-D tissue co-culture system to study the functional link between ASC-mediated ECM remodeling and endothelial cell sprouting. Human ASCs isolated from lipoaspirates were seeded into collagen type I-embedded microwells. Subsequently, a confluent monolayer of human umbilical vein endothelial cells (HUVECs) was seeded on top of these cultures, either immediately or after 7-days of ASC pre-culture, and angiogenic sprouting was quantified. Interestingly, immediate seeding of HUVECs led to pericyte-like association of ASCs with the endothelial monolayer and corresponding basement membrane deposition and reduced endothelial sprouting. In contrast, ASC pre-culture prior to HUVEC seeding dramatically increased angiogenic sprouting. Importantly, this effect was largely due to ASC-dependent matrix metalloproteinase (MMP) rather than VEGF secretion as MMP, but not VEGF inhibition abrogated these effects. The physiological relevance of these results to angiogenesis *in vivo* was tested using *s.c.* implantation of the varied constructs into immunocompromised mice. Collectively, our results suggest that the pro-angiogenic capability of ASCs is not only determined by their secretion of pro-angiogenic factors, but also their proteolytic remodeling of the surrounding ECM. Insights gained from this study will help improve current therapeutic strategies targeting angiogenesis during health and disease.

### Bioprinting Complex Cartilaginous Structures with ECM-based Bioinks

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**Introduction:** Bioprinting is an emerging technology in personalized medicine capable of producing patient specific implants. Current polymeric bioinks however do not provide the complexity of the native extracellular matrix (ECM), which contains a vast array of growth factors and matrix molecules. To address this shortcoming, a ECM-based bioink was developed and patient-sized auricular and nasal cartilages were printed.

**Methods:** Clinical BioCartilage<sup>®</sup> (Arthrex, USA) were mixed with gellan gum and alginate to form a bioink. This bioink was further mixed with chondrocytes in 6x10<sup>6</sup>/ml concentration. Extrusion printing using a Biofactory<sup>®</sup> (RegenHU, Switzerland) was carried out with a 410 micron needle and a printing speed of 800 mm/min. A thermo-reversible support was co-printed as a temporary sacrificial structure also serving as a cation reservoir to initiate crosslink of the bioink. Rheological, mechanical and biological properties of the bioink were characterized.

**Results:** Cartilage structures were printed in high resolution and in clinically relevant sizes. The bioink illustrated shear thinning and fast cessation of flow. Furthermore, the bioprinted structures had 224 kPa ± 7.0 kPa young's modulus. Cell viability was high (>80%) after printing and the presence of cartilage particles increase proliferation. MRI measurements of the printed constructs and volumetric images with diffusion coefficients were compared to native cartilage.

**Discussion & Conclusions:** The presented bioink allows reconstruction of particles in new structures with anatomical and functional characteristics mimicking the native tissue.

### Therapeutic Effects of Thermosensitive Ferulic Acid-loaded Hydrogel on Oxidative Stress-induced Damage in CISD2-deficient Cardiomyocytes

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CISD2 (synonyms ZCD2, Noxp70, Miner1, ERIS), a member of CDGSH iron sulfur domain protein family, is a mitochondria outer membrane protein. Recent studies have demonstrated that CISD2 deficiency causes mitochondria dysfunction and leads to cell death that may have a strong correlation with cardiovascular diseases. Ferulic acid (FA) is an excellent antioxidant and has been proven to have ability to prevent reactive oxygen species-induced diseases. In the present study, we aimed to evaluate the possible therapeutic effects of FA on CISD2+/+, -/- murine induced pluripotent stem cells (miPSCs)-derived cardiomyocytes under hydrogen peroxide (H2O2)-induced oxidative stress and the feasibility of use the thermosensitive chitosan-based hydrogel containing FA for tissue regeneration.

In the study, characterization of CISD2+/+, -/- miPSCs were evaluated by the alkaline phosphatase activity, embryoid body formation, mRNA gene expression, immunostaining, western blotting and transmission electron microscopy. After differentiation, the results showed that the mitochondria reactive oxygen species content of CISD2-/- miPSCs-derived cardiomyocytes were significantly higher than that of CISD2+/+ miPSCs-derived cardiomyocytes. The results of cell viability assay and calcein stain demonstrated that 500 µM of FA might be the optimal concentration to treat CISD2+/+, -/- miPSCs-derived cardiomyocytes. In the H2O2-induced oxidative stress model, the apoptosis level were significantly decreased when post-treatment of FA-loaded hydrogel on CISD2-/- miPSCs-derived cardiomyocytes. The biocompatibility of FA-loaded hydrogel has been demonstrated *in-vivo*. The results of the study suggest that the thermosensitive FA-loaded hydrogel may treat CISD2-/- miPSCs-derived cardiomyocytes from the damage caused by oxidative stress and have potential application for tissue regeneration.

### Gelatin-based Injectable hydrogels for Enhanced Angiogenesis

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**Objectives:** While injectable biomaterials are attractive for soft tissue regeneration, inadequate vascularization of the injectable constructs has long been a barrier, leading to necrosis or volume reduction after implantation. In this work, we developed a three-step process to synthesize novel injectable gelatin-derived hydrogels that are capable of controlling growth factor delivery to induce fast angiogenesis.

**Methodology:** In our approach, tyramine was first introduced into gelatin chains to provide enzymatical crosslinking points for gel formation after injection. Next, heparin, a polysaccharide with binding domains to many growth factors, was covalently linked to the tyramine-modified gelatin. Finally, vascular endothelial growth factor (VEGF) was incorporated into the gelatin derivative by binding with the heparin in the gelatin derivative. A chicken chorioallantoic membrane (CAM) assay and animal experiments were performed to evaluate *in vivo* bioactivity of the VEGF released from the hydrogels.

**Results:** The gelatin-based injectable biomaterials were successfully synthesized and characterized using FTIR, toluidine blue assay, gelation time measurement, mechanical property test, and *in vitro* enzymatic degradation. An *in vitro* release study and bioactivity assay indicated that the VEGF was released in a sustained manner with high bioactivity for over 3 weeks. *In vivo* implantation experiments shows deeper and denser cell infiltration and angiogenesis in the heparin-modified gelatin/VEGF gels were observed than in the controls after being subcutaneously injected in the dorsal side of mice for 2 weeks.

**Conclusions:** A gelatin-based injectable hydrogel was synthesized. The gelatin derivative/VEGF is an excellent injectable delivery system for fast inducing angiogenesis of soft tissue regeneration.

### The Development and Effect of BMPs Loaded Chitosan Based Sponge as a Delivery Carrier for Bone Regeneration

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The chitosan for pharmaceutical applications has been steadily increased because of its biocompatible, biodegradable, mucoadhesive and nontoxic natures. In present study, we have synthesized new carboxylic anhydride-conjugated glycol chitosan for new drug carrier. Hexanoic glycol chitosan (HGC), hydrophobically modified glycol chitosan, was synthesized by controlled N-acetylation of glycol chitosan. The HGC was evaluated degrees of acetylation (DA), kinetic behavior and effects of released drugs for practical applications in drug delivery systems. Aqueous solutions of HGC, has been mixed with both growth factors, bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-7 (BMP-7). The solution of containing HGC and growth factors was lyophilized and was made into sponge as a drug delivery carrier. HGC sponges conjugated with BMPs were employed for verifying the effect of bone regeneration via following assays, alkaline phosphatase activity (ALP), alizarin red staining (ARS) and reverse transcriptase-polymerase chain reaction. The release behavior of BMPs carriers significantly affects the efficacy of osteogenesis. In preliminary test, HGC sponge containing example drug, neutral red, was changed to hydrogel and slowly released to 30% of initial doses during 1 month. BMPs released from HGC sponges stimulated into osteogenic differentiation of bone marrow human mesenchymal stem cell than the controls in ALP and ARS while showing their higher values in BMP-2 than in BMP-7. These results were confirmed by gene expression, BMP-2, CBFA1, Runx2, osteocalcin, displaying similar trend. We are studying the effects of BMPs loaded sponge *in-vivo* calvarial defect rat model for bone healing. HGC sponge may serve as a versatile carrier for increasingly a candidate for tissue engineering.

### Characterization of Microvesicles (MVs) Derived from Human Adipose Mesenchymal Stem Cells Cultured in Normoxic and Hypoxic Conditions

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Mesenchymal stem cells (MSCs) are considered effective therapeutic agents inducing the functional improvement in the repair of injured tissues, not only through direct engraftment and differentiation, but also through their paracrine activity. Increasing evidences show that besides the secretion of trophic factors, the release of microvesicles (MVs) constitutes an alternative paracrine mechanism adopted by MSCs. In this preliminary study, we aim to carry out a detailed characterization of MVs released by human adipose-MSCs to study their possible involvement in tissue regeneration. The isolation of MVs is based on repeated ultracentrifugation steps of the conditioned medium of MSCs cultured in both hypoxic and normoxic conditions, followed by characterization of the resulting pellets enriched in MVs through electron microscopy, flow cytometry, western blot, mass spectrometry, and microRNA expression analysis. We observed that the MSC-derived MVs showed highly different distribution of size and shape. MVs positive for the expression of CD81 and Alix were detected in both culture conditions. The hypoxic condition enhances the release of MVs enriched in those microRNA that are typically associated to pro-angiogenic and pro-survival signals and to the macrophage

polarization. The recognition of MVs as carriers of genetic information has opened a new era in the field of cell biology. The use of MSC-MVs instead of whole living cells could therefore offer a series of advantages, both in term of safety and easy of handling.

#### Effects of the Cell Population on the Histological Properties of Cylindrical Fibrin-Agarose Nerve Substitutes

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**Background:** Generation of mechanically stable three-dimensional nerve substitutes may contribute to bridge critical peripheral nerve gaps, and fibrin-agarose hydrogels have been used in tissue engineering with promising results. The aim of this study is to elaborate and evaluate a nanostructured cylindrical fibrin-agarose nerve substitute (FAH).

**Methods:** Cellular (c-FAH) and acellular (a-FAH) nerve substitutes were generated using human fibrin and 0.1% type VII-agarose in a plastic compression nanostructure reactor. Cellular nerve substitutes (c-FAH) contained human adipose-derived mesenchymal stem cells. Both FAH were kept in culture and analyzed after 2, 4, 8 and 16 days for histological and ultrastructural analyses. Cell proliferation, cytoskeleton remodeling and extracellular matrix (ECM) synthesis were analyzed by immunohistochemistry.

**Results:** The histological analysis revealed changes in the morphology and behavior of the cells, which digested the surrounding biomaterial in a-NFAH and c-FAH. Cell proliferation and cytoskeleton remodeling differed between a-NFAH and c-FAH, with high cell proliferation after 16 days. A progressive synthesis of proteoglycans, glycoproteins and collagen was observed in the c-NFAH in comparison to a-FAH. Ultrastructural analysis confirms the degradation of the scaffold and the formation of interconnected cavities, but the cylindrical structure was preserved.

**Conclusions:** This study demonstrates the possibility to elaborate nanostructured nerve constructs with metabolically active cells and essential ECM molecules. This c-NFAH could be used for tissue engineering applications including peripheral nerve repair, but *in vivo* analyses are necessary to confirm this hypothesis.

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#### Study of a Tropoelastin-containing Dermal Scaffold for Wound Healing

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Burn injuries are among the most physically and psychologically debilitating injuries within approximately 6.6 million patients suffer from severe burns globally. The aim of our study is to characterize a novel, recombinant tropoelastin (10% w/v)-containing dermal substitute and examine its potential for improving wound healing.

Tropoelastin-containing scaffold was provided by Elastagen (Australia). The surface morphology was examined using a scanning electron microscopy, pore size and porosity were analysed using ImageJ. Proliferation of human dermal fibroblasts (HDFs) was assessed via counting cell number and cell infiltration into the scaffolds was examined by DAPI staining. Wound healing was examined in an established mouse model over 4 weeks. Angiogenic response to dermal scaffolds with/without tropoelastin was assessed using the

IVIS<sup>®</sup> *in vivo* imaging system and AngioSense 750EX fluorescent probe. Skin biopsies were collected at d14, d28 for histological and immunohistochemical analysis.

We found that tropoelastin served to increase pore size and elasticity compared to control and the increased open space contributed to the HDFs ingrowth. Tropoelastin-incorporated scaffold accelerated HDF proliferation and also supported active cell migration and infiltration into the scaffolds. In the mouse model, the scaffold with tropoelastin accelerated early stage angiogenesis by 2 weeks, as evidenced by increased angiogenesis fluorescent radiant efficiency in live animal imaging and the expression of endothelial cell adhesion marker CD146. Moreover, scaffold with tropoelastin decreased wound contraction compared to open wounds but had similar contraction to skin grafts and control scaffold. We conclude that including tropoelastin in the dermal substitute promotes wound repair through enhanced vascularization.

#### A Continuous Solvent- and Oil-free Method to Prepare Injectable Pegylated Fibrinogen Cell-laden Microparticles

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Injectable biomaterials with *in situ* cross-linking reactions have been suggested to minimize the invasiveness associated with most implantation procedures. However, problems related with the rapid liquid-to-gel transition reaction can arise because it is difficult to predict the reliability of the reaction and its end products, as well as to mitigate cytotoxicity to the surrounding tissues. An alternative minimally invasive approach to deliver solid implants *in vivo* is based on injectable microparticles, which can be processed *in vitro* with high reliability, while showing low cytotoxicity. Their delivery to defects can be performed by injection through small diameter syringe needles. We present a new methodology for the continuous, solvent- and oil-free production of photopolymerizable microparticles containing encapsulated human dermal fibroblasts. A precursor solution of cells in photo-reactive PEG-fibrinogen (PF) polymer was transported through a transparent injector exposed to light-irradiation before being atomized in a jet-in-air nozzle. Shear rheometry data provided the cross-linking kinetics of each PF/cell solution, which was then used to determine the amount of irradiation required to partially polymerize the mixture prior to atomization. The partially polymerized drops fell into a gelation bath for further polymerization. The system was capable of producing cell-laden microparticles with high cellular viability, with an average diameter between 88.1 µm and 347.1 µm, and a dispersity between 1.1 and 2.4, depending on the parameters chosen.

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#### Highly Porous Particles for Cell Recruitment and Delivery in Bone Tissue Engineering

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In this study we have investigated the ingrowth of cells into highly porous particles. Tissue engineering particle-based systems have great potential as scaffolds, since they are injectable, removing the need for open surgery. Lack of vascularisation afflicts many cell-laden scaffolds, as after implantation an insufficient nutrient supply often results in mass cell death within the scaffold. Particle-based systems would provide the necessary porosity to allow vascularisation.

We have investigated how human embryonic stem-cell derived mesenchymal progenitors (hES-MP) respond to porous microparticles. Both degradable and non-degradable polymer particles were produced with 80% interconnected porosity through a double emulsion method. The poly-High Internal Phase Emulsion (poly-HIPE) based particles had a size distribution of 100–1000  $\mu\text{m}$ . Additionally, monodispersed particles were produced via a fluidic device. When cultured with hES-MPs, particles were found to form large agglomerations as the cells bound them together after just a few days. These aggregates continued to grow in size until all particles had combined into one structure.

Staining showed that the scaffold appeared to direct the hES-MPs towards an osteoblast lineage. This differentiation allowed cells to penetrate throughout the porous particle. The morphology of the cells within the pores appeared to change over time to resemble that of osteocytes. Osteocyte formation would be consistent with the expected hypoxic environment within the centre of the cell-particle agglomeration. The binding of the particles by cells shows that it would be possible to create a continuous scaffold, with tissue-like properties, from individual particles over a period of time.

### Mimicking Cell-cell Interactions with Bioactive Fragments of N-cadherin Within Poly Ethylene Glycol Dimethacrylate Matrix for Accelerating Neural Differentiation of Mouse Embryo Stem Cell

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Bioactive signaling utilizing advanced tissue engineering approaches to optimize artificial extracellular matrices is expected to lead to further improvements in the restoration of neurological function after central nervous system injury. N-cadherin (NCAD) is a major adhesion molecule involved in the development and plasticity of the central neuron system, and promotes migration and neurite extension in the adult neural tissue. Using polyethylene glycol dimethacrylate (PEGDM) hydrogels containing continuous gradient of NCAD peptide (HAVDI) to optimize the NCAD peptide concentration (CNCAD) for mES survival, neurite extension and gene expressions of mature neural markers. Characterizations of the CNCAD showed a linear increase in peptide concentration for 94 to 537  $\mu\text{m}$  peptide range without significant changes to the young's or shears modulus, swelling ratio, and mesh size. Alkaline phosphate (ALP) levels were reduced 30 to 50% on between 189 and 467  $\mu\text{M}$  CNCAD compared with the ALP level of mES. Gene expressions of nanog and oct3/4 (pluripotent stem cells markers) were shown 20% on 94 to 537  $\mu\text{M}$  peptide range at Day 3 neural differentiation compared with mES. TUJ1 gene expression was significantly increased by 6 fold on 292 and 467  $\mu\text{M}$  CNCAD, compared with 94  $\mu\text{M}$  CNCAD at day 6, and lead the longer neurites at 290  $\mu\text{M}$  compared to other concentrations test. These results indicated that CNCAD, between 292 and 467  $\mu\text{M}$ , are optimal concentrations for enhancing neural differentiation of mES.

### Synergistic Cues of Growth Factors and Micro-Fibers on MSCs in Collagen Gels

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The extracellular matrix (ECM) maintains tissue phenotype through stimuli including contact guidance, anisotropic mechanical properties, and bioactive signals. Therefore, toward engineering of functional tissues, development of a 3D environment that recapitulates key ECM characteristics is essential. Toward this goal, we explored the synergistic roles of aligned electrospun networks and localized growth factors (GFs) on ligament differentiation of mesenchymal stem cells (MSCs) within a model system where cell migration, proliferation, and differentiation were not impeded by a dense electrospun fiber layer.

Aligned PCL/chitosan (core/shell) fibers were co-axially electrospun and then functionalized with fibroblastic growth factor-2 and growth and differentiation factor-5 through covalent conjugation or physisorption. Resultant bioactive fibers were embedded within collagen gels containing MSCs, and assayed for mRNA markers of ligament differentiation. After 7 days, expression of scleraxis and collagen 1 $\alpha$ 1 were up-regulated in fiber/collagen composites containing conjugated-GF, while tenomodulin expression was elevated when exposed to composites containing GF-physisorbed fibers. In contrast, incorporation of unbound GFs within the collagen gel phase of composites did not enhance expression, perhaps due to rapid diffusion or deactivation, while markers were suppressed when growth factors were presented only in the culture media. We postulate that GF localization to the fibers enhanced the expression of ligament markers via the close proximity of GFs and contact adhesions.

Our ongoing work involves the development of thicker composites that maintain sparse bioactive fiber distribution. If successful, these composites could offer unique platforms for the study of synergistic cues and the development of ligament and other aligned tissues.

### A New Method for Differentiating Adipose Stromal Cells into Adipocytes

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Obesity (BMI  $\geq 30 \text{ kg/m}^2$ ) is increasing problem world wide. It is characterized by an increase in adipocyte size and number, changes in adipocytokine secretion (including TNF $\alpha$  and IL-6) and recruitment of macrophages into tissue. These changes lead to chronic inflammation, which is related to various diseases such as type 2 diabetes and cardiovascular diseases. There is an urgent need and interest to study adipose tissue and develop strategies for controlling it.

*In vitro* cultures are fastest and easiest tool to study adipocytes. *in vitro* adipogenesis is often induced by isobutylmethylxanthine, dexamethasone and insulin, but the method has long culture time and uneven differentiation of cells. It has also been criticized for the cells not being responsive.

In our method we differentiate human adipose stromal cells (hASC) first 3 days with bioactive active extract, which contains various adipocytokines. Within three days, cells cultured in the extract accumulate fat evenly resembling young adipocytes. For the following 10 days, cultures are switched to serum free medium supplemented with adipogenic compounds. At day 13, mature adipocytes are obtained judged by the accumulation of triglycerides, measured with Adipored, normalized with WST-1, expression of adipocyte genes (e.g. PPAR $\gamma$ , Adiponectin, AP2, Glut4) and functionality of the cells, including responses to insulin exposure: Akt phosphorylation, glucose uptake and inhibition of lipolysis.

This adipocyte differentiation method reproduces natural human adipogenesis and is useful for identifying inhibitors or agonists of cell signaling pathways and for studying compounds for their ability to stimulate or inhibit adipogenesis.

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### Human Pluripotent Stem Cell Culture on Hydrogels Grafted with Nanosegments for Feeder-free Culture

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Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are an attractive prospect for

tissue engineering and regenerative medicine. The tentative clinical potential of hiPSCs and hESCs is restricted by the use of mouse embryonic fibroblasts as a feeder layer. The development of feeder-free cultures using biomaterials having specific nanosegments will create lower the cost of production without introducing xenogenic contaminants. We investigated human ESC (H9) and human iPSC culture on biomaterials with different elasticity and grafted with different nanosegments. We prepared dishes coated with polyvinylalcohol-co-itaconic acid (PVA-IA) hydrogels having different elasticity ranging from a 3.7 kPa to 30.4 kPa storage modulus by controlling the crosslinking time in crosslinking solution, and grafted with several ECM-derived cell-adhesion peptides. The cell binding domain of vitronectin was grafted onto the PVA-IA substrates. The hESCs and hiPSCs cultured on the most stiff substrates (e.g., storage moduli more than 30 kPa) tended to differentiate after five days of culture, whereas the hESCs and hiPSCs cultured on relatively softer substrates of 12–25 kPa maintained their pluripotency. Only a few small or no colonies of hESCs were observed on the softest substrates (10 kPa). Therefore, these results indicate that cell culture substrates with the optimal elasticity can maintain the pluripotency of hESC and hiPSC culture. It is concluded that both the physical and biological properties of biomaterials affect the *ex vivo* expansion of HSPCs as well as culture of hESCs and hiPSCs.

#### Development of Techniques for Pancreatic $\beta$ -cell Engineering

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$\beta$ -cell therapy, through pancreas or islet transplantation, is thought to be a curable treatment approach to establish a long-term, stable euglycemia in diabetic patients. Many studies have explored methods to obtain large amounts of functional  $\beta$ -cells or islets from different sources to address the limited organ availability. Most studies indicated that  $\beta$ -cells significantly lost insulin secretion during *in vitro* expansion, and re-differentiation efforts resulted in limited capability to produce insulin or to reverse hyperglycemia *in vivo*. We hypothesize that cultured  $\beta$ -cells could recover their ability to secrete insulin in a bioengineered environment that is similar to their natural pancreatic tissue. Accordingly, we developed a biological scaffold by electrospinning a blend solution of pancreatic extracellular matrix and synthetic polymer, and matched its properties to that of the native pancreatic tissue including mechanical properties, microstructure. In parallel, we expanded de-differentiated porcine islet cells by dissociating isolated islets and placing them in long-term culture. We observed a distinct sub-population of small-sized cells in the confluent islet cell layer after 20 days and found that these cells were positive for insulin immunostaining, and expressed insulin mRNA as determined by RT-PCR. Although, these small-size cells had a relative weak capability to secrete insulin when compared to native  $\beta$ -cells, it is expected that the function of these cells could be improved greatly if cultured on a pancreas-like scaffold with an appropriate culture medium.

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#### Thyroxine Significantly Increases Type II Collagen Content and Functional Measures in Engineered Cartilage

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**Problem:** The clinical application of engineered cartilage (EC) is consistently limited by poor mechanical (functional) properties.

Collagen content is also notoriously low in EC, and is a likely cause of poor functional properties. Thyroxine (T4) has been reported to stimulate type X collagen in hypertrophic chondrocytes.

**Hypothesis:** Collagen content and functional measures of EC cartilage will be increased by (T4).

**Methods:** Donor-matched sets of self-assembled EC were generated from culture-expanded rabbit articular chondrocytes. One sheet from each set was used as control, with 25 ng/ml T4 added to the culture medium of experimental sheets.

**Results of T4 treatment: Composition.** Total collagen was increased  $2.1 \pm 0.8$  fold ( $p < 0.01$ ,  $n = 7$ ) at 1 month and  $2.1 \pm 0.4$  fold ( $p < 0.001$ ,  $n = 5$ ) at 2 months when normalized to DNA content. Changes in glycosaminoglycan content, thickness, and collagen-crosslinking, were not significant. **Gene expression.** COL2A1 was increased  $2.8 \pm 1.6$  fold at 1 month ( $p < 0.01$ ,  $n = 7$ ). No differences for COL1A2 or COL10A1 were detected. Densitometry on Coomassie blue gels showed that type II collagen was increased  $2.2 \pm 1.8$  fold at 1 month ( $p < 0.05$ ,  $n = 6$ ). Type X collagen was below the level of detection in all constructs. **Functional measures.** At 1 month, Young's modulus was increased  $3.8 \pm 3.6$  fold ( $p < 0.01$ ,  $n = 6$ ) and the 5% tangent modulus increased  $1.6 \pm 0.4$  fold ( $p < 0.01$ ,  $n = 6$ ). **Damage resulting from frictional-shear.** At 2.8 MPa normal stress, damage was  $0.3 \pm 0.3$  fold that of controls ( $p < 0.05$ ,  $n = 5$ ) at 2 months. The coefficient of friction did not differ from controls.

**Conclusion:** T4 increases type II collagen accumulation and improves functional measures of EC.

#### A Modular, Three Dimensional *In Vitro* Kidney Perfusion Bioreactor

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Chronic kidney disease, a precursor to renal failure, affects over 20 million people in the United States. Tissue engineering offers a critical expansion to traditional benchtop and *in vivo* methods for studying the causes of end stage renal disease. Three dimensional, *in vitro* models allow for dynamic perturbations under conditions that more closely mimic the physiological environment. The goal of this research was to establish a modular, 3D kidney culture system. Our approach used rapid prototyping and soft lithography to fabricate a perfusion bioreactor system. To provide a versatile and stable culture surface the bioreactor utilizes a porous, silk protein scaffold. The silk sponge is infused with a collagen-Matrigel extracellular matrix and seeded with cells under static conditions before culture in the bioreactor. After placement in the device, the established layer of immortalized human renal epithelial cells experience consistent shear stress across the entire culture surface, as simulated using COMSOL modeling, to mimic the mechanosensory effects in healthy and disease states. This modular system combines the fluidic control of traditional 2D microfluidics with the capacity to mimic physiological complexity achieved with 3D tissue engineering. Physiologically relevant *in vitro*, human kidney models can be used, in conjunction with traditional methods, to gain expanded insight into kidney disease, nephrotoxicity and drug reactivity.

#### Skeletal Muscle Thin Films: A Tool for Understanding the Effect of 2D Extracellular Matrix Cues on Myotube Density, Alignment and Contractility

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While the process of myogenesis is well understood *in vivo*, there is a need for new tools that enable a better understanding of how to engineer uniaxially aligned, functional skeletal muscle *in vitro*. To address this, we have engineered a muscular thin film (MTF) assay that allows us to correlate extracellular matrix (ECM) protein cues to myotube formation and twitch stress. Specifically, we differentiate C2C12 myoblasts on microcontact printed ( $\mu$ CP) lines of ECM proteins patterned on a thin film of polydimethylsiloxane (PDMS), thus integrating a layer of myotubes with a PDMS film. These MTFs contract upon electrical stimulation and cell-generated twitch stress is



calculated from the bending of the MTF. Results showed that a significantly greater density of myotubes differentiated on laminin as compared to fibronectin, collagen IV, and collagen I lines. Next, we examined myotube formation on 20, 50, 100, and 200  $\mu\text{m}$  line widths of LAM with line spacings of 10, 15, 20, and 30  $\mu\text{m}$  and found that uniaxial myotube formation was achieved on widths less than 200  $\mu\text{m}$  and spacings greater than 15  $\mu\text{m}$ . MTFs grown on line widths  $\geq 50$   $\mu\text{m}$  exhibited twitch stresses  $> 10$  kPa. These results demonstrate that the skeletal MTF assay is a viable tool for elucidating how ECM composition and micropatterning influence muscle formation and contractile function. Future work will focus on understanding how micropatterned ECM cues influence myogenesis and contractility of primary human myoblasts and establishing the MTF assay as a clinically relevant platform for *in vitro* drug screening.

#### Adipogenic Cell Sheets to Recreate *In Vitro* Adipose Tissue Microenvironments

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Cell sheet (CS) engineering, taking advantage of cellular self-matrix organized as in native tissue, has been largely explored, including by us, for different purposes [1–3]. Herein we propose for the first time, the use of human adipose stem cells (hASCs)-derived CS to create adipose tissue analogues with different levels of maturation. hASCs were cultured on UpCellTM thermo-responsive dishes for 1, 3 and 5 days under basal conditions previously established by us [3]. The influence of pre-differentiation time and respective cell number, over CS stability and differentiation was assessed. Mechanically robust CS were only obtained with 5 days pre-differentiation period. Adipogenesis was followed along the culture assessing the variation of expression of mesenchymal (CD73, CD105 but not CD90) and adipogenic (PPAR $\gamma$ , FABP4 and LPL) markers by flow cytometry, immunocytochemistry and RT-PCR. Increased ratio of differentiated cells was achieved for longer pre-differentiation periods, while maturation degree was modulated by the maintenance medium. Independently of the overall CS differentiation/maturation level, 3D constructs were fabricated by stacking and further culturing 3 CS. Thus, by varying the culture conditions, different 3D adipose tissue-like microenvironments were recreated, enabling future development of new tissue engineering strategies, as well as further study of adipose tissue role in the regeneration of different tissues.

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#### An Irradiation-Injection Approach to Study Tendon Stem Cell Differentiation in a Mouse Model

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Tendinopathy is a prevalent tendon disorder that affects millions of Americans and costs billions of healthcare dollars every year. Current clinical treatments for tendinopathy are largely palliative because the precise cellular and molecular mechanisms of the disorder are not defined. Previously, using an *in vitro* model we showed that the leading cause of tendinopathy is the aberrant differentiation of tendon stem cells (TSCs) into non-tenocytes when subjected to mechanical over-loading [1]. Under identical conditions, the resident tendon cells or tenocytes did not differentiate into non-tenocytes *in vitro* [2]. However, it is not clear whether tendon cells (TSCs and tenocytes) *in vivo* also behave in a similar fashion. Therefore, in this study we used a novel irradiation-and-injection approach to determine whether native TSCs could be eliminated or reduced by irra-

diation thus enabling tracking of GFP tagged TSCs in the injected region. Histology results showed that these injected GFP-TSCs differentiated into non-tenocytes after intensive treadmill running. This approach can reveal the precise fate of TSCs *in vivo* and can offer more insights to the mechanisms causing tendinopathy. Our results indicate that the irradiation and injection approach has the following advantages: It can be used successfully to obtain pure TSCs *in vivo* without potential cell contamination from tenocytes. It is an effective approach to determine the role of TSCs in tendon homeostasis and tendon pathology or tendinopathy. This approach is feasible and can be routinely used in laboratories to perform basic science research.

#### Fibroblast Behavior in an Injectable Covalently Cross-Linkable Gelatin Matrix Incorporating Select Growth Factors

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An injectable matrix capable of replacing lost stroma, and stimulating the proliferation and migration of endogenous fibroblastic cells, could enable the early treatment of an array of musculoskeletal defects, including those in the annulus fibrosus of intervertebral disk. Gelatin, which has adhesion ligands for fibroblasts and endothelial cells and the ability to undergo enzymatic degradation, was conjugated with hydroxyphenyl propionic acid (Gtn-HPA) in order to enable independent tuning of the gelation time and degree of covalent cross-linking *in vivo* by horseradish peroxidase and peroxide (1). NIH 3T3 fibroblasts seeded in Gtn-HPA and exposed to the cross-linking process survived with  $95 \pm 1\%$  viability. The proliferative and migration behavior of the cells was compared when the following growth factors were incorporated into the Gtn-HPA: TGF- $\beta$ 1, FGF-2, PDGF-BB, EGF. On day 7, Gtn-HPA incorporating TGF- $\beta$ 1 or FGF-2 increased the cell numbers for  $\sim 2$  folds compared to controls, in a proliferation assay. A migration assay, designed as "annulus-core" system in which cells migrate from an annular cell-seeded type I collagen gel into growth factor-loaded Gtn-HPA cores, demonstrated increases in the cell number, clustering, and depth of migration into the Gtn-HPA when TGF- $\beta$ 1 was incorporated into the Gtn-HPA. The results demonstrate the promise of this injectable gelatin-based matrix for treatment of select early-stage musculoskeletal defects/ruptures.

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#### Evaluation of the Immunomodulatory Potential of a Chitosan-graft-poly( $\epsilon$ -Caprolactone) Copolymer

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Recently we synthesized a copolymer consisting of poly( $\epsilon$ -caprolactone) chains chemically grafted on a chitosan backbone (CS-g-PCL) and we showed that it can support the growth of Wharton's jelly Mesenchymal Stem Cells providing a material with potential in cardiovascular applications [1]. An essential part of the biocompatibility assessment includes the investigation of the immune response against the biomaterial indented for *in situ* regeneration. The aim of the current study is to evaluate the potential of CS-g-PCL to modulate different functions of innate and adaptive immune response under various stimuli. Specifically, we study the effect of CS-g-PCL on the profile of secreted cytokines in spleen cultures and on the proliferation of T and B lymphocytes. Additionally, we analyzed the influence of the biomaterial in the polarization of bone marrow-derived macrophages (BMDM), since the transition from M1 to M2 phenotype is beneficial for tissue remodeling upon biomaterial implantation. The results show that although CS-g-PCL does not induce an immune response, it induces specific effects on immune cells. CS-g-PCL shows anti-inflammatory action in BMDM culture by reducing the

interferone- $\gamma$  inducible levels of interleukin-12/23 without affecting the interleukin-4 inducible Arginase-1 levels. Similar reduction is observed in the levels of interleukin-12/23 in splenocytes, which can be partially explained by the secretion of IL-10. Our contribution to the immunomodulatory evaluation of CS-g-PCL aiming to improve its biocompatibility, is significant for tissue engineering applications.

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### Investigation of T Lymphocyte Dynamics in an *In Vitro* Model of the Lymph Node Stroma

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In the lymph node (LN), T lymphocytes perceive multiple signals that influence cell shape changes, mode of migration, and activation status. Particularly, during inflammation, lymphatic vessel dilation leads to increased flow, stromal cell proliferation, and changes in the cytokine milieu that further promote guided lymphocyte-antigen encounters. While this suggests that LN stromal cells regulate the quality and type of immune response, the physical mechanisms by which the stromal network directs lymphocyte migration and education are largely unknown. Therefore, we developed an *in vitro* model of the LN paracortex to investigate the interdependence of T cell migration on stromal cell-mediated microenvironmental cues. Fibroblastic reticular cells (FRCs), lymphatic endothelial cells, and dendritic cells harvested from LNs and bone marrow of C57BL/6 mice were mixed in collagen-I and loaded into a PDMS microdevice for 3D culture within physiologically relevant geometries and flow conditions. We first primed stromal cells with SIINFEKL, the MHCII-immunodominant peptide from ovalbumin (OVA), in the presence or absence of IFN $\gamma$  and TNF $\alpha$ . Then, we added fluorescently labeled CD8+ OT-I cells to the device, which specifically recognize the SIINFEKL-MHCI complex, and with live-imaging tracked OT-I cell migration and interaction frequency over time. Under inflammatory conditions, the presence of FRCs enhanced OT-I cell interactions, in part by matrix remodeling and expression of adhesion molecules ICAM-1 and VCAM-1. The *in vitro* model provides important and complimentary insights compared to that of traditional cell culture or animal models, and has the potential to improve our understanding of how the LN stroma modulates adaptive immunity.

### Mechanical Stretch-Induced Alternation of Poly(N-isopropylacrylamide) Gel Modified PDMS Surface Properties

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Based on a hypothesis that poly(N-isopropylacrylamide) (PIPAAm) hydrogel modified poly(dimethylsiloxane) (PDMS) surface (PIPAAm-PDMS) properties are modulated by applied mechanical stress, PIPAAm-PDMS was prepared as temperature-responsive cell culture surface by using electron beam irradiation method. Widely used PDMS was selected as a model elastic substrate in this experiment. To prove a hypothesis, uniaxially stretched and unstretched PIPAAm-PDMS surfaces were evaluated by a variety of surface analysis methods. FT-IR/ATR method revealed that graft polymer density of PIPAAm-PDMS was determined to be 9.9  $\mu\text{g}/\text{cm}^2$ . By modifying PDMS with PIPAAm hydrogel, contact angle decreased from 110° (PDMS) to 78° (unstretched PIPAAm-PDMS) at 37°C. AFM image of PIPAAm-PDMS surface suggested uniform coverage of PDMS surface with PIPAAm component. By applying 20% of uniaxial mechanical stretching stress to PIPAAm-PDMS, the contact angles increased from 78° to 83° (stretched PIPAAm-PDMS). The stretched and unstretched PIPAAm-PDMS surfaces showed temperature-dependent contact angles alteration. The thickness of the graft PIPAAm hydrogel layer decreased from 700 nm to 480 nm. Considering that temperature-responsive cell

culture surfaces with thinner PIPAAm hydrogel layer shows more hydrophobic property, 1, 2 that PIPAAm-PDMS surface properties were altered by the applied mechanical stretching stress. Cell attachment assay also suggested that the stretched PIPAAm-PDMS was more cells adhesive. These results possibly suggested that PIPAAm-PDMS surface properties are controllable by dual stimuli such as temperature change and the degree of applied mechanical stress.

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### Enhancing the Articular Phenotype of Dedifferentiated Equine Chondrocytes by Expression of Pluripotency Factors

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*In vitro* culture expansion of chondrocytes is essential for generating sufficient cell numbers for transplantation. However, extended culture can lead to loss of the articular phenotype, with reduced expression of Col2A1. The pluripotency transcription factors Oct4, Sox2, Klf4 and c-Myc (OSKM) have been shown to reprogram fibroblasts into chondrocytes. In this study we show that lentiviral vector expression of OSKM in dedifferentiated equine chondrocytes enhances the articular chondrocyte phenotype in 3D cultures resulting in high levels of extracellular matrix synthesis.

Monolayer expanded chondrocytes were transduced with a lentiviral vector co-expressing Oct4, Sox2, Klf4 and c-Myc (OSKM) or separately with a lentiviral vector expressing Green fluorescence protein (GFP). Transduced chondrocytes were induced in 3D pellet cultures for 14 days with media containing transforming growth factor beta 3 (TGF $\beta$ -3). Glycosaminoglycan (GAG) was quantified using Dimethyl methylene blue (DMMB). Immunohistochemical staining was performed on paraffin embedded sections for collagen type II and collagen type X and QPCR was also performed to quantify transcripts of the same targets.

Toluidine blue staining for GAG showed intense staining in chondrocytes expressing OSKM. The total DNA and GAG content was significantly higher in the OSKM transduced cells compared to untransduced chondrocytes ( $p < 0.05$ ). Immunohistochemical staining for collagen type II showed diffused extracellular matrix in OSKM transduced cells compared to the chondrocytes controls with Collagen X expression evident in all conditions. mRNA expression levels of *col2a1* and *col10a1* were significantly higher in OSKM expressing cells indicating redifferentiation of the chondrocytes with ectopic expression of the transcription factors ( $p < 0.0001$ ).

### The Effect of Immobilized Antibody Density on iPS Cell Rolling Behavior in Cell Rolling Column

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**Introduction:** We have been proposing a label-free cell separation device called "cell rolling column"<sup>1</sup>, in which, cells selectively roll on antibody-immobilized column surfaces. The aim of this study is to investigate the effect of the immobilized antibody density on cell rolling behavior, culminating in optimal cell separation. Induced pluripotent stem (iPS) cells were infused into polymer coated microfluidic channels immobilized with antibodies to stage-specific embryonic antigen 1 (SEA-1), known as a pluripotency marker for murine iPS cells.

**Materials and Methods:** Poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-nitrophenyl oxycarbonyl poly(ethylene glycol) methacrylate (MEONP)] (PMBN)<sup>2</sup> were synthesized with different monomer feed ratios of MPC:BMA:MEONP of 30:60:10 (PMBN10), 33:66:1 (PMBN1) and 33:67:0 (PMBN0). Microfluidic channels were coated with PMBN by cast method to immobilize anti-SSEA-1 antibody. Immobilized antibody density was evaluated using <sup>125</sup>I-labeled antibodies. Undifferentiated mouse iPS cells suspended in phosphate buffered saline were infused into the channels at the shear stress ranged from 0.2 to 4.6 dyn/cm<sup>2</sup>, and cell rolling speed was measured under a phase contrast microscope.

**Results:** The antibody density was increased with increasing MEONP ratio. At high shear stress conditions, every cell was drifted and no cell rolled on each polymer surface. However, cells started to roll on PMBN10, PMBN1 and PMBN0, below the shear stresses of 2.6, 0.9 and 0.7 dyn/cm<sup>2</sup>, respectively. On PMBN10, a flow cytometry assay revealed that the rolling cells expressed more SSEA-1 than the drifted cells.

**Conclusions:** These results indicate that the proper immobilized antibody density and shear stress are important for iPS cell rolling.

### Cell Type Specific Microspheres Incorporating Growth Factors as Injectable Cell Carriers for Tissue Repair

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The detection, isolation and sorting of cells holds an important role in cell therapy and regenerative medicine. Also, injectable systems have been explored for tissue regeneration *in vivo*, because it allows repairing complex shaped tissue defects through minimally invasive procedures.

Here, we report the use of polymeric microspheres to simultaneously isolate and enrich specific cell types that can be also used as an injectable cell carrier system to form small tissue constructs *in situ*. The rationale was to functionalize the particles with antibodies to target specific cell types and release growth factors (GFs), first to increase the number of cells growing over the particle surface and then induce the differentiation of these cells by a sustained release from the particle core.

GFs loaded microspheres of photocrosslinkable chitosan were fabricated using a flow focusing microfluidic chip. The droplets produced were crosslinked by UV while flowing inside a Tygon tube before being collected into an eppendorf. Monoclonal antibodies against cell surface antigens specific to endothelial cells and stem cells were immobilized on the surface of the microspheres. Experimental results showed that the microfabricated spheres provide suitable surfaces to capture a target cell type and subsequent expansion of the captured cells. The aggregation of the functionalized microspheres has been also shown to successfully form 3D robust structures upon injection into a mold.

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### Developing Electropun Scaffolds to Influence Mammalian Cell Phenotype; Building an *In Vitro* Model of the Human Bronchial Airway

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We report a programme of research aimed at addressing the shortage of *in vivo*-like 3D *in vitro* models of the bronchial mucosa. Our work employed electrospinning as a method to produce defined, porous 3D matrices. We created fibres of the non-degradable polymer polyethylene terephthalate (PET) with diameters ranging from nanometre to micrometre. We found that the nanofibre scaffold closely resembled the basement membrane of the bronchiole mucosal layer, and epithelial cells cultured at the air-liquid interface showed polarized differentiation. The microfibre scaffold mimicked the porous submucosal layer of the airway into which lung fibroblast cells showed good penetration. Epithelial and fibroblast cells were co-cultured with enhanced epithelial barrier formation observed. In addition, we electrospun PET scaffolds composed of uniaxially orientated nanofibers and evaluated this topography's effect on Human Airway Smooth Muscle (HASM) cell adhesion, alignment, and morphology. The fibre's orientation provided contact guidance enabling the formation of fully aligned sheets of smooth muscle. Moreover, smooth muscle cells cultured on the scaffold presented an elongated cell phenotype with altered contractile protein levels and distribution; cells cultured on this scaffold responded to the bronchoconstrictor bradykinin. Using a commercially available bioreactor system, we co-cultured all three cell-types to provide an *in vitro* model of the airway wall over an extended time period. We highlight the potential of this model for *in vitro* diagnostic studies investigating important inter-cellular cross-talk mechanisms or assessing novel pharmaceutical targets, by providing a relevant platform to allow the culture of fully differentiated adult cells within 3D, tissue-specific environments.

### Novel Polyisocyanopeptide Hydrogels for Rapid Vasculogenesis

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A new class of material biomimetic hydrogel consisting of fibrillar oligo(ethylene glycol) polyisocyanopeptides (PIC) was studied as 3D scaffold for cell culture and vascularization. This novel thermo-responsive super hydrogelator mimics the biomechanics of the ECM, showing strain stiffening under mechanical deformation. In the PIC matrices structural control at the nano-meter level can be achieved (fiber bundle size, pores size etc.) and also modular introduction of desired handles e.g. GRGDS (Gly-Arg-Gly-Asp-Ser) with 2–55 nm separation and gel stiffness ranging from 10–1000 Pa. The vascularization of PIC gels with human bladder smooth muscle cells and human umbilical vein endothelial cells co-cultures readily occurred in soft (< 200 Pa) PIC gels with GRGDS in 10–23 nm separation. In the blends of fibrin with naked PIC, vasculogenesis occurred in a low percentage of fibrin. 3D cultured structures could be removed from PIC gels by cooling the gels below their transition temperature, allowing easy extraction of the formed vascularized network. The rate of tissue formation could be tuned by varying the density of the integrin GRGDS units and/or altering the strain stiffening biomechanics of the PIC matrices. The host cells penetrated and built up ECM in the PIC matrices *in vivo*; naked PIC stayed in place during 2 weeks; GRGDS units induced faster degradation rate. In conclusion, PIC gels represent a very simple biomimetic system ideal for 3D cell culturing in a defined synthetic environment; PIC gels were injectable and could be used as an alternative vehicle for cell delivery in the future.

### Newly Developed Culture Method Providing Efficient Oxygen Supply to a Three-dimensional Artificial Tissue

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By overlaying cell sheets on resected tissue as a vascular bed, we have attempted to fabricate artificial cardiac tissues in a bioreactor. However, upon the fabrication of these tissues, oxygen supply to the tissue was assumed to be insufficient, because culture medium used for *in vitro* culture dissolves a lower amount of oxygen than blood. Therefore, this study attempted to supply a sufficient amount of oxygen to the vascular bed by using oxygen carriers. The activity of the vascular bed was assessed by an ATP-dependent luciferin-luciferase luminescence imaging system.

A resected section of femoral tissue with a connectable artery and vein was put onto a tissue culture chamber and served as a vascular bed. The vascular bed was then perfused in a custom-made bioreactor. This study used perfluorocarbon (PFC) emulsion, which is an effective oxygen carrier. Oxygenated PFC with luciferin-containing culture medium was continuously infused into the vascular bed from the artery side. The amount of ATP production was observed as a metabolic activity index by measuring bioluminescence imaging (BLI) signal intensity.

Without PFC-perfusion, the oxygen level decreased gradually as observed by the decrease of BLI signal intensity; however, BLI signal intensity in the vascular bed increased with oxygenated PFC-perfusion. The increased intensity of BLI was found to be greater than that of the control medium-perfusion, indicating that the metabolic activity and ATP production of the vascular bed increased by supplying oxygen from the oxygen carrier.

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#### Hyaluronic Acid Down-regulates Interferon Signalling in an Injured Bovine Intervertebral Disc

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Low back pain is a major cause of disability for people between 20 and 50 years old that imposes a serious socio-economic burden. Therefore, the current focus of regenerative medicine is on identifying molecular markers for designing specific therapeutics. Previously, we have identified the anti-proliferative IFIT3 and IGFBP3 as downstream targets of the inflammatory cytokine IFN $\alpha$ 2b signalling pathway in the human annulus fibrosus (AF). In this study, injection of hyaluronic acid (HA) hydrogel was hypothesized to have an anti-inflammatory effect on this signalling pathway using the bovine disc as a model system. Inflamed bovine organ culture was used to test the anti-inflammatory effect of HA hydrogel on the IFN $\alpha$ 2b signalling pathway and the downstream targets, IFIT3 and IGFBP3. Discs harvested from seven young calves were cultured for three days with or without 100 U/ml IFN $\alpha$ 2b. Experiments included intact disc, injured disc, injured disc treated with a cross-linked HA hydrogel, injured disc treated with IFN $\alpha$ 2b, and injured disc treated with IFN $\alpha$ 2b and HA hydrogel. PCR analysis was carried out to check the gene expression of IFN $\alpha$ 2b signalling molecules in extracted AF cells. Additionally, immunohistochemistry was used for protein expression. Relative gene expression data was statistically analyzed with one way Anova, where  $p < 0.05$  were considered significant. The results showed that HA significantly down-regulates IFN $\alpha$ 2b receptors, STAT1/2, JAK1, IFIT3 and IGFBP3. Protein expression analysis also confirmed the PCR results. Hence, it was concluded that HA plays an anti-inflammatory role in the IFN $\alpha$ 2b signalling pathway resulting in down-regulation of anti-proliferative IFIT3 and IGFBP3.

#### Early Detection Of Structural Abnormalities and Cytoplasmic Accumulation of TDP-43 in Tissue-Engineered Skins Derived from ALS Patients

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Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset disease characterized by the selective degeneration of motor neurons (MNs) in the central nervous system (CNS). Current diagnosis of ALS is based on clinical assessment of related symptoms which appear late in the disease course after degeneration of a significant number of MNs. As a result, the identification and development of disease-modifying therapies is difficult. Novel strategies for early diagnosis of ALS, to monitor disease progression and to assess response to existing and future treatments, are urgently needed.

Due to the common embryonic origin of both skin and neural tissues, many neurological disorders, including ALS, are accompanied by skin changes that often precede the apparition of neurological symptoms. We have developed a unique tissue-engineered skin model (TES) derived from symptomatic, sporadic and familial ALS patients as well as pre-symptomatic FALS patients carrying a known pathological DNA mutation. TES were generated from isolated keratinocytes and fibroblasts and analyzed using different biochemical, immunohistological and molecular methods. Our ALS-TES presents a number of striking structural and molecular features, uniquely seen in patients-derived TES, including extracellular matrix (ECM) disorganization and cytoplasmic TDP-43 inclusions, a pathological signature found in the majority of ALS cases in the affected regions of the CNS.

Consequently, our ALS-TES could represent a renewable source of human tissue to better understand the physiopathological mechanisms underlying ALS, facilitate the identification of disease biomarkers for early diagnosis and disease monitoring, as well as provide a unique tool for the development of drug screening assays.

#### Calcium Phosphate/Mesoporous Silica Bone Cement Containing Osteogenic Growth Peptide

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Calcium phosphate (CaP) cements have been widely investigated as bone graft substitutes due to its excellent self-setting ability, biocompatibility and osteoconductivity. In addition, mesoporous materials have also been studied for application in medical devices due to their large surface area, which is capable of loading numerous biological molecules. This study aimed the development of bone cement based on CaP and mesoporous silica (mSi) functionalized with synthetic peptide, osteogenic growth peptide (OGP). The mSi-based particles were prepared by sol-gel process. After adsorption of OGP into mSi particles, the cement was prepared in a suitable liquid-to-powder ratio: mSi-OGP,  $\beta$ -tricalcium phosphate, monocalcium phosphate monohydrate and solution PEG400. The surface area and porosity of the mSi were characterized by adsorption-desorption of Nitrogen (BET) and transmission electronic microscopy (TEM). Compressive strength (CS) of the cement was evaluated after the periods of 1 and 7 days soaking in simulated body fluid (SBF). The release profile *in vitro* of OGP peptide from cement in SBF was monitored by fluorescence spectroscopy. BET patterns confirmed the porosity of the silica particles. TEM images showed the hexagonal arrangement of mesopores. CS revealed an increase in strength after 7 days ( $1.95 \pm 0.20$  to  $2.81 \pm 0.45$  MPa). After this period, a fracture of the cement can be observed by scanning electronic microscopy. The release profile of OGP peptide showed that the peptide content was not released until 7 days. According to results, the CaP/mSi cement containing OGP peptide can be a potential material for bone graft substitutes.

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### Target-Specific Systemic Delivery of Bioengineered Mesenchymal Stem Cells for The Treatment of Liver Diseases

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Mesenchymal stem cells (MSCs) have been widely explored for innovative cell therapies due to their ability to differentiate into diverse lineages and secrete a variety of favorable cytokines. Among various routes of cell administration, the target-specific systemic delivery of MSCs via intravenous injection is quite attractive for the selective and effective treatment of intractable diseases. However, after systemic delivery, most of MSCs are entrapped in the lung, resulting in the delivery of MSCs to the undesired site and the low efficacy of cell therapy. Here, we exploited hyaluronic acid (HA) for the liver target-specific systemic delivery of genetically engineered MSCs (eMSCs) expressing TRAIL and IL-12M. We conjugated N-terminal amine group of wheat germ agglutinin (WGA) to aldehyde-modified HA for the surface modification of eMSCs. The successful synthesis of HA-WGA conjugate was confirmed by gel permeation chromatography, circular dichroism, and ELISA/Bradford assay. The cytotoxicity of HA-WGA conjugate and the incorporation of HA-WGA conjugate into the cellular membrane of eMSCs were assessed by MTT assay and confocal microscopy. After surface modification of eMSCs with HA-WGA conjugate, the liver target-specific delivery of eMSCs/HA-WGA conjugates was investigated via *ex vivo* bioimaging with an IVIS<sup>®</sup> imaging system and a fluorescence microscope. Currently, we are investigating the therapeutic effect of target-specific eMSC therapy for the treatment of liver diseases. We will discuss the feasibility of the target-specific eMSC therapy via intravenous injection for the treatment of liver diseases such as liver cirrhosis and liver cancer.

### Adipose Derived Stem Cell Therapy for Skeletal Muscle Regeneration

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**Introduction:** Skeletal muscle has a remarkably efficient regeneration capacity. However more extensive injuries that can lead to an irreversible fibrosis, scarring and even loss of muscle function and are beyond muscle self-regeneration ability. The aim of this study was to evaluate *in vivo* skeletal muscle regeneration capacity after injection of adipose derived stem cells (ADSCs) in an acute injured skeletal muscle tissue.

**Material and Methods:** Mouse primary ADSCs were isolated from subcutaneous fat tissue, expanded and characterized by immunostaining and FACS analysis. Crush injury of the tibialis anterior (TA) muscle was performed in 12-week-old C57BL/6 mice, followed by ADSCs injection. Injured muscles were analyzed for gene and protein expression by RT-PCR and immunohistochemistry. The functional outcome was assessed by myography.

**Results:** Morphological and functional changes in the regenerating muscles were compared after 1 and 2 weeks post injury. We observed, that a single intramuscular injection of ADSC into crushed TA muscle of mice resulted not only in incorporation of the ADSCs in damaged area, but also along the remaining healthy myofibers. Furthermore, the cell-injected muscles showed improved contractility in comparison to the collagen-injected controls in the organ bath studies.

**Conclusions:** Autologously transplanted ADSCs into acute damaged skeletal muscle accumulate at the injury site and remain there during remodeling phase. Despite lack of ADSCs differentiation into myofibers, they have positive influence on muscle contractility and regeneration process.

### Cartilage Repair using Human Embryonic Stem Cell-derived Chondroprogenitors

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We developed a 14-day culture protocol under potential GMP, chemically defined conditions, to generate chondro-progenitors from human embryonic stem cells (hESCs) (1). Chondrogenic cells derived from hESCs were encapsulated in fibrin gel and implanted in osteochondral defects in the patella groove of nude rats and cartilage repair was evaluated by histomorphology and immunocytochemistry. Genes associated with chondrogenesis were upregulated during the protocol and pluripotency related genes were downregulated. Aggregation of chondrogenic cells was accompanied by high expression of SOX9 and strong staining with safranin O. Culture with PluriSln1 was lethal for hESCs, but was tolerated by hESC-chondrogenic cells and no OCT4 positive cells were detected in hESC-chondrogenic cells. iPSCs were also shown to generate chondro-progenitors in this protocol. Repaired tissue in the defect area implanted with hESC derived chondrogenic cells was stained for collagen II with little collagen I, but no collagen II was observed in the fibrin only controls. Viable human cells were detected in the repair tissue at 12 weeks. The results show that chondrogenic cells derived from hESCs, using a chemically defined culture system, when implanted in focal defects were able to promote cartilage repair (2). We also investigated the transcriptome of hESCs and chondrogenic cells using mRNA-seq and confirmed the characteristic chondroprogenitor phenotype of hESCs-chondrogenic cells derived under our chondrogenic protocol.

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### Nanoparticles Versus Conductive Polymers for Enhancing Conductivity in Fibrous Scaffolds for Skeletal Muscle Tissue Engineering

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There are currently no viable procedures to regenerate large volume defects in skeletal muscle. Tissue engineers have investigated using topographical and electrical cues to enhance myoblast development on scaffolds *in vitro*. Both conductive nanoparticles and conductive polymers have shown promising results when combined with polycaprolactone (PCL) in electrospun scaffolds to promote muscle cell proliferation and differentiation. The objective of this study was to evaluate and compare scaffolds that use conductive nanoparticles and conductive polymers in fibrous scaffolds for skeletal muscle tissue regeneration. Scaffolds were fabricated through electrospinning PCL solutions with either multi-walled carbon nanotubes (MWCNT) or polycaprolactone-polypyrrole (PCL-PPy) copolymer added to improve scaffold conductivity. The fibers were collected on a spinning mandrel at 3000 RPM. Sections of fibrous scaffolds from PCL, PCL-MWCNT, and PCL-PPy groups were measured for their conductivity and seeded with human mesenchymal stem cells. Metabolic activity was measured on days 1, 3, and 7 after seeding using a PrestoBlue cell viability assay. The addition of the MWCNT and the PPy both caused a significant increase in conductivity over scaffolds with PCL alone. Metabolic activity increased over time in all groups, but metabolic activity increased the most in the PCL-PPy group, which may indicate the superiority of conductive polymers over nanoparticles for myoblast biocompatibility. In future studies, we will evaluate how conductivity and mechanical properties of these scaffolds affect cell morphology and gene expression. In addition, *in vitro* experiments will determine how electrical stimulation can be used to promote cell differentiation and protein production.

### A New Approach for 3D Tissue & Organ Fabrication Inspired From Orthopedic Surgery

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Fabrication of transplantable 3D tissue or organ *in vitro* is one of the major goals in regenerative medicine. Several scaffold-free systems have been developed to avoid potential side effects caused by scaffold mainly used to build three-dimensional tissue construct. They seemed to be still unable to produce fine structures without contamination from exogenous biochemical materials.

Inspired from bone fracture treatments in orthopedic surgery, we established a simple method to fabricate 3D scaffold-free cell construct. This method uses spheroids and temporal fixator which enable placement of various types of three-dimensional cells into desired xyz positions without need of hydrogels or biochemical reactive materials. We also developed a robotic system for scaffold-free cell construction.

By using this "Bio 3D printer", we successfully fabricated cartilage, blood vessels, liver, and so on. Also some of pile-lines are already start *IN VIVO* study.

Near future, we may be able to build living organs for autologous transplantation by using this scaffold free Biofabrication system. And this multi-cell construct may be useful research tools for drug development.

### Tuning Bone Morphogenetic Protein-Material Interactions with Diluent Buffering Capacity and pH

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Bone morphogenetic proteins (BMPs) potently stimulate the formation of bone and enhance the repair of challenging musculoskeletal indications such as long bone nonunions, open tibia fractures, and lumbar spine fusions. The FDA approved device INFUSE combines BMP2 with an absorbable collagen sponge to retain the protein at the implantation site, yet only 20% of the loaded protein remains after 1 week. We investigated the use of high surface area microporous calcium phosphate (CP) granules for sustaining BMP release and began by examining the interactions of BMP2 with CP under various buffer conditions. We found that when BMP2 was applied to the granules in a low pH, high buffering capacity diluent 74% of the protein was in a tightly-bound fraction requiring treatment with guanidine for extraction, compared to 54% when the protein was applied in a low buffering capacity diluent. In addition, fluor-tagged BMP aggregated at the surface of granules when loaded in a weak buffer, but exhibited a more uniform distribution when applied in a strong buffer. Interestingly, when applying BMP in the low pH, high buffering capacity diluent to carriers consisting of CP granules in a type I collagen sponge, the amount of protein in the tightly bound fraction (62%) was lower than when the protein was applied in the weak buffer (83%) but the distribution of fluor-BMP2 remained uniform within the granules. These results reveal an important role for diluent pH and buffering capacity in regulating the interaction of BMPs with CP-based carriers.

### Sandwich Culture with Bio-functional Hydrogels as a Three-dimensional Culture Technique to Induce Osteoblastic Differentiation of Mesenchymal Stem Cells

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The objective of this study is to investigate a three-dimensional (3-D) culture technique to induce osteoblastic differentiation of mesenchymal stem cells (MSC). To achieve the 3-D culture, MSC were maintained in a sandwich culture condition with bio-functional hy-

drogels. The bio-functional hydrogels were prepared through the immobilization of a cell signaling protein on poly(acrylamide) hydrogels. As the cell signaling protein, ephrin B2 of an Eph signal ligand was employed to induce the osteoblastic differentiation of MSC. A direct binding between ephrinB2 and EphB4 via the cell-cell contact is required to activate the ephrinB2-EphB4 signaling. In this study, a recombinant chimeric protein of ephrinB2 and IgG Fc domain (ephrinB2-Fc) was immobilized via protein A on the surface of hydrogels in an orientation-regulated manner that allows ephrinB2 to achieve the efficient ligand-receptor binding. Human MSC were sandwiched between the resulting bio-functional hydrogels and maintained in culture for 5 days. RUNX2 expression of MSC was measured by quantitative polymerase chain reaction to evaluate their osteoblastic differentiation. When maintained in the sandwich culture condition, MSC showed enhanced RUNX-2 expression, which is similar to those on the corresponding hydrogel in the two-dimensional (2-D) culture condition. The RUNX-2 expression could be modulated by not only the bottom hydrogels in the 2-D culture but also the upper hydrogels in the sandwich culture condition. This result suggests that cell signaling pathways for osteoblastic differentiation in MSC could be activated by immobilized ephrinB2 even in the sandwich culture condition.

### The Effect of Alginate Capsule Composition on Pancreatic Differentiation of Human Embryonic Stem Cells

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We have shown previously that calcium alginate encapsulation for differentiation of human embryonic stem cells (hESCs), results in efficient differentiation to insulin-producing cells. The unlimited supply of hESCs and the immunoisolation capability of alginate capsules, suggest this approach as an alternative treatment for type 1 diabetes. Islet encapsulation studies demonstrate that capsule composition has a significant effect on immunoisolation. However, capsule composition can modify the differentiation of encapsulated cells. In this study we are evaluating the effect of material properties on pancreatic differentiation of encapsulated hESCs. hESCs were encapsulated in barium alginate, crosslinked with 10, 15, 20, 50 or 100 mM BaCl<sub>2</sub>. AFM analysis confirmed increasing capsule stiffness with increasing BaCl<sub>2</sub> concentration. hESCs were differentiated by chemical induction and characterized after complete differentiation. Capsules of stiffness 4–7 kPa support cell viability and colony formation, in contrast to stiffer capsules. Pancreatic commitment was analyzed by PDX1 gene and protein expression. PDX1 gene expression was highest in the 4 kPa stiffness gel (10 mM BaCl<sub>2</sub>), and was significantly reduced as stiffness increased. Our study demonstrates that capsule material properties can significantly modulate the efficiency of chemically induced pancreatic differentiation. While specific encapsulation condition can significantly enhance the efficiency of chemical induction, in general higher crosslinking was found to be detrimental to viability and differentiation. Current work is underway for encapsulating hESCs in a high throughput alginate array platform. This will allow for more thorough investigation of the effect of substrate stiffness on differentiation, as well the signaling pathways which govern pancreatic differentiation.

### In Vitro Testing of the Neuroprotective Effect of Mesenchymal Stromal Cells in a 3D Model of Parkinson's Disease

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**Introduction:** Perfusion bioreactors are exploited as reliable models of tissue growth in controlled conditions. We previously

adapted [1] a miniaturized and optically accessible bioreactor for the interstitial perfusion of 3D cell-seeded scaffolds to assess the ability of mesenchymal stromal cells (MSCs) to protect neuronal-like cells against 6-hydroxydopamine (6-OHDA) in a dynamic model of Parkinson's disease. Here we focused on the adaptation of experimental protocols to 3D applications under perfusion.

**Materials and Methods:** For both SH-SY5Y cells and MSCs from rat bone marrow, seeding conditions on polystyrene scaffolds were optimized. To find the best protocol for oxidative damage, SH-SY5Y cells were incubated with 6-OHDA in static or dynamic conditions and their metabolic activity was evaluated by Alamar Blue<sup>®</sup> assay. To study the ability of MSCs to improve the survival of damaged neuronal-like cells, the scaffolds were kept in static or dynamic conditions, then cell lysates were analyzed by Western Blotting.

**Results:** A homogenous cell distribution was obtained on the scaffolds. Alamar Blue<sup>®</sup> assay suggested that the incubation in dynamic conditions enhances 6-OHDA toxicity. MSCs improved the survival of damaged SH-SY5Y cells, with the better performances for the cells in the bioreactor.

## Reference

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## Buccal Epithelial Cell to Urothelial Cell Transdifferentiation

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In bladder tissue engineering, there is a need for an alternate epithelial cell source for patients with bladders compromised by disease or cancer. Since buccal mucosa is already used in urology-based surgical applications, buccal epithelial cells have been proposed as a possible surrogate cell source. The aim of this work was to investigate the potential to transdifferentiate buccal epithelial cells into bladder epithelial (urothelial) cells by first identifying and then implementing a set of candidate transcription factors.

Epithelial cells were isolated from buccal mucosa tissue obtained with patient consent and ethical approval. To investigate the role of PPAR $\gamma$  in inducing urothelial-type differentiation, cultures were treated with 1 $\mu$ M troglitazone (TZ; PPAR $\gamma$  activator) and 1 $\mu$ M PD153035 (PD; EGFR-TK inhibitor) for up to 6 days; a method (TZ/PD) known to induce terminal differentiation of urothelial cells. For transdifferentiation experiments, a retroviral-based transduction system was used to produce buccal epithelial sub-lines overexpressing the genes of interest.

Compared to urothelial cells, buccal epithelial cells showed low expression of PPAR $\gamma$  transcript and showed minimal expression of FOXA1 or GATA3 in response to TZ/PD. GATA3 overexpression and subsequent TZ/PD treatment resulted in an upregulation of FOXA1, but did not promote urothelial-type differentiation. PPAR $\gamma$ 1 overexpression resulted in increased expression of FOXA1 and further TZ/PD treatment of the cells caused an upregulation of both FOXA1 and GATA3.

Although neither GATA3 nor PPAR $\gamma$ 1 overexpression promoted the full transcription factor profile necessary for urothelial-type transdifferentiation, each promoted the expression of downstream genes thought to play a critical role.

## Bioreactor Growth Factor Regimen Tuning for Bone Progenitor Cell Seeding, Coating, and Differentiation on Resorbable Poly(propylene fumarate) Scaffolds

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We wish to optimize a growth factor dosing regimen for the production of bone extracellular matrix (ECM) on resorbable poly(propylene fumarate) (PPF) bone tissue engineering scaffolds. The ECM is secreted by bone marrow-derived human mesenchymal stem cells. In the present study, basic Fibroblast Growth Factor (FGF-2) (5 ng/ml), Platelet Derived Growth Factor (PDGF-bb) (40 ng/ml) and Epidermal Growth Factor (EGF) (20 ng/ml) doses were chosen based on our experience or consensus in the literature for their proliferation potential. Bone morphogenetic protein (BMP) 4 (50 ng/ml), 6 (50 ng/ml) and 7 (27 ng/ml) in the presence of the following osteogenic substances: dexamethasone (10<sup>-7</sup> M),  $\beta$ -glycerophosphate (10 mM) and ascorbic acid (50  $\mu$ g/ml) have been chosen for similar reasons in regards to bringing about MSC differentiation to bone. We also studied these growth factors at 10X concentration to determine the dose effect. Proliferation was analyzed through MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay, Scanning Electron Microscopy (SEM) and toluidine blue staining, whereas, differentiation was analyzed via alizarin red S staining and assay, alkaline phosphatase (ALP) staining and assay, and SEM. Initial proliferation study findings suggest that a combination of EGF, PDGF-bb and FGF growth factors at 1X concentration promotes significantly higher proliferation than other growth factor-containing groups in a time period of 1 week. Our differentiation study results suggest that alkaline phosphatase production increases from day 14 to day 21 in all the groups containing BMP's and the osteogenic media group, whereas mineralization is highest in the BMP-7 group at 1X concentration.

## Skeletal Muscle Engineering with Cellulose Nanowhiskers

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Skeletal muscle is a specialized tissue with a limited self-repair capacity. Significant injuries result in fibrosis, which can lead to functional impairment in patients. Free muscle transfer remains the gold standard treatment of severe muscle injury but is limited in scope. In order to efficiently control the myogenic differentiation of potential precursor cells for tissue engineering applications, it is critical to understand the balance of topographical and mechanical cues necessary for differentiation.

Cellulose nanowhiskers (CNWs) are high-aspect ratio biocompatible cellulose nanocrystals. Constructs of polyelectrolytes and CNWs have been made to obtain thin-film scaffolds whose topographical and mechanical properties can be tuned independently.

Multi-layered polyelectrolyte films were constructed using alternating layers of chitosan (Chi) and polystyrene sulphonate (PSS) and their stiffness tuned by the progressive incorporation of CNWs. The response of C2C12 myoblasts plated onto these surfaces monitored. Film stiffness was measured using AFM nanoindentation on PBS-hydrated films.

Films terminated in aligned CNWs were able to align C2C12 cells: normalized to the mean angle of the long axis relative to the x-axis, aligned cells showed standard deviation of 20.4 $^\circ$  while on unaligned cellulose nanowhiskers cells had a standard deviation of 42.2 $^\circ$ . Film stiffness was directly proportional to CNW content, ranging from 82 kPa  $\pm$  1.8 kPa in PSS/Chi only films to 4.9 MPa  $\pm$  0.34 MPa in Chi/CNW only films.

Our study has demonstrated the ability to engineer substrates to influence myoblast growth. This provides beneficial knowledge for the future application of CNWs tailored to function as a tissue engineering scaffold.

## Directing Immune Cell Responses using Nanopatterned Interfaces

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In order to maximise clinical efficacy in TERM therapies, materials need to elicit a favourable immune response *in vivo*. Historically this has been achieved by producing an “immune-inert” material, however in order to maximise the future potential of TERM therapies, materials that can more directly influence the immune system are required. Here, we describe the fabrication of immunomodulatory materials capable of directing immune cell activation, highlighting the example of an “artificial immune synapse” engineered on the nanoscale.

Recent advances in super-resolution microscopy have suggested that immune cell receptors are clustered on the cellular surface at the nanoscale, however it is not yet clear what role this clustering plays in cellular activation. Using gold nanoparticles fabricated through block co-polymer micellar lithography, we have fabricated functionalised nanoarrays which present immune-stimulatory ligands with precise nanoscale order to investigate cellular response to the nanoscale arrangement of stimulatory ligands.

Cellular responses in representative human adaptive and innate immune cells have been investigated; specifically, we highlight T cells and NK cells, stimulated with anti-CD3 or anti-CD16 respectively. In both cell types a decrease in key signalling and activation markers occurred as inter-ligand spacing increased from 25 to 100 nm (T cells  $p < 0.01$ , NK cells  $p < 0.001$ ). These studies suggest that immune cell signalling can be controlled by the precise spatial organization of receptor-ligand engagement on a sub-100 nm scale, offering a fundamental insight into immune cell signaling requirements and suggesting immunomodulation within TERM may be facilitated by nanofabrication techniques.

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#### Extracellular Matrix Hydrogel for Treatment of Ulcerative Colitis

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Ulcerative colitis (UC) affects over 500,000 individuals in the United States and current therapies are directed only at symptomatic remission. Definitive treatment for UC (colectomy) is invasive, associated with high morbidity, and diminishes quality of life. Effective treatment of UC requires mucosal repair that is subsequent to inflammation reduction and restoration of epithelial barrier function. Extracellular matrix (ECM) hydrogels can induce an *in-vitro* shift in macrophage phenotype towards an anti-inflammatory cytokine profile but it is unknown whether this will occur *in-vivo* and, if so, whether the response would be robust enough to treat colitis. The objective of the present study was to determine the efficacy of ECM for restoration of colonic mucosa in a rat model of UC.

UC was induced in rats by administering 5% dextran sodium sulfate (DSS) in drinking water for 7 days. Rats were divided into treatment (ECM hydrogel) and no treatment control groups ( $n=3$ /group). ECM treatment was provided via daily enema for 7 days, and animals were sacrificed on day 8. Criteria for disease activity included weight change, colon length, and histologic scoring. The present study showed that ECM hydrogel remains adherent to the colon for 12–24 hours and results in markedly diminished signs of UC and restoration of mucosal architecture. Treated rats gained weight more rapidly and had an increased colon length compared to control animals. Histologic scoring also showed ECM diminished inflammatory activity in the colonic mucosa. These results suggest that an ECM hydrogel is a potential therapeutic for individuals with UC.

#### N-acetylcysteine Reduces Fibrosis and Improve Both Muscle Regeneration and Function in Compression Induced Injury

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During severe muscle injury, perturbations to the muscle micro-environment by reactive oxygen species (ROS) can lead to muscle damage, fibrosis, and disability. Therefore, identifying ways to preserve muscle microenvironment may decrease fibrosis and improve functional recovery. Here, we study the effects of N-acetylcysteine (NAC), a known antioxidant, on muscle regeneration in response to injury. Adult female Lewis rats were subjected to compartment syndrome (CS) injury and half received NAC (150 mg/ml) and the other half received equal volume of PBS for three days after injury. NAC treated rats had a significant increase in muscle function compared with PBS treated rats accompanied by a significant decrease in tissue fibrosis, determined by Masson's trichrome staining. In addition, NAC treated rats had higher Pax 7, MyoD, PECAM, KDR, and CD146 gene expression, measured using RT-PCR. These results suggest that treatment of skeletal muscle injuries with antioxidants, such as NAC, may be a viable option for prevention of long-term fibrosis and scar formation and improve muscle recovery and function.

#### Differentiation and Regeneration by Sustained Release of Testosterone and Estradiol: An *In-vivo* Bone Tissue-Engineering Study

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**Introduction:** Bone tissue engineering appears to be a promising solution for reconstruction of bone defects. This is why, regenerative strategies to provide potent pro-osteogenic signals for available population of multipotent stem cells recently became a major area of interest. This study investigated whether the *in-vivo* osteogenic differentiation potential of adipose-derived mesenchymal stem cells (ADMSCs) is enhanced by sustained release of testosterone and 17 $\beta$ -estradiol.

**Methods:** Sixty Sprague-Dawley rats were randomized and divided into five experimental groups. For the surgical procedure, biparietal bone defects (8 mm in diameter and 2 mm in thickness) were created. Poly (2-hydroxyethyl methacrylate)-Gelatin Cryogel based scaffolds was used as the vehicle system for testosterone and/or 17 $\beta$ -estradiol loaded nanoparticles and ADMSCs. The rats were sacrificed at the end of weeks 6 and 12, and their calvariae were harvested for histologic and micro-computed tomographic ( $\mu$ CT) evaluation.

**Results:**  $\mu$ CT evaluation of scaffold with testosterone, estradiol, and ADMSCs revealed the highest median value, and the difference was significant compared with the blank defect group. Histologic samples demonstrated a significant difference between experimental groups for bone defect repair at the end of weeks 6 and 12. The group containing scaffold, testosterone, estradiol and ADMSCs had the highest median score at week 12, which was significantly higher than scores for the empty scaffold and ADMSCs group and the blank defect group.

**Conclusion:** Testosterone and 17 $\beta$ -Estradiol appears to be promising agents for future cell-based bone tissue-engineering studies.

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#### Human Aortic Valve Interstitial Cells Phenotype is Modulated by Substrate Stiffness and is Associated with Discrete Cellular Rigidity

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Aortic valve calcification is a widely diffused pathology caused by aortic valve interstitial cells (aVICs) conversion into myofibroblasts and calcium-depositing cells. Here we investigated the role of substrate elasticity on human aVICs differentiation toward pathologic phenotypes. Thin Polyacrylamide gels (PAAg) served as tunable stiffness substrates (elastic module  $3.4 \pm 2.6$  [Low];  $16.3 \pm 3.7$  [Mid-Low];  $22.5 \pm 5.1$  [Mid-High];  $58.3 \pm 8.8$  [High] kPa, mean  $\pm$  SD validated by atomic force microscopy, AFM) for aVICs seeding ( $2E+3$  cells/cm<sup>2</sup>) and culture for 48 hours, before fixation and either staining for immunofluorescence or AFM cell rigidity assessment. Computer-assisted cell morphometry showed an increasingly spread cell shape on substrates with Mid-high/High vs. Low/Mid-Low elasticity (cell area,  $\mu\text{m}^2$ :  $566 \pm 64.8$  [Mid-High] and  $692.3 \pm 83.3$  [High] vs.  $293.8 \pm 42.2$  [Low] and  $320.1 \pm 38.8$  [Mid-Low]; mean  $\pm$  SE;  $P < 0.01$ , 1-way ANOVA;  $n > 6$ ), accompanied by an increased number of focal contacts (stained by anti-Vinculin, -Paxillin). The increase in polymerized  $\alpha$ SMA onto stiffer PAAg suggested a myofibroblast conversion. Cellular rigidity showed a striking difference between cells plated onto stiffer vs. more compliant matrices (8.2 fold increase High vs Low,  $P < 0.001$ ; Wilcoxon). Culturing aVICs onto substrates of incremental stiffness determined translocation of YAP transcription factor from the cytoplasm to the nucleus, thus suggesting the involvement of the Hippo pathway in hard-substrate-dependent mechanotransduction. These effects were reversible by treating cells with inhibitors of cytoskeleton polymerization (Y27632 and Blebbistatin). These results establish, for the first time a direct cause-effect relationship between mechanical compliance of the substrate, cellular biophysical characteristics, and mechano-sensing driven transcriptional pathways mediated by cytoskeleton signaling in cells of the human aortic valve.

#### Injectable Gelatin Matrix to Enable Reconstruction of Deep Brain Lesion Cavity with Endogenous Neural Stem Cells

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Neural stem cells (NSCs) found endogenously in the brain can promote regeneration after injuries/diseases without the complications/risks of cell transplantation. However, they have to be carefully supported and guided in fluid-filled deep brain lesion cavities where physical structures for supporting NSCs and instructive cues for engaging NSCs are absent. Addressing these deficiencies to harness the potential of endogenous NSCs necessitates the use of injectable biomaterials since deep lesion cavities are completely surrounded by sensitive brain tissue. In a rat model for intracerebral hemorrhage (ICH), we injected gelatin-hydroxyphenylpropionic acid (Gtn-HPA) matrix into deep brain lesion cavities at 2 weeks after ICH to re-establish physical structure in the cavity space. The injected matrix was well tolerated, maintained a tight interface with the apposing brain tissues and conformed to various shapes across different lesion cavities. We further loaded the Gtn-HPA matrix with epidermal growth factor (EGF). Immunostaining for the NSC marker, nestin, showed that with the injection of EGF-loaded Gtn-HPA matrix, a massive population of endogenous NSCs migrated beyond the lesion boundary to populate the cavity space. In contrast, all other treatments (i.e., aCSF, Gtn-HPA matrix alone and a bolus of EGF solution) only led to a few endogenous NSCs, which remained confined around the lesion cavity. Notably, the infiltration of endogenous NSCs into the lesion cavity was accompanied with statistically significant neurological improvements relative to all other treatment groups. Overall, the reconstruction of deep brain lesion cavity by endogenous NSCs shows promise in promoting tissue and function recovery in the injured brain.

#### An Injectable Multifunctional Dual-Phase Microsphere-Reinforced Gelatin Matrix Permissive of Mesenchymal Stem Cell Infiltration for Musculoskeletal Soft Tissue Repair

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This study developed a novel strategy using a dual-phase multifunctional injectable gelatin-hydroxyphenyl propionic acid (Gtn-HPA) composite: preformed 8 or 16 wt% Gtn-HPA (stiff, degradation-resistant) microspheres in a 2 wt% (compliant, more rapidly degradable) Gtn-HPA matrix. The properties of the 2 wt% Gtn-HPA enable: 1) the filling of defects with the composite prior to the *in vivo* covalent cross-linking of the matrix, the rate and degree of cross-linking of which are independently controlled by horseradish peroxidase and peroxide, respectively; 2) bonding of the matrix to the microspheres; 3) the ready migration of endogenous cells into the composite; and 4) the incorporation of select growth factors for their ready release to serve as chemoattractants and mitogens. The results of a 3D migration assay showed that both the cell number and migration distance in the dual-phase gel system were comparable with the 2 wt% mono-phase Gtn-HPA, but significantly higher than 8 wt% mono-phase Gtn-HPA (into which few cells migrated). Our results also showed that the dual phase gel system had comparable degradation resistance and prolonged drug releasing profile with 8 wt% mono-phase Gtn-HPA, which were significantly higher than 2 wt% mono-phase Gtn-HPA. In addition, the compressive modulus of the dual-phase gel system was  $5.3 \pm 0.4$  kPa and  $19.3 \pm 2.6$  kPa when 8 wt% and 16 wt% Gtn-HPA were used as the bead phase, respectively, which was 4–13 fold higher than that of 2% mono-phase ( $1.5 \pm 0.06$  kPa). These results support the continued study of a novel injectable dual-phase Gtn-HPA composite for regenerative repair of defects/ruptures in musculoskeletal soft tissues.

#### Injectable Hybrid Hydrogel for Mesenchymal Stem Cell Delivery, from PEG-based Multifunctional Hyperbranched Polymers

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Stem cell therapies have been attracted much attention for the last few decades in the field of regenerative medicine and tissue engineering. Increased evidence indicates that the traditional 2-dimensional (2D) cell culture systems cannot fully mimic the natural tissue microenvironment, advanced 3-dimensional (3D) extracellular matrix (ECM) replacements are required not only for developing our understanding on stem cell behavior, but also for the clinical application. Here, an injectable PEG-based multifunctional polymer was developed via RAFT homopolymerization of the di-vinyl monomer of PEGDA. The rapid gelation (within two minutes) can be achieved between the polymer and thiolated hyaluronic acid at physiological condition due to the high degree of multi-acrylate functionality of this polymer. In addition, by simply varying the synthesis recipe such as the reaction time and the ratio of the chain transfer agent (CTA) to monomer, tunable polymer molecular weight, acrylate-functionality degree and the cyclized/hyperbranched molecular architecture can be finely controlled. Thus the 3D microenvironment (e.g. chemical and mechanical properties) of this hydrogel can be easily adjusted by altering the polymer characters and the crosslinking conditions. Primary adipose-derived stem cells (ASC) were embedded into the *in situ* crosslinked hydrogel, and the cell behavior such as the morphology, viability, metabolic activity, proliferation, phenotype and paracrine secretion profile were fully evaluated. The results suggested the hydrogel maintained the good cell viability and stemness, and increased the secretion level of certain growth factors and cytokines, which provide this injectable hydrogel

delivery system a decent potential for stem cell therapy and tissue regeneration applications.

### L-Glutamic Acid Derived Biodegradable Polyurethanes

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Due to their biocompatibility, chemical versatility, and excellent mechanical properties, polyurethanes (PUs) have been extensively used in various implantable devices. With the intensive growth of tissue engineering and regenerative medicine, there has been an increased interest in the design of biodegradable PUs. In this study, new biodegradable PUs were synthesized by two shot condensation polymerization of polycaprolactone diol and hexamethylene diisocyanate, and extended with a novel glutamic acid ester derivative (2S)-bis(2-hydroxypropyl) 2-aminopentanedioate (HPAP). HPAP was synthesized by Fischer esterification method, from the starting material L-glutamic acid. L-glutamic acid is the most natural abundant L-isomer of glutamic acid, and has wide range of applications in biodegradable polymers and hydrogels, therapeutics, neurotransmitter substances. Mass analysis of HPAP; calculated for C<sub>11</sub>H<sub>21</sub>NO<sub>6</sub> (M+) 264.1443, and found 264.1447 by HRMS. The biodegradable polymer (PU-HPAP) had weight average Mw of 33100 g/mol and radius of gyration 1420 nm, obtained from Berry-Plot of static light scattering measurements. Presence of HPAP provided chain stiffness and increase in Tg from -56°C to -50°C. Enzymatic degradation mechanism of ester backbone was extensively investigated by lipase. The degradation products did not show any cytotoxic effect on L929 cells by MTT assay. Degradations of polymers were observed via surface erosion, which implies that water diffusion into the matrix was slower than hydrolysis. This new biodegradable polyurethane and its derivatives have promising outcome for tissue scaffold applications to provide drug delivery up on surface.

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### Enhanced Osteoblast Proliferation on Poly-HEMA Hydrogel Loaded with Nanocrystalline Hydroxyapatite and Arginine-Rich-RGD Amphiphilic Peptide

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Traditional orthopedic implant materials lead to orthopedic problems including low biocompatibility and insufficient prolonged osteointegration. Herein, we aimed to develop biomimetic poly(2-hydroxyethyl) methacrylate (pHEMA) hydrogels loaded with amphiphilic peptide nanoparticles (APNPs) containing the arginine-rich-RGD (Arg-Gly-Asp) sequence conjugated with C18 fatty acid and nanocrystalline hydroxyapatite (nHA) to improve orthopedic implant biocompatibility.

The pHEMA hydrogel composites were synthesized by sonicating the 2-hydroxyethyl methacrylate (HEMA) monomer with nHA, APNPs, and 2,2'-azobisisobutyronitrile (AIBN) initiator. Briefly, NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> solution was mixed with NH<sub>4</sub>OH to adjust the basic pH, followed by the slow addition of Ca(NO<sub>3</sub>)<sub>2</sub>. The HA precipitates were centrifuged and rinsed with water three times. The nHA were

then prepared by heating at 200°C for 20 hours in a 200 ml Teflon liner. Next, the APNPs were dissolved in water and mixed with 70%(v/v) of HEMA, nHA and AIBN. Finally, this solution was sonicated in water at 60°C for 14 min, and the pHEMA hydrogels were prepared. In the cell experiments, human osteoblast cells (3500 cells/cm<sup>2</sup>, Omega C-12760) were seeded on plain pHEMA, nHA-loaded pHEMA, APNPs-loaded pHEMA, and nHA/APNPs-loaded pHEMA. The cells were allowed to grow for three days, and cell densities were evaluated by the MTT (3-(4, 5-dimethylthiazolyl)-2,5-diphenyltetrazolium-bromide) assay.

Results showed that the nHA/APNPs-loaded pHEMA hydrogel had high biocompatibility and could enhance osteoblast proliferation. Thus, we conclude that this nHA/APNPs-loaded pHEMA construct could be an innovative coating agent on various bone implants to enhance biocompatibility. Long term osteoblast functions, such as calcium deposition, and alkaline phosphatase synthesis will also be presented.

### Bioactive Nanocomposite Spheres with Proved Shape Memory Capability in Bone Defects

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Bioactive materials promote a bone-like apatite surface layer in physiologic conditions, an important step for successfully bonding with host tissue [1]. Bioactive chitosan nanocomposite spheres with shape memory capability and drug delivery function were successfully produced on biomimetic superhydrophobic surfaces. This preparation method led to spherical particles with controlled sizes and high drug load efficiency. The bioactive capability was achieved through the incorporation of bioactive glass nanoparticles (BGNPs) obtained from a sol-gel route. Spheres were prepared with different concentrations of BGNPs and genipin, a natural crosslinking agent. The successful development of bone-like apatite on the surface when the spheres were immersed in a simulated body fluid (SBF) was observed by SEM and EDS analysis. Higher apatite formation was observed for lower genipin concentration. Shape memory polymers represent a class of stimuli-responsive materials that offer mechanical and geometrical action triggered by an external stimulus [2]. The spheres presented shape memory behavior triggered by hydration with high values of shape fixity and shape recovery which enables their minimal implantation. The proof-of-concept was tested by introducing the spheres in a bone defect, where they showed a good geometrical accommodation in the implanted site. The bioactive spheres allowed the incorporation of a drug model and its effective release. Overall, the developed nanocomposite spheres showed great potential for bone tissue engineering in particular as a device to be implanted using minimal invasive procedures.

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### A Hydrogel System with Tunable Mechanical Properties to Control Mesenchymal Stromal Cell Differentiation

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It is well known that the differentiation of mesenchymal stromal cells (MSCs) can be controlled by adapting the mechanical properties of the matrix in which the cells reside. For this purpose, we have

developed a novel photocrosslinkable hydrogel platform consisting of methacrylated gelatin (GelMA) and poly(ethylene glycol) dimethacrylate (PEGDMA).

In this study, the concentration of GelMA remained constant at 5% w/v in order to have a constant availability of bioactive sites for cell attachment. The concentration PEGDMA was varied between 0% and 20% w/v, resulting in hydrogels with a compressive modulus ranging between  $0.46 \pm 0.15$  kPa and  $291 \pm 32$  kPa.

Human MSCs were incorporated in the gels and showed good viability. The cells were cultured in basic medium without differentiation factors for up to 7 days. Organization, as well as differentiation of the cells, depended on the mechanical properties of the gels. Soft gels resulted in elaborate organization of the MSCs and upregulation of the neural marker  $\beta$ 3-tubulin, while MSCs remained rounded and expressed the osteogenic marker ALP in stiffer gels.

This hydrogel platform offers a photopolymerizable, versatile, and tunable system to control the differentiation of human MSCs *in vitro*. Future studies will focus on a further elaboration of the differentiation of MSC within these gels, and the use of these gels for complex tissue engineering.

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### An Experimental and Computational Investigation of 3D Matrix Mechanics in Directing Stem Cell Lineage

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Robust control of mesenchymal stem cell (MSC) differentiation is vital for successful clinical application. It is clear that MSC fate can be directed by matrix stiffness and ligand chemistry; however, while the majority of work in this area has been carried out on 2D substrates, mechanotransduction is inherently different in the 3D environment presented by many matrices. Therefore, we sought to develop 3D, porous, recombinant elastin-like protein (ELP) matrices that allow independent control of stiffness and ligand chemistry for investigation of the role of these factors in directing MSC lineage in 3D. Porous matrices were formed using a sacrificial polymeric template, resulting in ELP matrices with a porosity of 60%. Matrix ligand chemistry was varied by altering the ELP amino acid sequence to include the adhesive ligands RGD or YIGSR at specific concentrations. Mechanical properties were controlled from 16–50 kPa by varying the ratio of the covalent crosslinker to reactive sites. Computational modeling was used to characterize the local mechanical environment presented to cells within the porous matrix, demonstrating that mechanical feedback from the matrix was dependent on material properties, pore geometry, and cell morphology, resulting in a range of cell-sensed stiffness from 6.4–50 kPa. Markers of osteogenic differentiation were found to be upregulated on the stiffest matrices after 7 days. In summary, we produced novel 3D porous matrices and developed computational models that reveal the local mechanical environment presented to cells. Ongoing work is investigating the interplay between ligand chemistry and mechanics in directing MSC lineage.

### Accurate Shape-designed Scaffolds for Human Auricular Tissue Engineering

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Engineered cartilage is a promising option for auricular reconstruction. Due to the lack of appropriate scaffolds, auricular reconstruction remains one of the greatest challenges in plastic surgery. The major technical bottlenecks that impede clinical translation of this approach is how to retain the accurate ear shape in skin-pressure environment after implantation. Although our group preliminary study confirmed the mechanical strength of PGA scaffold was enhanced by coating with polylactic acid (PLA), how to overcome its limitations in achieving desirable mechanical strength and accurate control over shape remains an unsolved problem. In this study, we improved the previous ear-shaped model. In experimental group, the ear scaffold used PCL mesh as inner core, which was wrapped with PGA fibers, and coated with PLA. And control group used the same weight PGA and PLA. Both group was seeded with microtia chondrocytes. Cell-material compound was tested by gross view, laser scanning, histology, immunohistology after been cultured *in vivo* and *in vitro*. The results shows that, the constructs in experimental group largely retained the original shape during cultured *in vitro* for 12 weeks, and formed ear-shaped cartilage-like tissues, which revealed a tissue structure with abundant cartilage extracellular matrices and mature lacuna. Additionally, the ear-shaped cartilage implanted in a nude mouse model for 12 weeks maintained not only in shape but also in size and flexibility. However, the control group's compound was wrapping, and lost its original shape. These results may provide a useful strategy for reconstructing cartilage tissue with complicated shapes such as external ear reconstruction.

### Physicochemical Polymer Properties Regulate Stemness and Therapeutic Potential of Human Mesenchymal Stem Cells

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Human mesenchymal stem cells (hMSCs) are a promising cell source for tissue engineering, but their therapeutic efficacy is severely reduced when derived from aging individuals or following *in vitro* expansion. In the present study, a discovery-driven approach was taken to identify the properties of synthetic biomaterial substrates that can improve stemness of hMSCs, and a systematic evaluation of the resulting phenotype was conducted. Our results indicate that increased stemness correlates with a pericyte-like, pro-angiogenic, and anti-inflammatory phenotype in 2D and 3D, both *in vitro* and *in vivo*. An investigation into the cell-material interfacial mechanism revealed that decreasing surface repellency abrogated the hMSC response but improved cell attachment. These data suggest that the surface repellency/adhesiveness can be optimized to promote cell-cell interactions while maintaining a basal level of cell-material interactions, thereby promoting the stemness and pericyte phenotype of hMSCs. This study yields mechanistic insights for methods to improve the stemness and therapeutic capacity of hMSCs through manipulation of polymeric cell culture substrates.

### Bioprinted Microfluidic 3-D Cancer Microenvironments for Detection of Histone Modification and Cell Migration

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Lung cancer is one the main cause of tumor related deaths in the World. Non-small lung cancer (NSCLC) accounts for more than 80% of all lung cancers. Epigenetic histone modifications controls cell growth and differentiation; deformities of these modifications are implicated in various cancer types. Histone modification has been identified as an important diagnostic and therapeutic target in various cellular pathogenesis including cancer. Microfluidic devices provide engineered solutions for biological research including 3-D cancer cell culture and clinical application towards personalized medicine. Cancer cell invasion is a multistep process playing a key role in cancer metastasis. It involves the modulation

of proteins such as cytoskeleton proteins and adhesion receptors. It also elicits the disruption of cell – matrix and cell – cell junctions, followed by the degradation of the local ECM, and migration into neighboring tissues. Here we generated microfluidic model provides a native like niche for lung cancer cells under dynamic culture conditions and a platform to detect histone methylation status in transcriptionally active methylation marks in H3 protein (H3K4, H3K36, and H3K79) in the circulating nucleosomes. Elevated level of H3K36 demethylation correlates with the over-expression of JMJD2A, and therefore, detection of H3K36me2/3 methylation levels in circulating nucleosomes may be predictive biomarker for early detection of cancer. In our model device (i) we generate cancer cell niches viable in their physiological pathology under continuous microfluidic flow, (ii) conditioned media collected from the terminal end is analyzed with nanoplasmonic platform for the nucleosomes and the related alterations in histone to correlate with culture growth.

#### Stimuli-responsive Macropore Generation Within Injectable Gelatin-based Hydrogels for Promoting Cellular Activities

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In tissue engineering, the porosity and pore interconnectivity of scaffolds play a pivotal role in cell ingrowth, vascularization, and nutrient diffusion for cell survival. Although injectable hydrogels have been considered as a suitable scaffold platform due to their mechanical and structural properties similar to extracellular matrix, their small mesh size limits the ability to recruit and infiltrate cells for tissue remodeling. In this study, *in situ* macroporous gelatin-based hydrogels were developed and the effect of porosity in hydrogels on cellular activities was investigated. Thermo-responsive gelatin particles (GPs) ranging from 40 to 300 um were fabricated and they were suspended in an aqueous solution of gelatin-PEG-tyramine (GPT), followed by the formation of hydrogels through the HRP-catalyzed crosslinking reaction. The porous structure and physico-chemical properties of hydrogels with or without GPs were characterized after incubation at 37°C. It was found that GPT/GPs hydrogels had a higher degree of porosity (85–93%) compared to only GPT (16%) hydrogels, and *in vitro* cell migration assay demonstrated that the macroporous gelatin hydrogels resulted in significantly enhanced cellular migration. Therefore, *in situ* forming hydrogel with macropores can be a promising candidate as an injectable bioactive scaffold for tissue engineering and regenerative medicine applications.

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#### Exposure to Reversine Affects the Chondrocyte Morphology and Phenotype *In Vitro*

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**Objectives:** The re-differentiation potential of healthy human chondrocytes (HAC), cultured for cell-based cartilage repair, compensates the expansion-dependent phenotypic changes. However patients undergoing resurfacing procedures often display osteoarthritic (OA) tissues, and OA-HAC show a severely reduced chondrogenic commitment. Reversine (Rev), a 2,6-disubstituted purine inhibitor of spindle-assembly checkpoints, converts differentiated mesenchymal cells to their undifferentiated precursors. Hence we hypothesized that Rev exposure could divert OA-HAC to more plastic cells, re-boosting their commitment.

**Methods:** HAC were enzymatically released from three osteoarthritic cartilage specimens (ICRS grade I/2), expanded for two weeks in standard medium, and treated with Reversine (Rev; 5 μM in 0.05%DMSO) or with DMSO alone for 6 days. Duplicate experiments were run per culture. Cell growth was assessed by the AlamarBlue™ assay. Cytoskeletal structure, endoproliferation and caspase-3-immunopositivity were assayed by fluorescence microscopy. The OA-HAC chondrogenic performance was evaluated by quantitative RT-PCR for GAPDH, Sox9, Aggrecan (Agg), type-II collagen (Col2), Ki67, CyclinD1, TGFβ-1, -2 and -3, Interleukin 1-β and -6 (IL-1β, IL-6).

**Results:** Rev-treated OA-HAC recovered polygonal morphology, reducing Ki67 expression and proliferation. Cell-cycle impairment accounts for altered cytoskeletal organization, endoproliferation and apoptosis, while a compensatory mechanism sustains the increased CyclinD1 transcript levels. Sox9, Agg and TGFs were over-expressed, but not Col2. Interleukin(s) transcripts were massively down-regulated. These events are dose-related and transient.

**Significance:** In monolayer a Rev-induced quiescent state forces the OA-HAC phenotype closer to the 3D-native one, unmasking a higher transcriptional activity for ECM components; these characteristics seem unleashed from a regained chondrogenic potential, although 3D-settings still need to be assessed.

#### ToF-SIMS Analysis to Study the Effect of Detergent Based Decellularization on the Surface Molecular Functionality of Biologic Scaffolds

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Detergents are frequently used to generate biologic scaffolds composed of extracellular matrix (ECM) due to their ability to solubilize cell membranes and dissociate DNA from proteins. The objective of this study was to determine the effect of different detergents, commonly used in decellularization protocols, upon a representative tissue, specifically the basement membrane complex (BMC) of porcine urinary bladder. Both structure and surface molecular functionality were investigated.

The BMC and underlying lamina propria were isolated and harvested from porcine urinary bladders and exposed to 3% Triton-X 100, 1% sodium dodecyl sulfate (SDS), 8mM CHAPS or 4% sodium deoxycholate for 24 hours. Time of flight secondary ion mass spectroscopy (ToF-SIMS) was utilized to characterize the surface molecular functionality of the BMC following detergent exposure. Principal component analysis (PCA) of positive ion spectra highlighted high mass peaks associated with detergent fragments observed on scaffolds exposed to SDS and deoxycholate. A phosphocholine peak, indicative of cell membrane, was observed in all samples, but to a greater extent with scaffolds not exposed to detergent. These results demonstrate the utility and suitability of ToF-SIMS to detect detergent fragments and examine molecular-level changes induced by the decellularization process. The present study examined the cell:matrix interaction of human urothelial cells seeded upon decellularized bladders and showed that detergent exposure affected cell proliferation and permeability of the cell monolayer. An understanding of the effects of detergent exposure on BMC structure, composition and surface molecular functionality will facilitate a rational strategy for successful *in vitro* and *in vivo* recellularization techniques.

#### Injectable Bioactive Bulking Agent for Vocal Fold Reconstruction

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Recently, the injection laryngoplasty techniques for managing of glottal insufficiency have gained popularity because of its initial low cost, minimal invasiveness, and low morbidity as compared to surgical repair method. A number of injectable substances have been adapted in the clinical practice, however, relatively short-term volume stability (by migration and resorption), inflammation, granuloma formation, and decreased vocal fold vibration are considered as limitations for practical use. In this study, we prepared growth factor (bFGF or HGF)-immobilized PCL microspheres as an injectable bioactive bulking agent which can provide a bulking effect (by microspheres) and stimulate smooth muscle in vocal fold (by the sustained release of growth factor) for effective treatment of glottal insufficiency. The growth factor release and adhesion/proliferation of vocal fold fibroblasts on the growth factor-immobilized microsphere were investigated. Preliminary animal study using a rabbit model to investigate the vocal fold reconstruction potential of the growth factor-immobilized microspheres was also carried out in this study [This work was supported by the National Research Foundation of Korea (NRF- 2014R1A2A2A04003979)].

#### **A Stimuli Responsive Liposome Loaded Hydrogel for the Controlled Release of Pro-angiogenic Therapeutics to Repair the Infarcted Heart**

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Since its discovery, Vascular Endothelial Growth Factor (VEGF) has been seen as a promising molecule for promoting neovascularization in the infarcted heart. However, results of myocardial clinical trials using VEGF delivery have generally been disappointing due to the short-lived effect and high instability of the protein when injected as a bolus. The use of thermosensitive liposomes embedded in a thermoresponsive hydrogel (denoted Lipogel) can permit controlled, localised treatment with the use of a minimally invasive hyperthermic stimulus.

In the present work, we investigated Lipogel's ability to recruit stem cells and then elevate their production of VEGF by controlling the release of a pro-angiogenic, small molecule drug Desferrioxamine (DFO) with an external hyperthermic stimulus. Initial cell recruitment was accomplished by the free release of Hepatocyte Growth Factor (HGF), inducing a migratory response in cells, followed by the delayed, temperol release of DFO from thermosensitive liposomes, resulting in a significant increase in VEGF expression. This delayed release could be controlled up to 14 days and enabled a dose responsive effect dependent on the time of administration of a hyperthermic pulse. Moreover, by changing the duration of the hyperthermic pulse, a fine control over the amount of DFO released was achieved.

The ability to trigger the release of therapeutic agents at a specific timepoint and control dosing level through changes in duration of hyperthermia enables sequential multi-dose profiles. This flexible platform system could aid in more physiologically accurate delivery of pro-angiogenic factors to the ischemic heart by finely controlling their scheduling and sequencing.

#### **Beneficial Impact of Co-culture on Hepatic Functions in Fluidized Bed Bioartificial Liver Bioreactor**

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Bioartificial liver (BAL) devices are designed to support liver failure patients waiting for organ transplantation. These require biologically active hepatocytes to cover the wide spectrum of liver functionalities. Primary hepatocytes dedifferentiate and lose their

roles after isolation from their *in vivo* environment. Here, we investigated the potential of liver cells co-culture - encapsulated in alginate beads and hosted in our lab developed fluidized bed bioreactor - in preserving hepatic functions *in vitro*.

Primary hepatocytes and non-parenchymal cells (sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells) were isolated from rat livers and phenotypically characterized. These cells were used to form spheroids according to a previously defined optimal co-culture ratio (hepatocytes/non-parenchymal cells). Produced spheroids were encapsulated in alginate beads and incorporated in the fluidized bed bioreactor where they were maintained for seven days. Hepatic activities were assessed. Specific staining (with cytokeratin-18, SE-1, CD68 and desmin antibodies) and confocal microscopy allowed the follow-up of the different cell populations and their locations in the spheroids.

Non-parenchymal cells, especially placed in the hepatocyte-constituted core of the spheroids, seemed to support liver functions over time in the fluidized bed BAL bioreactor. At day 7, albumin synthesis was about 3-fold higher in co-culture condition than in mono-culture one (respectively,  $98.9 \pm 5.9$  and  $32.9 \pm 12.3$   $\mu\text{g/day}/10^6$  hepatocytes). Similar enhancement was found for urea secretion; hepatocytes produced an amount 1.72-fold higher when co-cultured with non-parenchymal cells. Such approach should be translated to human cells for further BAL application.

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#### **Neurotrophic Factor Gradient Generation within Hydrogel Sheets for Differential Growth Guidance of Motor and Sensory Neurons**

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Delivery of motor- or sensory-specific neurotrophic gradients can potentially improve the specificity of the regenerated nerve and enhance the functional outcomes of peripheral nerve repair. Here we present a diffusion-based gradient platform that can generate centimeter-scale linear gradients of neurotrophic factors within hydrogel sheets. Using an organotypic rat spinal cord slice culture model, we examined the effect of neurotrophic factor gradient pattern (concentration range and gradient steepness) on the differential growth of motor vs. sensory nerves, and determined potential gradient characteristics for separating mixed nerves into motor and sensory pathways.

Gradients of glial-derived neurotrophic factor (GDNF) or nerve growth factor (NGF) were generated within collagen hydrogels using a diffusion-based gradient platform, incorporated into the organotypic culture, and the extents and rates of motor and sensory nerve outgrowth onto the hydrogels were recorded and analyzed.

We have successfully generated hydrogel strips with growth factor gradients using the diffusion-based gradient platform with tailorable length, concentration range, and gradient steepness of the neurotrophic factors. Various growth factors and hydrogels can be used with this platform. The most effective directional guidance of sensory neurons was achieved using hydrogel-delivered NGF gradients in a range of 0–0.1 ng/mL compared to gradients of 10 and 100-times greater concentration ranges.

This growth factor gradient delivery platform is valuable for optimizing motor vs. sensory nerve-specific guidance cues for nerve regeneration applications. It also allows for scalable and controlled patterning of gradients into hydrogel matrices that can be translated to conduit design for *in vivo* nerve repair.

#### **Development of an Improved Primary *in vitro* Model of the Human Small Intestine**

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The small intestine in our body represents the organ with the largest contact surface to the environment. Its main function is the absorption of essential nutrients, water and vitamins, and it is a barrier protecting us from toxic xenobiotics and pathogens. It provides an elegant system for stem cell studies as well as aspects of transport mechanisms and barrier functions.

In our study, we applied epithelial cells in a 3D *in vitro* culture system in order to mimic the microenvironment of the gut *in vivo*.

Intestinal crypts including stem cells were isolated from human small intestinal tissue samples and co-cultured on a decellularized porcine gut matrix together with intestinal fibroblasts. *In vitro* models were maintained under static and dynamic conditions for up to 14 days. Epithelial integrity was tested by FITC-dextran (4 kDa) and TEER-measurement. Models were further analyzed by cell proliferation assays, immunohistochemistry and electron microscopy.

Intestinal cells have formed a monolayer including all the differentiated cell types shown by, Mucin2, Villin, Chromogranin A, and Lysozyme immunohistochemistry. Electron microscopy depicted essential functional units of an intact epithelium such as microvilli, tight junctions and vacuoles. FITC-dextran and TEER-measurement proved tightness of the cell layer.

The development of an *in vitro* system based on human primary cells provides a promising tool for more predictive preclinical testing with pharmaceutical substances, probiotic active organisms or human pathogenic germs in infection studies. Further functional validation studies are necessary to show stable enzyme activity, transport function, and batch to batch consistency.

#### Enhancement of Neuronal Outgrowth by Gold Nanoparticles Decorated Polyurethane Nanofiber Scaffolds

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The main obstacle in regeneration process of peripheral nerve injuries is the limited ability of neuronal outgrowth which brings out the loss of neural sensory and motor functions. As in defect sizes more than 1–2 cm autogenic nerve grafts are not sufficient to maintain the continuous signal transduction, so tissue engineered nerve guidance channels would be efficient in large defects (1). Recently, scientists have focused on conductive materials in respect to the fact that electrical stimuli raises the amount of axonal elongation. In this study, to stimulate neuronal outgrowth, polyurethane (PU) nanofibers were prepared by electrospinning technique and gold nanoparticles of 50 nm were decorated on nanofiber surfaces by electrostatic interactions. In the next step, pheochromocytoma cells (PC12) were undergone neuronal differentiation on as-prepared scaffolds by electrical and mechanical stimulation. The effect of simultaneous mechanical stimulation between 0.5–2.50 Pa and electrical stimulation between 100–250 mV on optimal neuronal outgrowth was evaluated. As a result, it was observed that the integration of gold nanoparticles and simultaneous stimulation enhances neuronal outgrowth. This fact can be in close relation to conductivity of gold nanoparticles that upon decoration on PU nanofibers facilitated electrical communication between neuron-like cells throughout the scaffold. In conclusion, different from other studies, gold nanoparticles were decorated on PU nanofibers by a straightforward approach and neural guidance was assisted by simultaneous mechanical and electrical stimulations.

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#### Property-tunable Polymer Platform for Optimizing Cell-adhesive Peptide Function

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For long-term implantation of medical devices, such as vascular grafts, their sustainable function of both cellular-compatibility and antithrombogenicity is essentially important. It has long been a challenge for synthetic polymers for medical usage to balance this contradictory function. The polymer design to enhance higher cellular-compatibility can promote cellular adhesion and growth, although their thrombosis risk increases easily. The polymer design to highly inhibit thrombosis commonly result in disturbance of cellular organization at the implanted area. To balance such biocompatibility of medical device surface, extracellular matrix (ECM) proteins are one of ideal functional molecules that control cellular organization in our body. However, it has been technologically difficult to produce xeno-free ECM proteins in mass-productive manner for cheap and stable medical device coating material.

For cost-effective and stable production, we have focused on investigating ECM-derived short peptides that can partially replace the selective adhesion of ECM proteins (Biotechnol Bioeng. 2012 Jul;109(7):1808–16). In this paper, we deeply investigate the combinational effect of medically used polymer and short peptides, to clarify the commonly observed mismatch of polymer and its functionalizing molecules. To investigate such effect and changes on cell-compatibility at the surface of peptide-conjugated polymers, we have established a cell assay platform that can tune the polymer property by temperature or polymerization ratio. On such original platform, the peptide effect, and its combinational effect with the polymer property was investigated to create interaction effect map.

#### Nerve Growth Control by Optogenetic Stimulation

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There have been developed diverse kinds of methods to overcome the limited regenerative ability of peripheral nervous system (PNS) such as electrical stimulation, topographic guidance, and delivery of neurotrophic factors. Here, we explored the optogenetic control of nerve growth as a cell-specific alternative to electrical stimulation *in vitro*.

We developed the system for rapid screening of multiple optical stimulation parameters and applied it to the investigation of neurite outgrowth from dorsal root ganglia (DRGs). We found that illumination with 465 nm blue light increases neurite outgrowth from DRGs expressing light-sensitive ion channel channelrhodopsin 2 (ChR2) by three-fold as compared to unstimulated or wild-type (WT) controls. Moreover, we identified optimal stimulation frequency and duration, and finally correlated the enhancement of outgrowth to the total number of stimulation pulses. Finally, in a co-culture of WT and ChR2-expressing DRGs, we found directional outgrowth in WT DRGs in the presence of stimulated ChR2-DRGs positioned at a 10 mm distance, which is comparable to a large-gap PNS injury in a rodent model. These morphological changes in axonal growth are accompanied by an increased expression of nerve growth and brain derived neurotrophic factors (NGF, BDNF).

In addition to providing a high-throughput screening tool to identify optimal simulation conditions, our light-delivery platform system may allow for exploration of underlying electrophysiological mechanisms of axonal growth and aid in the design of future regenerative therapies for PNS injuries.

### Physiological and Biological Effects of Konjac Glucomannan Hydrogels on Human Tissue Engineered Skin

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We have established a three dimensional (3D) tissue engineered (TE) skin model populated with fibroblasts and keratinocytes to test the capacity of two hydrogels of konjac glucomannan (KGM); biodegradable from crosslinked KGM and non-biodegradable of graft conetwork hydrogels on the reepithelisation, stimulation of cells in dermal area and reduction on the rate of TE skin contraction. KGM is a plant heteropolysaccharides of D-mannose and D-glucose linked by  $\beta$ -1,4 glycosidic chains which stimulates fibroblasts proliferation and migration of both skin cells, fibroblasts and keratinocytes in a wound scratch assay. KGM therapeutic benefit on the inhibition of keratinocyte proliferation would be beneficial to wound healing by the reduction of skin contraction. In this study, we found that crosslinked KGM with higher EWC of (>90%) and free water properties significantly reduced the contraction of TE skin without disrupting the formation of the dermal and epidermal layers compared to graph-conetwork hydrogels. The differences in hydrogels' chemicals, swelling and water content properties confirmed using <sup>13</sup>C solid state NMR, DSC and FTIR showed that hydrogel properties significantly affect cellular viability and differentiation, which in turns reduced the contraction of TE skin

### Viability and Proliferation of Mesenchymal Stem Cells Derived from Four Different Dental Tissues in a Fibrin-agarose Matrix

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**Background:** For many years the goal was to develop biomaterials to promote cell proliferation. There are documents that describe the agarose fibrin matrix (AFM). However, there is little evidence of cells in combination with this matrix.

**Aims:** In this work we tested Mesenchymal stem cells (MSC) derived from different dental tissues with FAM.

**Methods:** FAM was cultured with MSC derived from, gingiva, apical papilla, pericorony tissue and dental pulp. We evaluated the proliferation of the cells in a fresh and in a cryopreserved matrix. Using a cell proliferation assay (MTT), we evaluated the cells after 7 days post-cultivation in the fresh and in the cryopreserved matrix. The MTT assay was evaluated at 24, 48, 72 and 96 hours.

**Results:** We show that FAM is easy to handle, allows homogeneous incorporation of the MSCs derived from all tissues. Dental pulp-derived MSC (DPSC) proliferated better in both conditions. The multipotency, the high proliferation rates and easy obtaining of the DPSC make them an attractive source of MSC for tissue regeneration.

**Conclusions:** Collectively our findings support the biological utility of the fibrin agarose matrix in combination with DPSC and their potential use in tissue regeneration.

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### Creating Thermo-Responsive Co-Electrospun Fibres for 3D Cell Growth

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The cell supply chain for regenerative medicine requires the development of new *in vitro* culture systems. Current methods to generate large quantities of cells for clinical use commonly use enzymatic digestion. However, this approach is not desirable for subsequent cell transfer to the body due to the destruction of important cell surface proteins and risk of contamination. Research has led to the development of thermo-responsive surfaces for the continued culture of mammalian cells with passaging achieved with a slight drop in culture temperature. Our aim was to generate a thermo-responsive 3D-fibrous scaffold using co-electrospinning. Thermo-responsive Poly (PEGMA<sub>188</sub>) polymer, was prepared by free radical polymerization, then co-electrospun with PLGA or PET polymers to form thermo-responsive fibres. Responsive behaviours were characterized by <sup>1</sup>H-NMR, XPS and WCA measurements. 3T3 mouse fibroblast cells, immortalized human mesenchymal stem cells and human cancer colon epithelial cells were seeded on these fibres. Subsequent cell viability assay were performed to measure the difference in cell number while changing the culture temperature. This study has demonstrated that co-electrospinning is a promising way to create fibres with thermo-responsive surfaces, able to support cell adhesion and proliferation at 37°C and cell detachment at 17°C. Importantly, cells were viable and proliferated in a similar manner to those cultured on control surfaces. These scaffolds were found to be a suitable substitute for conventional cell culture methods with a number of different cell types by supporting cell attachment, growth and detachment in appropriate numbers without any negative effect on the cultured cells.

### Ex Vivo Osteo-chondral Organ Culture

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The onset of osteoarthritis can be prevented by sustainable cartilage defect treatments. Pre-clinical screenings of novel biomaterials in an osteo-chondral environment are needed to evaluate cell-tissue-material-interaction and develop new chondral regeneration therapies.

We establish an osteo-chondral *in vitro* test system (ivTs) opening new opportunities in cartilage regenerative therapies.

Standardized porcine osteo-chondral explants (OCE) with reproducible cartilage defects were isolated and treated with cell free or cell loaded hydrogels or left untreated. OCE were cultured in custom made transwell systems. Cell vitality was analysed by live-dead-staining. OCE were characterized (immune-) histologically (Safranin-O, collagen II, aggrecan). Cartilage degeneration and defect regeneration was evaluated by sulphated Glycosaminoglycan (sGAG) release and extracellular matrix composition. Biomechanical push-out tests were performed to evaluate biomaterial-tissue integration.

Chondrocyte viability maintained over 28 days of *in vitro* culture. Cartilage like tissue formation activity was observed on top of calcified layer of untreated osteo-chondral defects. Hydrogels integrated into tissue of chondral defects. Total sGAG content decreased during *in vitro* culture in superficial and middle zone and reduced mechanical strength. Up to 30% of complete sGAG loss was released within 7 days independently of defect size.

Proteoglycan loss displays a symptom in early cartilage degeneration preceding to collagen II degradation.

Our ivTs is going to integrate biomechanical stimulation and hypoxic conditions supporting defect regeneration and diminish cartilage degeneration. The osteo-chondral ivTs is going to be

validated against *in vivo* studies representing pre-clinical standard to simulate cartilage defect conditions and studying processes in cartilage regeneration and degeneration pathways.

### Preparation of Gradient Decellularized Dermis-polymer Complex for Tissue Interlinking Device

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The connecting technology for the synthetic materials to living body has been necessary for preparing percutaneous devices, artificial blood vessels, and artificial ligament, etc. One of the most serious problems is formation of the distinct interface between synthetic materials and tissue. To overcome this problem, we tried to apply the tissue engineering technology. Our strategy is to develop a decellularized tissue-polymer complex with gradient polymer concentration (gradient complex), which has high affinity to the synthetic materials on one side and to the soft tissue on the other so as to interlink them. The decellularized dermis was prepared using sodium dodecyl sulfate (SDS). To prepare the gradient complex, we put the decellularized dermis into the methyl methacrylate (MMA) monomer, benzoyl peroxide and N,N-dimethyl-p-toluidine solution, and controlled the absorption time by pulling the dermis out of the solution at the certain speed. Then MMA was polymerized at room temperature for 12 hours. Using this process, we succeeded in obtaining a gradient dermis-poly (MMA) complex. The compression test showed that the mechanical strength of the complex changed gradually from dermis side to polymer side, indicating that the polymer exists in dermis with gradient concentration. As conclusion, we believe that this complex possesses high potential for the new type of soft tissue-synthetic materials interlinking device.

### Combining Sphingosine-1-Phosphate and Nanotopographical Signaling to Enhance Skeletal Muscle Maturation and Vascularization

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Healthy skeletal muscle is a highly regenerative, metabolically active, and organized tissue where the extracellular matrix is arranged as dense cables of aligned collagen fibers that provide structural and functional support. Engineered muscle grafts therefore need to be structurally ordered and well-vascularized while maintaining the capacity for myogenesis via a population of asymmetrically-dividing satellite cells. Here we have developed biomimetic nanopatterned PLGA substrates functionalized with sphingosine 1-phosphate (S1P), a potent angiogenic and myogenic factor, to enhance skeletal myoblast and endothelial differentiation. Primary mononuclear muscle cells cultured on these functionalized nanopatterned substrates were highly aligned and elongated, and displayed greater myogenic potential as evidenced by myosin heavy chain staining and by upregulation of markers for mature muscle including MyoG and Myh15. Interestingly, we found a biphasic dependence of skeletal muscle maturation on S1P concentration, with an observed optimal concentration of 175  $\mu$ M. We also found that S1P functionalization enhanced neovascularization potential, as evidenced by an increased expression of endothelial markers such as BS1, Ve-Cad and CD31 with increasing S1P concentration. Using Pax7Cre-GCaMP+ primary myoblasts isolated from transgenic mice, we quantitatively analyzed the contraction frequency using fluorescent imaging of Ca<sup>2+</sup> transients, and demonstrated the improved functionality of engineered tissue cultured on S1P functionalized nanopatterns.

### Response of Human Primary Oral Fibroblast on Micro-Pillared Silk Fibroin/Honey Membranes with Varied Mechanical Strength

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Oral fibroblasts play important roles in wound healing after tumor resection oral surgeries. To restore healthy microenvironment of oral mucosa, suitable bio-mimicking extracellular matrix (ECM) is in demand particularly to develop tissue engineered oral grafts. We incorporated topographical and mechanical cues to attain the required microenvironment. The micro-pillar structures were fabricated on silk fibroin (SF) and honey (1, 2, 4 and 6%) blended membranes by soft-lithography and physico-chemically characterized. The proliferation rate, cytoskeleton organization and Col III gene expression were analyzed after 4 days of primary oral fibroblast culture on membranes. Micro-pillars were of 7–8  $\mu$ m height having 10  $\mu$ m inter-space. With increased honey concentration young modulus of membranes were decreased upto  $9.03 \pm 1.71$  Mpa whereas elongation rate was increased upto  $193 \pm 2.37$  Mpa. It shows the membranes with high honey concentration are less stiff or soft in compare to membranes with lower concentration. The cellular proliferation and mitochondrial activity was increased with increased honey concentration on micro-pillared SF membranes. However, rate of collagen synthesis on membranes was initially decreased upto 2% honey concentration in comparison to control and then started increasing with high honey concentration (4 and 6%); it may be due to the changes in the stiffness and toughness of membranes. It shows membranes with good mechanical property may lead to high proliferation rate but not enough to create favorable microenvironment for proper functioning of cells. Hence, this study demonstrates that the extracellular microenvironment can be improvised by modulating topographical, compositional and mechanical cues of artificial ECM.

### Catch-and-release of Target Cells using Aptamer and Electrochemical Cell Detachment

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**Aim:** Sorting of specific cells from a heterogeneous suspension is indispensable in various applications such as regenerative medicine and cancer research. In this study, we developed the surface modified with oligopeptides and aptamers, with which cells can be selectively attached and then rapidly detached by the application of an electrochemical potential.

**Methods:** A zwitterionic oligopeptide, CGGGKEKEKEK, was designed for preparation of cell repulsive surface. This peptide spontaneously bonded to a gold surface via a gold-thiolate bond and formed a dense molecular monolayer by the electrostatic force between neighboring molecules due to the alternating charged lysine (K) and glutamic acid (E), making the surface cell repulsive. To selectively catch cells, the surface of peptide layer was further modified with an aptamer which has high affinity for Hep G2 cells. Cells selectively attracted on the surface were subsequently detached by electrochemically cleaving the gold-thiolate bond and releasing the peptide layer from the gold surface.

**Results:** When Hep G2 cells and NHDFs (normal human dermal fibroblasts) were exposed to the surface modified with the peptide, none of them attached on the surface. On the surface further modified with the aptamer, selective adhesion of Hep G2 cells was observed and the ration of Hep G2 cells (Hep G2/NHDF) increased from 50% to 88% after the single selection process. Almost all the Hep G2 cells attached on the surface were electrochemically detached within



5 min. Our results demonstrated that specific binding of aptamers and electrochemical cell detachment can be used for selective cell sorting.

### Functionalized Rosette Nanotubes as a Bone Regenerative and Anti-microbial Agent

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Bone has a limited capability to recover from large defects followed by traumatic bone loss, bone tumors or infections. Bone morphogenetic 7 (BMP-7), approved by the FDA for orthopedic applications, is one of the most important cytokines of the transforming growth factor beta family involved in skeletal development and bone remodeling. However, the amount of BMP-7 needed to induce bone growth is on the order of milligrams due to rapid clearance out of the body, which is extremely high and might cause a variety of adverse effects ranging from inflammatory responses to excessive bone growth. As a result, a novel delivery system need to be developed to prolong BMP-7 retention, thereby reducing such a high dose. In addition, due to the high cost of the production all BMP proteins, previous researchers have identified several peptide sequences, with similar abilities to promote bone cell growth but much lower cost, from the knuckle epitope of BMP proteins [1]. Furthermore, postsurgical bone infection is an emerging challenge as the number of bone surgeries increases in recent year. And short designed peptides could be potential candidates as antimicrobial agents to meet the challenge. In this study the use the aforementioned peptides were further improved for orthopedic applications by covalently connecting them with twin G<sup>A</sup>C base-derived rosette nanotubes [2,3].

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### Reducing Bacteria and Macrophage Density on Nanophase Hydroxyapatite Coated onto Titanium Surfaces without Drugs using Electrophoretic Deposition

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Reducing bacterial density on titanium implant surfaces has been a major concern because of the increasing number of nosocomial infections. Controlling the inflammatory response post implantation has also been an important issue for medical devices due to the detrimental effect of chronic inflammation on device performance. It has recently been demonstrated that manipulating medical device surface properties including chemistry, roughness and wettability can control both infection and inflammation. Here, the effect of nanophase (that is, materials with one dimension less than 200 nm) hydroxyapatite on bacterial adhesion and inflammatory responses (as measured by macrophage functions) *in vitro* was evaluated by modifying hydroxyapatite particle sizes and comparing such results to bare titanium and plasma sprayed hydroxyapatite coated surfaces currently used clinically today. Nanophase hydroxyapatite was synthesized in size ranges from 110–170 nm and was subsequently coated onto titanium samples using electrophoretic deposition. Results indicated that smaller nanoscale hydroxyapatite features on titanium surfaces decreased bacterial attachment in the presence of gram negative (*P. aeruginosa*), gram positive (*S. aureus*) and ampicillin resistant gram-negative (*E. coli*) bacteria as well as were able to control inflammatory responses, properties which should lead to their further investigation for improved medical applications.

### Development of a Culture System for Intestinal Organoids using Peptide-functionalized PEG-based Hydrogels

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Intestinal organoids are composed of budding crypts, epithelial cells and well-defined lumens. Organoids have gained interest as a tool to study intestinal biology and as a platform for drug screening because they recapitulate the complexity found *in vivo* (1). Organoids are cultured in ill-defined Matrigel-based hydrogels. Lot-to-lot variability or the presence of residual growth factors in Matrigel can affect the biological outcome. Therefore, the development of a well-defined matrix that can support the culture of organoids is of vital importance. PEG-based hydrogels are good candidates as synthetic matrices. They can be tailored to different applications by varying the stiffness, degradability and cell adhesion properties. Here we report our efforts to develop PEG-based hydrogels functionalized with a fibronectin-derived peptide (Synk-RGDS) or a collagen-derived peptide (GFOGER) for intestinal organoid culture. Preliminary results suggest that direct encapsulation of single cells obtained from mouse organoids in these PEG-based hydrogels results in formation of organoids similar to those that form in Matrigel. The PEG-GFOGER hydrogel showed higher number of organoids when compared to PEG-Synk-RGDS hydrogel. A two steps encapsulation procedure with varying stiffness and cell adhesion properties resulted in a completely different morphology. Mouse cells developed multicellular growth structures that resemble the villi of the intestine. Further analysis will be needed to confirm the identity of these structures. In summary, we were able to identify synthetic PEG-hydrogel culture conditions that allow single mouse cells to develop into three-dimensional structures that resemble mouse organoids.

### Development of Synthetic Extracellular Matrix Customized to Hybrid F1 Strain of Mouse Embryonic Stem Cells: Customization of Mechanical Property

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Recently, biomimetic, defined 3D synthetic extracellular matrix (ECM) for supporting maintenance of stem cell self-renewal *in vitro* in the presence or absence of leukemia inhibitory factor were established by the combination of poly(ethylene glycol) (PEG)-based 3D scaffolds with ECM analogs activating integrin heterodimers. However, the developed 3D synthetic ECM was restricted to the ESCs with inbred 129/Ola strain and there were no reports about development of 3D synthetic ECM customized to the ESCs with hybrid F1 strain. Accordingly, this research was conducted to customize mechanical property of 3D PEG-based scaffolds in the development of 3D synthetic ECM supporting self-renewal of ESCs with hybrid C57BL6 x DBA (B6D2) hybrid F1 strain. For these, hybrid B6D2F1 ESCs were cultured for 7 days in different mechanical properties of hydrogels constructed by combining vinylsulfone (VS)-functionalized 3-, 4- or 8-arm PEG of different concentration (7.5, 10, 12.5 or 15% (wt/v)) with dicystein-containing peptides with an intervening matrix metalloproteinase-specific cleavage site, and cellular proliferation assay and characterization were then conducted in the cultured ESCs. As the results, hybrid B6D2F1 ESCs showed the highest proliferation activity in 3-arm 10.0% PEG-based hydrogel and ESCs inside 3-arm 10.0% PEG-based 3D hydrogel showed sufficient transcriptional expression of self-renewal-related genes as ESCs on 2D MEF-coated or -free culture plate. Furthermore, alkaline phosphatase activity and expression of self-renewal-related proteins were detected strongly without any decrease in ESCs inside 3-arm 10.0% PEG-based hydrogel. These results demonstrate that mechanical property derived

from 3-arm 10.0% PEG-based hydrogel can support sufficiently self-renewal of hybrid B6D2F1 ESCs.

### Free Radical Polymerization for the Controlled and Facile Production of a Cell Repellent and Antifouling Surface in 2- and 3D Systems

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**Introduction:** Controlled radical polymerization techniques are versatile methods for providing surface functionalization with a wide variety of monomers; this has not been fully exploited in scaffold functionalization. Many of these reactions utilize high concentrations of potentially toxic catalysts and solvents. However, a modified form of ATRP, activators regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP), is performed in aqueous conditions and has a lower risk of toxic contamination.

**Materials and Methods:** Silicon wafers and poly-ε-caprolactone (PCL) were functionalized with a polymerization initiator, and the PCL-initiator was then electrospun into a fibrous mat. An antifouling bottlebrush coating of poly ethylene glycol methyl ether methacrylate (pOEGMA) was grafted from these 2D and 3D surfaces using ARGET ATRP. A biotinylated monomer and a drug releasing moiety were further incorporated into the bottlebrush conveying the potential for multi-functionalization through streptavidin-coupling and monomer selection. Antifouling properties were assessed with cell- and protein-binding assays.

**Results:** Reproducible polymerization was confirmed and characterized. A dry brush thickness on the 2D surface of 6.20 nm (+/- 0.04 nm) was observed with atomic force microscopy and spectroscopic ellipsometry and corroborated with X-ray photoelectron spectroscopy (XPS). Fluorescent labelling of histological sections, water contact angle measurement and XPS confirmed surface functionalization from 3D scaffolds. A profound antifouling effect was produced with negligible cellular adhesion at 7 days.

**Conclusions:** A versatile and effective antifouling coating of pOEGMA has been grafted from 2D- and 3D surfaces with applications in tissue engineering.

### Synthesis and Characterization of Injectable Bionanocomposite Hybrid Scaffolds with Responsive Control for Osteochondral Tissue Regeneration

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The regeneration of articular cartilage tissue has remained an elusive target due to the avascular and complex heterogeneous structure of hyaline cartilage. The major focus thus far has been in developing implantable scaffolds that require open surgery. However, recent efforts have sought to develop novel, minimally invasive therapies capable of forming *in situ*, and promoting close contact with existing tissue. Thermogelling polymers that have a lower critical solution temperature (LCST) close to body temperature, such as poly(N-isopropylacrylamide) (PNiPAAm), are promising candidates as injectable *in situ* forming hydrophobic hydrogels capable of delivering viable encapsulated cells to the defect. The next grand challenge in the development of injectable scaffolds is to endow them with spatiotemporally-varied and externally-controlled signalling to guide the regeneration of heterogeneous and multi-tissue defects. This research reports on the synthesis and characterization of novel injectable, dual-gelling bionanocomposite hydrogels composed of PNiPAAm, degradable polyamidoamine (PAMAM) crosslinking macromers, and functional hybrid inorganic iron oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles capable of responding to an external magnetic field to stimulate and control cell activity *in situ* spatiotemporally. The incorporation of amine-functionalized magnetic nanoparticles into

the injectable hydrogel system did not alter the LCST but did alter the kinetics of the chemical gelation process. Moreover, SQUID analysis demonstrated that the bionanocomposite hydrogels exhibit paramagnetic behaviour under strong magnetic fields, and tangential force measurements showed the efficacy of utilizing an externally-controlled magnetic field to mechanically stimulate encapsulated cells. Finally, the *in situ* delivery of viable cell populations encapsulated in the bionanocomposite hydrogel was demonstrated.

### The Model of Vascularized Three Dimensional Heart Tissue for Drug Evaluation

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**Background:** Creation of 3-D tissue model similar to a biological tissue using tissue engineering method are expected as an alternative approach to 2-D culture or animal test in a research for developing a new medicine. We have developed 3-D heart tissue with perfusable blood vessels *in vitro* using the cell sheet technology. Present study assessed the possibility as 3-D tissue model by performing drug evaluation in the heart tissues.

**Methods:** Cardiomyocytes co-cultured with endothelial cells were harvested as cell sheets from temperature-responsive culture dishes. Triple-layer cell sheets were overlaid on the vascular bed with an artery and a vein *in vitro*. Layered cardiac cell sheets were maintained in a bioreactor system.

**Results:** After perfusion culture, isoproterenol was administered to the engineered heart tissues from the artery. The drug efficacy was measured by bioluminescence imaging, electrical potential, and pressure. Bioluminescence imaging showed that adenosine triphosphate activity of the cardiac tissue improved by isoproterenol administration. Electrograms demonstrated increasing frequency after the isoproterenol administration. Moreover, blood pressure measurement indicated decreasing arterial pressure after the isoproterenol administration.

**Conclusions:** It is possible to analyze the pharmacological effects as metabolism and blood pressure as well as action potential by using our vascularized heart tissue model. We believe that *in vitro* vascularized 3-D tissue model will give a new possibilities for developing a new medicine.

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### Development of Three-Dimensional System for Culturing Notochordal Cells

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**Background:** The notochord plays a crucial role in the formation and patterning of axial skeleton. Recently, notochordal cells (NCCs) have drawn increasing attentions from developmental biology, stem cells research and tissue engineering as they are important in development and maintenance of NP. However, it is difficult to maintain their survival and phenotype in 2D culture for long term, making it difficult to understand their fate and functional maintenance during intervertebral disc development.

**Objective:** To develop a better culture system that is able to maintain the survival and phenotypes of NCCs.

**Methods:** NCCs were isolated from Foxa2<sup>mNE</sup>-Cre/Z/EG heterozygous embryos by identifying EGFP signal using FACS. FACS-sorted NCCs or notochord segments at the anterior, trunk and posterior regions were microencapsulated in type I collagen microspheres and cultured for up to 1 month before characterization for survival and phenotype maintenance.

**Results:** We demonstrated that FACS-sorted NCCs maintained their survival within the collagen microspheres for at least 4 weeks. However, most GFP cells showed elongated morphology. When the

notochord segments were cultured in collagen microspheres, clusters of round GFP positive cells co-expressing with major NCC markers were found after 1 month, suggesting that NCC phenotype was maintained. Longer period of culture and the role of niche cells present in the notochord in supporting the NCC maintenance are warranted.

**Conclusion:** Type I collagen microspheres present a potential 3D culture system for FACS-sorted NCCs or NC segments.

### P11a Nanofibrous Hollow Microspheres Support Chondrogenic Differentiation of Bone Marrow Mesenchymal Stem Cells and Enhance Cartilage Regeneration

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Bone marrow mesenchymal stem cells (MSCs) have been recognized as a viable cell source for cartilage regeneration. While TGF- $\beta$  mediated MSC chondrogenesis is well established in *in vitro* pellet culture, an appropriate cell and growth factor carrier is needed for clinical translation. We previously developed novel poly(L-lactic acid) (PLLA) nanofibrous hollow microspheres (NF-HMSs), which could be used as an excellent injectable chondrocyte carrier for cartilage repair. In this study, we hypothesized that these microspheres characterized by highly porous and nanofibrous structure with an open hole on the shell, could support the chondrogenic differentiation of MSCs and enhance cartilage tissue formation. To test this hypothesis, rabbit MSCs were mixed with NF-HMSs and induced towards chondrogenic differentiation *in vitro*. Aiming for future clinical application, we intend to avoid long-term *in vitro* induction and the deliveries of both TGF- $\beta$  and cells. In this work, a mixture of MSCs, NF-HMSs and TGF- $\beta$ 1 incorporated PLGA nanospheres was injected into the subcutaneous pockets of nude mice to generate neo-cartilage tissue *in vivo*. Both *in vitro* and *in vivo* results showed that TGF- $\beta$ 1 successfully induced chondrogenic differentiation of rabbit MSCs, which were demonstrated by positive staining of Safranin-O for GAG and immunohistochemical staining for collagen type II. Compared with MSCs alone group, histological examination and biochemical analysis revealed significant bigger size and higher quality of the cartilage tissue in NF-HMSs group. These results indicate that nanofibrous hollow microspheres are an excellent cell carrier to support chondrogenic differentiation of MSC and enhance cartilage tissue regeneration.

### Behavior of Bone Marrow Mesenchymal Stem Cells in Injectable Gelatin-Hyaluronan Matrices

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Treating cavitary defects contained within otherwise healthy tissue requires an injectable matrix capable of filling the defect and providing the functions of the lost extracellular matrix. Gelatin-hydroxyphenylpropionic acid (Gtn-HPA) and hyaluronic acid-tyramine (HA-Tyr) are injectable liquid formulations of natural biopolymer matrices that are capable of undergoing covalent cross-linking *in vivo*. Each of the two matrices possesses distinct attributes that may be desirable for their roles in cavitary defects: HA-Tyr offers relative mechanical robustness and the biological benefits of HA, while Gtn-HPA provides the necessary cell adhesion ligands to promote cell attachment and migration. We explored different HA-Tyr/Gtn-HPA matrix composites with the goal of identifying a ratio suitable for an osteogenic application. The mechanical properties, cell viability, proliferation, migration, and osteogenic differentiation potential were studied using goat bone marrow mesenchymal stem cells (bMSCs) incorporated in the gels containing

different ratios of Gtn-HPA and HA-Tyr. The data showed that in HA-Tyr rich gels, the gelation time was longer and compressive modulus was higher. While in Gtn-HPA rich gels, more cells were elongated and spread, and adhered to the matrix. Cell viability was greater and the cells proliferated faster in Gtn-HPA rich gels. In the migration assay employing PDGF-BB as a chemoattractant, a substantially greater number of cells migrated into the high Gtn-HPA ratio gels, while no cells entered the pure HA-Tyr matrix. In addition, larger and a greater number of cell nodules were observed and more calcium deposition was detected in the high Gtn-HPA ratio gels after 28 days of osteogenic induction.

### A Human Skin Equivalent Wound Model for Examining Novel Wound Healing Therapies

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**Objective:** The development of improved *in vitro* and *ex vivo* skin models is critical to advancing our understanding of human skin repair and regeneration. In particular, improved models are required to facilitate the development and pre-clinical evaluation of wound therapies. This is important as wounds are becoming an increasing burden to health care systems globally. Numerous difficulties are associated with the conduct of pre-clinical studies related to skin and wound repair. Pigs have previously been used due to their human-like mode of healing, but the expense and logistics related to their use also renders them sub-optimal. In view of this alternatives are urgently required to advance the field.

**Methodology:** The experiments reported herein were aimed at critically evaluating and refining a 3-dimensional *ex vivo* de-epidermised dermis (DED) human skin equivalent (HSE) model for the pre-clinical evaluation of novel wound therapies and reagents.

**Results:** Having established that wounded DED-HSEs do in fact "heal", we tested the effect of two novel wound healing therapies. Taken together the data demonstrate that these new models will have wide-spread application for the generation of fundamental new information on wound healing processes and will also hold potential in facilitating pre-clinical optimization of dosage, duration of therapies and treatment strategies, prior to clinical trial.

**Significance:** Of note, the utilization of these models will decrease our reliance on the use of animals for scientific experimentation, not just for wound healing studies, but also for the development and evaluation of consumer products directed at maintaining skin integrity.

### Hybrid Elastin-like Polypeptide-Polyethylene Glycol Hydrogels for 3D Cell Culture with Tunable Matrix Stiffness and Cell Ligand Density

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Protein engineered, biocompatible, elastin-like polypeptide (ELP) hydrogels have been used as biomimetic materials to study fundamental cell-matrix interactions and to achieve tissue regeneration. An advantage of this system is that the biochemical and mechanical properties can be easily and independently tailored; however, a disadvantage is that the lower critical solution temperature of ELP at  $\sim 34^\circ\text{C}$  leads to an opaque ELP hydrogel at physiological temperature. This opacity poses an obstacle to microscopic observation of the morphology and behavior of 3D encapsulated cells. To overcome this limitation, we designed a hybrid ELP-polyethylene glycol (PEG) hydrogel system which undergoes fast gelation in aqueous solution using the crosslinker tris(hydroxymethyl) phosphine (THP). Compared with pure ELP hydrogels, the incorporation of the hydrophilic PEG component results in smaller hydrophobic ELP aggregates at  $37^\circ\text{C}$ , as revealed by Coherent Anti-Stokes Raman Scattering (CARS) microscopy. This results in less light scattering and hence higher light transmittance and better imaging contrast at greater depths

into 3D cell cultures. In addition, we demonstrated that similar to pure ELP hydrogels, the matrix stiffness and cell-adhesion ligand density could be tuned independently in this hybrid ELP-PEG hydrogel. Encapsulated human fibroblasts demonstrated high viability (>98%) after 7 days of culture and adopted a more spread morphology in gels with lower matrix stiffness and higher RGD ligand concentration. The good light transmittance, excellent cytocompatibility, as well as independently tunable biochemical and mechanical properties make this newly designed ELP-PEG hybrid gel an ideal platform for future cell-matrix interaction studies.

#### Modulation of the Secretome of hBMSCs by Tailoring the Macromolecular Gradient In Hydrogels to Generate Tissue-to-Tissue Interfaces

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Tissue-to-tissue interfaces exhibit structural, biological and chemical gradients serving a wide range of physiological functions (e.g.:load transfer mediation between two adjacent tissues and sustaining cellular communications to retain tissue's homeostasis) [1]. Cells have the capacity to sense and respond to physical and chemical stimulus, performing better when cultured in three-dimensional (3D) environments. Spatial and temporal 3D gradient hydrogels (GHs) better resemble the natural environment of cells in mimicking their extracellular matrix [2]. We hypothesize that differential functional properties can be engineered by macromolecules modulation in 3D GH systems. The aim is to assemble the GHs and evaluate the suitability for human Bone Marrow Stem Cells (hBMSC's) encapsulation. GHs solutions were prepared by blend of macromolecules: hyaluronic acid (MW 851 KDa-1.15 MDa, Lifecore) and collagen type II (MW 110-200 KDa, Symatase) at different ratios. Hydrogels were fabricated into moulds; higher ratio solutions assembled at the bottom and two other solutions consecutively on top-of-each-other. FITC-labelling macromolecules confirmed the gradient construction. AFM proved the different young modulus along the gradient. hBMSC cultures, P3 at 106 cells/ml within GHs were observed under confocal microscopy by Live/Dead<sup>®</sup> staining showing good viability. Secretory cytokine measurement for pro-inflammatory and angiogenesis factors was performed using ELISA. 3D GHs platform made of different macromolecules showed to be a suitable environment for hBMSC's supporting high cell survival and exhibited biofunctionality. A suitable 3D platform to modulate the secretome of hBMSC's, concerning their pro-inflammatory and angiogenic secretion, can be achieved by tailoring the macromolecular gradient environment.

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#### Efficient Formation of Size-regulated Hepatocyte Aggregates on Oxygen Permeable Microwell Sheets and the Size-dependency of Their Metabolic Capacities

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Three-dimensional hepatocyte aggregates are expected as a useful *in vitro* model for a number of assays. One of the common issues for hepatocyte aggregate formation is critical loss of oxygen supply to the culture system, because of the high oxygen consumption of hepatocyte and of the low gas permeability of culture environment. Here we report efficient method to obtain size-controlled hepatocyte aggregates using an oxygen permeable poly-dimethylsiloxane

(PDMS)-based microwell sheet, which enables direct oxygenation to the culture environment. We prepared PDMS-based microwell sheet in different sizes and coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer to prevent cellular attachment. Rat hepatocytes were cultured and formed size-controlled aggregates on their surface. Our microwell enhanced inoculum cellular density up to four times higher and accelerated aggregation compare to the conventional polystyrene tissue culture plate surfaces (TCPS). Less than 88  $\mu\text{m}$  aggregates were overall higher functional than other sizes aggregates or monolayer culture in terms of several different sub-families of P450s. This highly-metabolic size ranges of hepatocyte aggregates are smaller than the limited size decided by the oxygen diffusion and consumption ( $\sim 150 \mu\text{m}$ ). In addition, irinotecan toxic assays showed interesting size-dependency and more than 52  $\mu\text{m}$  aggregates were effective for detoxification by CYP3A2. These results demonstrates the importance of the selection of hepatocyte aggregates sizes for accurate pharmacological and/or toxicological studies simply because observed metabolic rates of exogenous chemicals are the results of the diffusion, reaction and production of both the chemicals and their metabolite in the aggregates.

#### Improving the Multipotency of Mesenchymal Stem Cell During *In Vitro* Cell Expansion within the Grooves of a Micro-structure Pillar Array

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Multipotent stem cells such as mesenchymal stem cells (MSCs) have been extensively studied for the past decades to promote tissue repairs. Like any other cells, MSCs are home to 3-dimensional (3D) microenvironments where they constantly sense and respond to their environmental cues which governs their appropriate self-renewal and controlled differentiation. In regenerative medicine, donor MSCs are however culture on a flat substrate to facilitate cell expansion for achieving high number of cell population for the intended tissue repairs. The culturing on a flat substrate can possibly change their cellular morphology which affect their multipotency abilities. In this study, we investigated the effect of expanding MSCs within the grooves of micro-structural pillars (circles, rectangles and grills) on their multipotency. The grooves within the micro-structural pillars had demonstrate their capabilities to manipulate the MSCs morphology. Further investigation showed that the use of these MSCs expanded in different 3D micro-environment carried different capacities for osteogenic and chondrogenic differentiation. These findings gained us insights in creating novel cell culture platform for MSCs to improve their multipotency during cell expansion in the field of regenerative medicine.

#### *In Vitro* Models of SMCs Under Cyclic Mechanical Stimulation: a Comparative Study between 2D and 3D

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Herein, 2D (cell monolayers) and 3D (cellularized gels) cultures are directly compared as *in vitro* models for the investigation of the response of vascular smooth muscle cells (SMCs) to cyclic strain.

Human Umbilical Artery SMCs (HUASMCs) were cultured in monolayers and inside collagen gels cast in UniFlex<sup>®</sup> plates using specific molds and anchors. Uniaxial 7% cyclic strain was applied at 1 Hz for 2 and 5 days. For histology, 10  $\mu\text{m}$  sections were stained by Masson's trichrome. For immunofluorescence, samples were stained with phalloidin-rhodamine and DAPI. Western blot was used to detect  $\alpha$ -actin and calponin.

After 2 and 5 days of cyclic stimulation, an opposite cellular response was observed: cells aligned perpendicular to the strain direction in 2D cultures, parallel in 3D ones. The first behavior is quite peculiar since contrary to what occurs in arteries *in vivo* where SMCs align circumferentially, thus parallel to the strain, while it is similar to what happens during intima formation and in some pathological conditions. Moreover, the evaluation of the expression of  $\alpha$ -actin and calponin suggests that dynamic stimulation in 2D cultures had minimal influence on the synthesis of contractile phenotype markers while a significant effect could be observed for 3D models, pointing out that the dimensionality of the culture can strongly affect SMCs response to mechanical cues.

These results, together with further knowledge of SMCs behavior in different culture conditions, will contribute to the rational design of *in vitro* models conceived *ad hoc* for the investigation of specific physiological and pathological conditions.

### Engineering Regenerative Skeletal Muscle Tissues

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The ability to create engineered muscle tissues that mimic the structural, functional, and regenerative properties of native muscle would enable design of accurate *in vitro* models for pharmacological screening and promote regenerative therapies for muscle injury and disease. In this work, we aimed to engineer functional skeletal muscle tissues capable of regeneration *in vitro* and survival, vascularization, and maturation *in vivo*. We performed optimization of neonatal and adult rat-derived cells to prime them for myogenic fusion within 3D engineered muscle environment. Rapid generation of myofibers from these cells provided a homing environment and generation of a robust pool of quiescent muscle stem cells (satellite cells (SCs)). Functionally, both neonatal- and adult-derived engineered muscles exhibited contractile force generation comparable to that of native postnatal muscles. Following 2-week implantation in a mouse window chamber model, these muscle tissues underwent robust vascularization and maturation. In response to *in vitro* injury induced by cardiotoxin (CTX), SCs in the neonatal-derived engineered muscle underwent robust activation, proliferation, and differentiation resulting in the progressive regrowth of muscle and a successful recovery in force generation and calcium handling, assessed non-invasively by using a calcium-indicator, gCaMP6. In contrast, adult-derived engineered muscles underwent only a moderate recovery when supplemented with growth factors. *In vitro* post-CTX functional recovery of different engineered muscles was found to directly correlate with the starting abundance of Pax7+ SCs, with lower initial SC numbers found in neonatal- vs. adult-derived muscles. Our studies demonstrate requisite roles of SC maintenance in engineering of functional and regenerative engineered muscles.

### Hypoxia and Load Enhances GDF6 Driven Discogenic Mesenchymal Stem Cell Differentiation But Alters Matrix Composition in Tissue Engineered Nucleus Pulposus Constructs

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**Introduction:** The niche of the intervertebral disc (IVD) is a harsh microenvironment that is subjected to mechanical load and hypoxic conditions. Investigation of these two environmental factors on cells to be used in repair/regeneration is important in assessing the potential efficacy of novel cell-based IVD therapies.

**Aim:** To investigate whether exposing adipose derived MSCs (AD-MSCs) supplemented with GDF6 to hypoxia (2%) or load (0.04 MPa, 1 Hz, 1 hour) or a combination of the two enhances discogenic differentiation and matrix formation.

**Methods:** Human AD-MSCs (n=3) were seeded in type I collagen hydrogels on Bioflex<sup>®</sup> culture plates and separated: 1). Normoxia; 2). Normoxia+load; 3). Hypoxia; 4). Hypoxia+load.

Hydrogels were cultured in a differentiating media containing GDF6 (100 ng/ml) and loaded groups exposed 0.04 MPa, 1 Hz, 1 hour/day.

QPCR analysis of discogenic differentiation markers was undertaken, along with analysis of sGAG content (DMMB). Additionally, micromechanics of the constructs were analysed using AFM.

**Results:** AD-MSCs treated with GDF6 exposed to all groups resulted in enhanced discogenic differentiation compared to normoxia. Hypoxia + load increased PG content as shown by the greatest sGAG and upregulation of ACAN. However, the combination of treatments upregulated COL2A1, resulting in the lowest ACAN:COL2A1 ratio. Micromechanical analysis showed that constructs treated in hypoxia + load had a stiffer matrix composition than constructs cultured in normoxia.

**Discussion:** Whilst hypoxia and load enhance discogenic differentiation, the matrix composition and micromechanics are stiffer compared to constructs in normoxia. Therefore in addition to gene expression, these characteristics must be analysed to ensure that there is synthesis of an appropriate functional matrix.

### Chemically Defined Synthetic Substrates for Controlled Self-assembly of Stem Cell Aggregates

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Recent studies show that three-dimensional (3D) aggregates of stem/progenitor cells can self-assemble into organoids resembling native organs in their composition, structure, and function. However, pervasive use of ill-defined matrices (e.g., Matrigel) in current approaches<sup>1</sup> limits reproducibility and impedes understanding of mechanisms dictating organoid assembly. Here we used chemically defined substrates composed of self-assembled monolayers (SAMs) to exert spatiotemporal control over a human embryonic stem cell (hESC) aggregate self-assembly process.

hESCs were cultured on mixed SAMs of COOH- and OH-terminated alkanethiols adsorbed in various patterns onto gold surfaces. Cyclic RGDfK and RGDfC (cycRGD) peptides were coupled to COOH groups using carbodiimide chemistry to form non-labile amide linkages and labile thioester linkages, respectively, between peptide side chains and SAMs.

Dynamic cell-substrate adhesion was a strong determinant of self-assembly. Patterned substrates presenting cycRGD via labile thioester linkages enabled reproducible self-assembly of initially 2D hESC populations into 3D aggregates, while substrates presenting cycRGD via non-labile amide linkages prohibited self-assembly. Modulating cycRGD density and Rho kinase signaling changed self-assembly kinetics, suggesting cellular adhesion and contractility are critical to this process. Shape of resulting aggregates was controlled by geometry of initial 2D patterns. Ongoing studies indicate that the interior structure of self-assembled aggregates is fundamentally distinct from that of aggregates generated by forced centrifugation during lineage-specific differentiation. The tunability and reproducibility of this method will allow for critical improvements upon current methods for organoid formation.

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### Dynamic Shape Changes Have a Greater Effect on Myogenic MSC Differentiation than Static Shape Generated by Biomaterials with Differentiation-supporting Nanoscale Stiffness

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A key stepping stone for developing mesenchymal stromal cell (MSC)-based therapies is to better direct MSC fate. An emerging method for steering MSC differentiation is controlling cell shape. Other methods to induce MSC lineage commitment use biomechanical forces and biomaterials with lineage-specific elasticity. However, little is known how biomaterial and biomechanical cues control cell shape, and how this regulates differentiation. As a model system, we used cyclic stretch for dynamically inducing elongated MSC shapes, which are associated with myogenic differentiation. We compacted soft collagen type I hydrogels into fibrous sheets with a fine-tuned nanoscale stiffness known to additionally support myogenic differentiation. This established a system of two competing biophysical cues with dynamic and static effects on shape. Calculating quantitative shape descriptors, we demonstrated that biomaterials dictated specific baseline MSC morphologies in a substrate type- and concentration-dependent way. However, the effects of cyclic mechanical stretch dominated over biomaterial-associated effects. Upon relieving cyclic biomechanical tension, MSCs reversed back towards the biomaterial- dictated shape, suggesting that biomechanical forces can override the effects of biomaterials on shape. Shape and expression of myogenic markers significantly correlated, which indicated their intertwined nature. MSCs experiencing mechanically-induced dynamic shape changes expressed higher levels of specific marker genes than MSCs with static shapes defined by biomaterial cues. Comprehensively, this suggests that dynamic shape changes are more effective than static shape in promoting myogenic differentiation. A promising future avenue may focus on developing biomaterials which harness the *in vivo* dynamic biomechanical environment for shape instructive signals.

#### Self-Assembled Peptide Gels for Intervertebral Disc Tissue Engineering

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Lower back pain (LBP) has been estimated to cost the UK economy £12 billion per year. Aetiology varies but intervertebral disc (IVD) degeneration is strongly associated with LBP. IVDs consist of the gelatinous nucleus pulposus (NP) surrounded by the tougher annulus fibrosus. The main IVD role is to dissipate loads from the spine. With age, a degeneration cascade occurs within the IVD which often leads to LBP.

Due to ineffective current treatments coupled with an aging population, a new approach to LBP management is urgently required; cell-based therapies are regarded to hold particular promise. Therefore a self-assembling peptide hydrogel (SAPH) was researched for its potential as a cell delivery system and scaffold for NP tissue engineering. SAPHs are readily modifiable, biocompatible and biodegradable<sup>3</sup>. The culture of bovine NP cells and the more clinically relevant human bone marrow mesenchymal stem cells (h-BMMSCs) in the SAPH were investigated.

Rheology determined that the SAPH was injectable and had comparable mechanical properties to the native NP. Encapsulated bNPCs displayed high viability, produced the appropriate extracellular matrix components (ECM) and maintained the NP phenotype. When GDF-64 was used to induce a NP-like phenotype from h-BMMSCs, the SAPH supported their culture and NP-associated ECM components were deposited. Interestingly when h-BMMSCs were cultured in SAPHs in absence of a NP inducing growth factor, gene expression profiles determined that cells were differentiated towards a NP-like phenotype. This suggested that the SAPH had a crucial influence on determining h-BMMSC fate towards a discogenic phenotype.

#### Surface Modification of a POSS-Nanocomposite Material to Enhance Cellular Integration for Tissue Engineering Applications

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Polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane (POSS-PCU) is a nanocomposite material with increasing applications in tissue engineering. Integration of synthetic implants can be problematic but can be improved by various topographical modifications. In this study, the POSS-PCU nanocomposite was modified by dispersion of the porogens; sodium bicarbonate (NaHCO<sub>3</sub>), sodium chloride (NaCl) and sucrose onto the material surface. The principle aim was to increase surface porosity, thus providing additional opportunities for improved cellular and vascular ingrowth. To assess the effect of the porogen addition, the material's mechanical strength, surface chemistry, and wettability were assessed and any change in surface porosity was characterised by scanning electron microscopy (SEM) and permeability experiments. Finally a small scale *in vivo* experiment was performed in a mouse model to examine the hypothesis that an increased surface porosity would facilitate improved cellular and vascular integration. As expected results demonstrated a good correlation between the size of the porogen used and increased porosity. No alteration in surface chemistry and wettability was detected with only modest changes in mechanical properties. Permeability experiments revealed improved pore interconnectivity in each dusted sample. *In vivo* experiments demonstrated greatly improved cellular in growth and integration with host surrounding tissues including vascular ingrowth. These experiments represent a positive advancement for POSS-PCU, and since the level of porosity required depends greatly on the desired application, this technique increases the options available for current and future POSS-PCU applications in tissue engineering.

#### Engineering Biointerfaces to Select and Enrich Cancer Stem Cells

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Cancer stem cells (CSCs) have been identified and validated in solid tumors and cell lines which exhibit capacities for self-renewal, proliferation, and tumor initiation. However, the most common method for CSC selection utilizes fluorescence label or magnetic-activated cell sorting requires antibody and complex procedure, and mostly yet low number of viable cells.

CD133 and CD44 are most widely used markers on the selection of CSCs, besides, Hyaluronan mediated CD44 interaction promotes tumor progression has been observed. Herein, series of multilayer films with hyaluronan (HA) were used to engineer biointerface and to mimic CSC niches.

Layer-by-layer deposition of multilayer films was monitored by quartz crystal microbalance. MTT test and live/dead assay were used to evaluate toxicity of cells on multilayer films. Cell morphology and colony formation on different surfaces were investigated. Furthermore, after culture on different multilayer films, cancer stem cell markers expression was determined by using flow cytometry and the drug-resistance of doxorubicin were also evaluated. It is showed that cells culture on engineer biointerface displayed best colony formation ability, high CSCs marker expression and higher chemo-resistance than control group. Therefore, this study designed a label-free selection and enrichment surface for CSCs may serve as a new strategy for cancer drug testing.

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#### Decellularized Sphincter Extracellular Matrix for Skeletal Muscle Restoration

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The majority of the patients with stress urinary incontinence (SUI) suffer from sphincter muscle dysfunction due to muscle and/or nerve damage. The long-term efficacy of SUI therapies is often limited and accompanied by significant side effects putting the focus on alternative therapies. To address the newly discovered importance of cell-extracellular matrix (ECM) interactions, we investigated the use of ECM-derived sphincter muscle to improve skeletal muscle engineering. Previously, we established a simple mechanochemical approach for the decellularization of whole sphincter apparatus (*i.e.* including smooth and skeletal muscles) maintaining the biochemical composition of the native tissue. Whole acellular urethra bioscaffolds were microdissected and cryo-sectioned to obtain ECM-derived bioscaffolds containing only smooth or skeletal ECM or both. ECM-derived gels were produced through acidic pepsin partial digestion. Muscle progenitor cells (MPCs) were used to repopulate these ECMs. Through H&E staining, calcein AM assay and Ki-67 immunostaining it was confirmed that MPCs adhered, spread and proliferated in the ECM-derived bioscaffolds and gels. Importantly, the urethra ECMs provided the adequate environment for the fusion of muscle fibers expressing desmin, MyHC and  $\alpha$ -actinin, as evidenced through immunofluorescence staining and RT-PCR gene analysis. The acellular urethra ECMs produced here provided the essential biochemical and topographical cues for MPCs to fuse and produce new skeletal muscle fibers. These results will be used to define the optimal matrices for skeletal muscle engineering. We envision that this approach represents a simple “off-the-shelf” product for SUI-related therapies. *In vivo* studies using nude mice will be performed in the near future.

#### Cyclic Uniaxial Strain on a Chip: a Novel Platform to Generate Functional 3D Cardiac Microtissue

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*In vivo*, cardiomyocytes undergo a cyclic mechanical strain deriving from the beating of the heart, which is known to play a role in activating mechano-sensitive signaling pathways. Thavandiran *et al.* recently showed that cardiac differentiated pluripotent stem cells can functionally self-assemble in mature cardiac micro-constructs after exposure to uniaxial auxotonic stress [1]. Such result confirmed the importance of mechanical stimulation for the maturation of the cardiac tissue.

In this study, we aimed at generating novel perfused microfluidic devices which allow fine microenvironmental control over the mechanical stimulation to engineer reproducible and highly functional 3D cardiac microtissues. To this purpose, we developed an innovative PDMS-based micro-bioreactor able to recapitulate the physiological strains experienced by cells in the native myocardium (range 10–15%). Briefly, an array of posts was implemented to confine and culture cell-laden gels, and a pneumatic actuation system was embedded to induce uniaxial cyclic strains to the 3D constructs (generated by neonatal rat or human pluripotent-derived cardiomyocytes).

Stimulated cardiac constructs expressed higher levels of Cx43 and myosin light chain 2v compared to the 3D static culture condition, suggesting superior electrical connection among neighboring cells and cardiac maturation. Moreover, the cyclic mechanical stimulation promoted the spontaneous synchronous beating of the constructs. Electric pacing experiments further demonstrated the superior maturation of the stimulated constructs, thus confirming the suitability of the platform for both high-throughput pre-clinical drug screenings and physio-/pathological states investigations.

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#### Using Tissue Engineered 3d Co-culture Models to Investigate the Effects of Photochemical Internalisation on Nervous System Cells

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Photochemical Internalisation (PCI) is a novel drug delivery technology in which low dose photodynamic therapy (PDT) can selectively rupture endo/lysosomes by light activation of membrane-incorporated photosensitisers, facilitating intracellular drug release in the treatment of cancer. For PCI to be developed further, it is important to understand whether nerve damage is an impending side effect when treating cancers within or adjacent to nervous system tissue. Our previous work used tissue engineered 3D co-culture models to investigate PDT treatments that could destroy cancer cells without killing neurons [1], and revealed the biochemical pathways responsible for this phenomenon [2]. These engineered neural tissue models maintain neurons and glial cells within a spatially and mechanically relevant 3D environment in which cells can interact with each other and adopt phenotypes that mimic their behaviour *in vivo*. The aim of this study was to investigate the effects of PCI agents on nervous system cells in 3D co-culture. Cells were organised within collagen hydrogels and treated with combinations of photosensitisers, chemotherapeutic agents and light. The sensitivity of neurons and glia to PCI treatments was investigated and compared to relevant cancer cells in order to identify approaches that minimise nerve toxicity.

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#### Biomimetic Polyurethane Scaffolds Guiding the In Vitro Behavior of Cardiac Stem Cells

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Cardiac regeneration is guided by cardiac progenitor cells (CPCs) and affected by the cardiac extracellular matrix (ECM). Laminin-1 (LN1), typical of developing heart, is over-expressed in pathological heart, promotes CPC proliferation and viability [1]. Scaffolds for myocardial Tissue Engineering (TE) should display biomimetic properties respect to cardiac ECM and elastomeric properties.

In this work, a thermoplastic polyurethane (PU) was synthesized from poly( $\epsilon$ -caprolactone) diol (Mn=2000 Da), 1,4-budandiisocyanate and L-lysine ethyl ester dihydrochloride [2]. Bi-layered scaffolds with 0°/90° lay-down pattern were prepared by additive-manufacturing technique [2]. Functionalisation was performed in two steps: 1) acrylic acid grafting/polymerization by Argon Plasma treatment; 2) carbodiimide-mediated grafting of LN-1 and gelatin (G). PU scaffolds showed a mean fibre diameter of 152 ± 5  $\mu$ m and mean spacing of 505 ± 5  $\mu$ m. FTIR-ATR analysis of protein-coated scaffolds showed higher intensity of the absorption bands at 3370 cm<sup>-1</sup> (-OH and -NH stretching) and 1650 cm<sup>-1</sup> (amide I).

Contact angle decreased from 90° for PU to 60–65° for G- and LN1-PU. XPS analysis showed successful acrylic acid grafting/polymerization and protein grafting. Scaffolds were degraded *in vitro* by lipase (0.3 mg/ml) in 3 weeks. CPCs proliferation on LN1-PU scaffolds was higher than on PU and G-PU scaffolds, increasing from 8,18% on day 7 to 11,8% on day 14. LN1-functionalization stimulated CPC differentiation into cardiomyocytes and endothelial cells. Dynamic culture also affected CPC behaviour.

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### A 3D Culture System for Osteoarthritis Chondrocytes

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Chondrocytes from cartilage tissues of osteoarthritis (OA) are abnormal cells that lost their function in maintaining the cartilage matrix. These cells are described as “dedifferentiated”. Similarly, dedifferentiation is also observed in functioning mature chondrocytes upon monolayer culture, and it greatly affects the outcome of engineered tissues. In order to engineer functional cartilage tissues, a 3D culture platform with the ability to resume the phenotype of dedifferentiated cells and support redifferentiation is of great importance. We have developed in our lab a collagen microencapsulation technique that entraps cells in a condensed nanofibrous collagen meshwork, forming solid cell-collagen microspheres, which are a robust template for both mature cells and differentiating cells. Specifically, this system has been shown to support chondrogenic differentiation of stem cells and preserve the phenotypic characteristics of the subsequent chondrogenic lineages. These results prompted us to hypothesize that the system could also be appropriate for OA chondrocytes. In this study we aim to investigate the maintenance of chondrocytic phenotypes of chondrocytes isolated from OA patients in the 3D collagen microsphere system by taking reference to pellet and monolayer cultures. Results has been shown to be beneficial for OA chondrocytes phenotype maintenance and redifferentiation. OA Chondrocytes cultured in such way have a better expression of chondrogenic markers, especially in expressing SOX9, which is the master regulator in chondrogenesis. With such benefits of collagen microencapsulation, chondrocytes isolated from OA patients could possibly be turned back to functional cells and serve various purposes such as drug screening models, pathology models and even implantable constructs.

### Fabricating Glycosaminoglycan-based Microribbon-like Hydrogels as Injectable Macroporous Scaffolds for Tissue Engineering

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Hydrogels have been widely used for fabricating tissue engineering scaffolds. However, most hydrogels are nanoporous, whereas macroporosity is desirable for efficient nutrient diffusion, cell proliferation and matrix deposition. Second, few hydrogels developed-to-date allow independent tuning of niche cues. This makes it difficult to decipher complex cell-niche interactions. To overcome these limitations, we have recently reported a method to fabricate gelatin hydrogels into microribbon-like structures, which can be subsequently crosslinked into 3D macroporous scaffolds for direct cell encapsulation. The goal of this study is to develop a method for fabricating microribbon-like hydrogels based on various glycosaminoglycans (GAGs) including Hyaluronic acid (HA) and chondroitin sulfate (CS) and heparin sulfate (HS). We first wet-spin maleimide functionalized polymer precursors with various chemical compositions (gelatin, PEG, HA and CS) into isopropanol bath, which was crosslinked by Dithiothreitol (DTT). The formed microribbons were then treated with aminoethyl methacrylate to allow

photocrosslinking among the microribbons to form 3D macroporous network. Additional biochemical ligands could be added onto microribbon surface via maleimide groups of polymer precursors before wet-spinning. Our fabrication process led to successful formation of microribbon-like hydrogels using HA, CS and HS. The mechanical stiffness, biochemical cues and macroporous of the microribbons and the formed scaffolds could be tuned independently. Unlike conventional hydrogels, these microribbon-based scaffolds could sustain up to 50% cyclic deformation without failing, and supported adipose-derived stem cell adhesion, spreading and proliferation in 3D. We envision these microribbons-based biomaterials could provide a novel injectable macroporous scaffold for engineering a broad range of tissue types.

### A Biomimetic Approach for Engineering Stratified Organization of Articular Cartilage by Recapitulating Biochemical, Biomechanical and Geometrical Factors Involved in Cartilage Tissue Development

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Articular cartilage is a multifunctional tissue with a highly organized structure which provides a lubricating surface for the gliding joint and a load bearing matrix attached to the underlying bone. During native cartilage tissue development, changes in biochemical, mechanical, and geometrical factors direct the formation of stratified structure of articular cartilage. In this study we attempted to simulate the zonal organization of articular cartilage by recapitulating biochemical, mechanical and geometrical factors involved in the tissue development. To this end human mesenchymal stem cells (hMSCs) were encapsulated in acrylate-functionalized lactide-chain-extended polyethylene glycol (SPELA) gels simulating cell density, stiffness and geometrical properties of different layers of articular cartilage. hMSCs encapsulated in a soft gel (80 kPa) and cultivated with a combination of TGF- $\beta$ 1 and BMP-7 growth factors differentiated to the superficial zone phenotype of articular cartilage. hMSCs encapsulated in a gel with 2.1 MPa stiffness and cultivated with a combination of TGF- $\beta$ 1 and IGF-1 differentiated to the middle/deep zone phenotype. hMSCs encapsulated in a 320 MPa gel, reinforced with nanofibers aligned perpendicular to the gel surface and loaded with TGF- $\beta$ 1 and HA showed the hypertrophic phenotype of calcified zone. The results of this work can potentially lead to the design of more clinically effective multilayer grafts mimicking the biochemical, biomechanical and geometrical properties of the native tissue for treatment of articular cartilage defects.

### Design and Characterization of an Injectable Scaffold with Controlled Gelation for Nucleus Pulposus Therapy

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Reversal of intervertebral disc (IVD) degeneration can have a potentially monumental effect on spinal health. As such, the goal of this research was to create an injectable cellularized alginate-based nucleus pulposus (NP) that will restore disc function. It is well documented that NP cells lose phenotype rapidly in standard 2D culture, and previous work by this lab has shown that NP cell loaded alginate hydrogels maintain cell phenotype under hypoxic culture. However, typical alginate gelation occurs instantaneously and limits injectability while increasing void space. Therefore, this study utilized a 2:1 ratio of CaCO<sub>3</sub> to glucono- $\delta$ -lactone (GDL) as an alginate cross-linker to slow gelation and increase potential as an injectable therapy.

Using the 2:1 ratio previously described, gelation testing was performed on 1% alginate with concentrations of 30, 60, and 90 mM CaCO<sub>3</sub> and 15, 30, and 45 mM GDL, respectively, with 10% CaCl<sub>2</sub> as the control crosslinker. Alginate construct characterization for all concentrations was performed via ultimate and cyclic compressive



testing over a 28 day degradation period in PBS, dehydration and swell testing, and albumin release kinetics.

Overall, the 30 and 45 mM GDL alginate concentrations presented the most viable option for use in further studies, with a gelation time between 10–30 minutes, low hysteresis over control, low percent change in thickness and weight under both PBS degradation and re-swell conditions, and stable mechanical properties over 21 days *in vitro*. Forthcoming studies include cell incorporation and response to the slow-gelling alginate, and further release kinetic quantification with varying protein sizes.

#### Evaluation of Gelatin-hydroxyapatite-alginate Microcapsule as a Building Block for Bone Tissue Engineering

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Microcapsulation have been used successfully for delivering cells to repair tissues. Microcapsules have been recently considered as building blocks in modular tissue engineering approach. In this study, alginate (A)-based microcapsules containing gelatin (G) and nano hydroxyapatite (nHA) were prepared by electrostatic method and used for encapsulating osteoblast-like cells (MG63 cells). Effect of the concentrations of hydrogel components (A: 0.5- 1.5%, G: 0-5%, nHA: 0- 1%, W/V percentage) were examined on proliferation and differentiation behaviors of the cells by using a full factorial experimental design technique (two levels with center point). MTT assay method was used to determine cell proliferation. The cell behavior inside microcapsules was evaluated by alizarin red for calcium deposition as well as Alkaline Phosphatase activity (ALP) of the cells. The MG63 cells showed a significantly higher proliferation (more than 24-fold) when the cells encapsulated in G-nHA microcapsules were cultured in T-flask as compared to the cells cultured in microcapsules without G and nHA. The results of alizarin red staining and ALP activity assay revealed that, in the presence of nHA, the microcapsules not only provide a substrate for cell growth but can also enhance the osteoblast-like cell differentiation. The results also showed that the parameters of nHA concentration and amount of G/A have maximum effects on ALP and cell proliferation, respectively. The study demonstrated that the G-nHA-A microcapsule has potential to be used in modular bone tissue engineering.

#### A 3d In Vitro Functional Model of Hepatogenic Differentiation

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End stage liver disease (ESLD) is a global health problem and clinical applied therapies are far from being satisfying. Even *in vitro* testing for new therapies is not easy to establish because mature hepatocytes in culture lose their specific function. In the last ten years the use of pluripotent stem cells as alternative source of differentiated cells is increasing.

We investigated the differentiation potential of human ASC (adipose derived stem cells) toward the hepatocyte phenotype on a 3D commercially available collagen scaffold in order to model the *in vivo* microenvironment, where cells are thought to maintain their function and differentiation. Differentiation was induced by selected growth factors and 2D cell plastic culture was used as control.

Differentiation was verified by detection of specific markers at different time points. PCR analysis after 2 months of culture revealed the presence of HNF4 $\alpha$  and CYP3A4. Immunostaining showed the presence of Thy-1, cKit and Ft13 on cell surface. Furthermore, intracellular albumin and cytokeratin18 was distributed throughout the scaffolds. Albumin was also found in the medium, indicating the capacity of the cells to release it. These markers were present in a higher amount as compared to the differentiation in 2D.

In future, this model system may be used (i) to test different types of drugs *in vitro* and (ii) to investigate its potential for liver regeneration in animal models of ESLD.

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#### Fusion Protein Tethered Calcium Phosphate Substrates for Bone Tissue Engineering

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The seek for biomaterials that facilitates initial cell adhesion followed by an enhanced osteogenic differentiation of stem cells is one of the main goals of bone tissue engineering. Here we designed a fusion protein to allow the tethering onto calcium phosphate based substrates, while exposing cell binding domains. For this purpose, the protein was engineered to contain the osteocalcin and fibronectin domains, allowing for excellent anchorage onto the calcium phosphate substrate while exposing the fibronectin domains. The designed substrates allowed increased initial cell adhesion and attachment, presenting spread morphology in the presence of the tethered protein. Due to the presence of osteogenic domains, at a later stage, osteogenic genes and proteins were significantly up-regulated. When implanted *in vivo* in a rat calvaria model, the results confirmed higher levels of new bone formation in the fusion protein containing substrates. Collectively, these results indicate that the use of fusion proteins such as osteocalcin-fibronectin that link via calcium phosphate recognition is an efficient tool to generate biomaterials with cell-attractive and osteogenic capabilities, showing its potential for bone tissue engineering.

#### Human Platelet-Rich Plasma Enhances Proliferation and Synthesizes sGAG Production of OA Chondrocytes in a Tissue-Engineered Construct

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Platelet-rich plasma (PRP) is a pool of endogenous biological growth factors that can improve the healing and regenerative capacity of multiple orthopaedic tissues. PRP supplemented biocompatible scaffolds is one of the current topic of interest in the field of musculoskeletal regenerative medicine. The goal of this study was to characterize the effect of human PRP in proliferation and sulfated-glycosaminoglycan (sGAG) production of osteoarthritic (OA) chondrocytes within a tissue-engineered (TE) construct. Collagen type I scaffolds were prepared by incorporating PRP (COL I/PRP) in a 1:1 ratio. 100 mg/mL CaCl<sub>2</sub> was added to PRP while incorporating into the COL I scaffold. Human osteoarthritic cartilage-derived chondrocytes were encapsulated in COL I/PRP scaffolds and cultured in basal media without fetal calf serum (FCS) for 21 days compared with a control COL I construct. The results indicate that PRP increases the proliferation rate of OA chondrocytes significantly from 1 to 7 days during *in vitro* expansion in monolayers compared to FCS controls and with a mild stimulatory effect in COLI/PRP constructs as observed from the DNA quantification and metabolic activity. Higher levels of PDGF-BB (6.4 ng/mL) and bFGF (0.2 ng/mL) were observed in PRP when quantified with a multiplex assay, which might be factors influencing the proliferation rate. Concurrently, OA chondrocytes accumulate significant levels of sGAG in a COL I/PRP as equally observed in control COL I scaffolds. Our findings

suggest PRP in replacement of FCS for *in vitro* expansion of OA chondrocytes and their role in sGAG production for cartilage TE applications.

### 3D Brain-on-a-Chip with an Interstitial Level of Flow and its Application as an *in vitro* Alzheimer's Disease Model

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**Introduction and Objectives:** *In vitro* models for neurologic diseases have been highly demanded for better understanding of pathology and strategies for treatment of the diseases such as Alzheimer's disease; however, there have been limits in mimicking *in vivo* microenvironment on *in vitro* system. Here, we developed an *in vivo*-mimicking microfluidic 3D brain model with an interstitial level of flow by combining concave microwell arrays with an osmotic micropump system.

**Materials and Methods:** 3D micro-spheroidal neural tissue (neurospheroid) was formed on concave microwell arrays of microfluidic chip. The interstitial level of flow which is about 0.25  $\mu\text{L}/\text{min}$  was provided by the osmotic micropump system. With this platform, we investigated the effect of flow and amyloid- $\beta$  (A $\beta$ ) on neurospheroids.

**Results:** The group cultured with flow showed larger neurospheroids and more robust neural network between neurospheroids on the chip comparing to the group cultured without flow. And the group treated with A $\beta$  showed destructed neurites and synapses while the control group showed intact neurite and synapses which indicates the neurotoxic effects of A $\beta$ .

**Conclusion:** We developed a brain-on-a-chip that creates 3D cytoarchitecture and interstitial flow providing *in vivo*-like microenvironment which has a great potential as an *in vitro* brain model. As such, this platform could fill the gap between traditional *in vitro* neural cell culture models and *in vivo* brain studies, serving as a more reliable tool for studying neurological disease pathology and treatment strategies as well as drug screening applications.

### Enhancing the Transdifferentiation Process of Human Dermal Fibroblasts into Skeletal Muscle Cells using Small Molecules and Extracellular Matrix Components

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*In vitro* microphysiological systems can be used as tools for understanding human physiology or pathophysiology and for toxicological or pharmacological studies. Existing *in vitro* models of human skeletal muscle lack the physiology and functional output of native muscle [1]. Here, we utilize the ability of the MYOD1 transcription factor to induce transdifferentiation of human dermal fibroblasts (HDFs) into skeletal muscle. Our objective was to significantly enhance this process in order to derive sufficient cells and form 3D biosynthetic skeletal muscle tissues. *In vivo* myogenesis is both regulated by particular signaling pathways (WNT, TGF $\beta$ ) and the surrounding ECM [2]. We hypothesized that molecular pathway manipulation and ECM constitution would significantly alter the transdifferentiation process. Transdifferentiation was evaluated based on the number and size of multinucleated cells, sarcomeric organization, and functional characteristics (cell contractions, calcium signaling and membrane depolarization characteristics). Transdifferentiating cells were treated with small molecule inhibitors or ligand activators of particular signaling pathways (WNT, TGF $\beta$ , RTK). The cells were also seeded on substrates coated with collagen, laminin, fibronectin, silk fibroin and vitronectin. We identified a specific combination of small molecule inhibitors, protein ligands and growth factors along with particular ECM components which significantly improved the transdifferentiation process.

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### Substrate Stiffness Affects Cellular Reprogramming Efficiency via Increase in Mesenchymal-to-Epithelial Transition and Stemness Markers

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The stiffness of hydrogels has been attracting great interest in the biomedical engineering because the stiffness of hydrogels can be used as a potent regulator of cell fate decision. Here, we report that the stiffness of hydrogels promotes the reprogramming of mouse embryonic fibroblasts into induced pluripotent stem cells (iPSCs). We prepared cell culture substrates of various stiffnesses (0.1, 1, 4, 10, and 20 kPa) using a polyacrylamide (PA) hydrogel. We found that culture on a soft substrate plays an important role in inducing cellular reprogramming via activation of mesenchymal-to-epithelial transition (MET) and enhancement of stem cell marker expression. Our results suggest that physical signals from a soft substrate can be used as a potent regulator to promote cell fate changes associated with reprogramming into iPSCs, which may lead to effective and reproducible iPSC production and have various applications in iPSC-based therapy and regenerative medicine.

### Cobalt-doped Bioactive Glasses: Using Hypoxia to Influence Mesenchymal Stem Cell Behaviour

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**Objectives:** Low oxygen tension is a critical component of the mesenchymal stem cell (MSC) niche and has been implicated in the regulation of lineage commitment. Cobalt is a known hypoxia mimicking agent through activation of the hypoxia inducible factor (HIF-1) pathway. Here, increasing amounts of cobalt were incorporated into the bioactive glass (BG) composition  $3\text{SiO}_2:0.07\text{-P}_2\text{O}_5:(1.4\text{-X})\text{CaO}:1.6\text{ Na}_2\text{O}^*\text{X}$  (X=CoO) by substitution with calcium (0%, 1%, 1.5% and 2 mol%) to evaluate the influence on hMSC behaviour and stemness. Upon contact with biological fluids, BG partially degrades which releases ions from the glass network, thereby modifying the local ionic microenvironment.

**Methods:** Human bone marrow-derived MSCs (hMSC) were cultured in control (CTL) or cobalt-doped BG (CoBG) conditioned medium for up to 21 days. The impact of CoBG on HIF1 $\alpha$  protein abundance, hMSC metabolic activity and differentiation capacity were determined.

**Results:** CoBG conditioned medium triggered HIF1 $\alpha$  protein accumulation in hMSCs in a cobalt concentration-dependent manner. Glycosaminoglycan production and histological assessment of hMSC pellets indicated a reduced chondrogenic differentiation in presence of CoBG dissolution products compared to CTL and 0%CoBG. In accordance, the expression of the stem cell marker *Oct4* was increased when compared to CTL. Preliminary results suggested that exposure to CoBG influenced cell cycle progression by modification of p21WAF1/CIP1 protein expression.

**Conclusion:** In presence of CoBG dissolution products, hMSCs appeared to maintain an undifferentiated phenotype while the chondrogenic lineage commitment was reduced in a cobalt concentration-dependent manner. These findings offer exciting insights into

how hMSC behaviour and stemness can be regulated by cobalt containing biomaterials.

### Artificial Thymus: A Tissue Engineering Strategy

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The thymus is the central organ responsible for the generation of T lymphocytes (1). Various diseases cause the thymus to produce insufficient T cells, which can lead to immune-suppression (2). Since T cells are essential for the protection against pathogens, it is crucial to promote de novo differentiation of T cells on diseased individuals. The available clinical solutions are: 1) one protocol involving the transplant of thymic stroma from unrelated children only applicable for athymic children (3); 2) for patients with severe peripheral T cell depletion and reduced thymic activity, the administration of stimulating molecules stimulating the activity of the endogenous thymus (4). A scaffold (CellFoam) was suggested to support thymus regeneration *in vivo* (5), although this research was discontinued. Herein, we propose an innovative strategy to generate a bioartificial thymus. We use a polycaprolactone nanofiber mesh (PCL-NFM) seeded and cultured with human thymic epithelial cells (hTECs). The cells were obtained from infant thymus collected during pediatric cardio-thoracic surgeries. We report new data on the isolation and characterization of those cells and their interaction with PCL-NFM, by expanding hTECs into relevant numbers and by optimizing cell seeding methods.

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### Use of Hydroxyapatite Particles to Selectively Modulate Cell-Matrix Interactions in Perfusable Agarose-Based Constructs for Musculoskeletal Regeneration

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An engineered osteochondral construct should accommodate the disparate cell-matrix interactions required for chondrocyte and osteoblast differentiation. Fortunately, modular tissue engineering allows the specification of scaffold architecture at multiple scales. Here, we describe the selection of naturally-derived biomaterials to drive cell behavior in agarose-based microbeads and in the interstices between microbeads once they are assembled into perfusable constructs.

Rat bone marrow stromal cells were encapsulated within non-aggregating spherical microbeads consisting of agarose (Ag) +/- nano (1.5 or 3 wt%) or micro-scale (3 wt%) hydroxyapatite (HA) particles. Constructs were cast in gelatin molds containing microbeads in agarose +/- nano or micro HA carrier gels.

Cell viability in microbeads was >95% at days 7, 14 or 21 and not affected by HA particle size or concentration. While Ag + micro HA and Ag-only microbeads cultured in osteogenic medium both secreted osteocalcin, cell spreading was only evident in the former. Coomassie Blue staining also showed qualitatively greater protein content in Ag + micro HA microbeads.

In constructs, histology showed cell migration from the microbeads to Ag + micro/nano HA carrier gels in static culture and when perfused in Kiyatec bioreactor chambers, respectively. Little to no interstitial migration was observed for Ag-only carrier gels, even when containing Ag + HA microbeads.

These preliminary results suggest that HA's protein adsorptive capacity can promote cell adhesion when incorporated in agarose, a material whose non-adherent properties we have previously harnessed for chondrogenic microbeads. HA could therefore be used to regionally modulate cell adhesion within an agarose-based, modular construct to allow the maintenance of osteoblastic and chondrocytic phenotypes simultaneously.

### In Vivo Repair of Rat Caudal Annulus Fibrosus with Injectable, Cell-Seeded Collagen Gel

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**Purpose:** The annulus fibrosus (AF) can become damaged in many ways, allowing the nucleus pulposus (NP) to herniate. Therefore, it is essential to repair the damaged AF to prevent subsequent degeneration. We have developed an injectable collagen gel that has been successful in rat tail models [1,2]. This study observes the effect of seeding these gels with primary cells on AF repair.

**Methodology:** Athymic rats were prepared for surgery and anesthetized according to IACUC protocols. AF of the Cd3-Cd4 disc was exposed and punctured with an 18 gauge needle. Each defect was treated with a collagen gel or left punctured. Treated groups received cell-seeded or acellular crosslinked collagen gel. Each gel was then exposed to blue light for 40 s. Discs were evaluated for NP hydration and disc height.

**Results:** Samples treated with cell seeded collagen gels exhibited higher NP sizes over all other groups at five weeks post-surgery. Greatest NP sizes in all groups were exhibited at week one, with cell seeded gels being highest at 33% of healthy adjacent. Histological slides showed a GAG-rich cap sealing the outermost layer of the AF defect in cell-seeded gel samples.

**Significance:** The presence of cells enhanced repair of IVD defects. As such, crosslinked collagen gels seeded with primary ovine AF cells were effective at repairing the AF in rat caudal IVDs *in vivo*.

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### Biological Substrates to Prevent Dedifferentiation of Osteogenically Induced Mesenchymal Stem Cells

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Prior to transplantation, mesenchymal stem/stromal cells (MSCs) can be induced toward the osteoblastic phenotype using a soluble cocktail. However, the optimal induction duration is unknown and there is little evidence of differentiated MSCs directly participating in bone formation, suggesting that MSCs may revert to an undifferentiated phenotype upon transplantation. Cell-secreted decellularized extracellular matrices (DMs) represent a promising strategy to confer bioactivity and direct cell fate through the presentation of a physiologically relevant milieu. Therefore, we examined the effect of induction duration on cementing the osteoblastic phenotype of MSCs, and the capacity of DMs to preserve the phenotype upon withdrawal of induction. Increasing the duration of induction did not preserve the osteoblastic phenotype after the removal of the osteogenic stimulus. Regardless of induction duration, up to 6 weeks, MSCs exhibited up to a 5-fold reduction in osteoblastic markers within 24 hours following stimulus withdrawal. We then demonstrated that seeding osteogenically induced MSCs on DMs sustained the osteoblastic phenotype of MSCs by preserving up to 2-fold more calcium deposition than tissue culture plastic, together with significant increases in VEGF secretion. DMs sustained MSC phenotype at

least partially by increasing actin cytoskeletal tension via the ROCK II pathway. MSCs deployed *in vivo* on DMs exhibited significant increases in cell survival when measured with bioluminescence imaging. These results underscore the rationale for deploying MSCs into a bone defect site using DMs to preserve the *in vitro*-acquired osteoblastic phenotype to accelerate the process of bone repair.

#### Cytoskeletal Tension Mediates Adipocyte Dysfunction in a 3D *In Vitro* Model of Obesity

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Obesity affects 35% of American adults and with associated comorbidities (type 2 diabetes, cardiovascular disease and stroke) being some of leading causes of preventable deaths. On a tissue level, obesity is defined by adipocyte hypertrophy, fibrosis and inflammation. This adipocyte hypertrophy leads to local regions of hypoxia within the adipose tissue. Hypoxia inducible factor 1 alpha (HIF1 $\alpha$ ) increases activity of many pro-fibrotic factors including lysyl oxidase (LOX). LOX crosslinks collagen resulting in matrix stiffening and subsequent tissue fibrosis. Matrix stiffness and degradability have been demonstrated to affect cell fate, ultimately by how the cell spreads on the surface. A major pathway involved in how cells sense and respond to changes in matrix properties is the RhoA-GTPase/Rho-associated coiled coil-containing kinase (Rho/ROCK) pathway. In this study, we link the effects of altered cell shape/cellular tension to adipocyte dysfunction independent of inflammation.

We have developed a 3D adipocyte model within a collagen gel where upon exposure to hypoxia cells undergo a cell shape change from round to spread. This is accompanied by pathological changes to adipogenic genes (PPAR $\gamma$ , adiponectin), HIF1 targets (LOX, VEGFA) and collagens (Col1, Col6). Pharmacologic inhibition of myosin (downstream of ROCK) by blebbistatin, or LOX by beta-aminopropionitrile, prevents the cells from spreading into the matrix and tempers the pathological changes to gene expression. Furthermore, these results are not apparent in 2D cultures as cells cannot deposit and remodel the matrix in the same manner, demonstrating the importance of 3D models for the study of dynamic disease models.

#### Recapitulation of the Tumor Microenvironment *In Vitro* using a Novel Perfusion Bioreactor: Towards Personalized Tumor Models

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Solid tumors are heterogeneous both within a single tumor as well as between different patients. Heterogeneity can exist in the tumor cell phenotype as well as in the microenvironmental composition and host immune response, with best treatment options often determined empirically. *In vitro* tumor models derived from patient biopsies could enable testing of patient-specific therapeutic strategies. While much research has been devoted to developing such systems, numerous challenges remain in developing models that can faithfully predict patient response, since many micro-environmental factors that are known to affect drug efficacy have failed to be recapitulated *in vitro*.

To address this challenge, we have developed a flexible, mid-throughput platform for long-term, multicellular 3D culture of primary cells that, with perfusion, maintains the complexity of the *in vivo* tumor microenvironment. Our meso-scale bioreactor allows the setup of multiple scaffolds in an easy-to assemble 24-well plate format, as well as the optimization of different culture conditions in terms of perfusion velocity, scaffold material and exogenous cytokines and growth factors.

We used our system to culture primary cells isolated from a murine breast cancer model (MMTV-PyMT). Cells were seeded in porous collagen scaffolds supplemented with hydrogel-based matrices under perfusion, with conditions that we have optimized for maintaining

consistent cellular composition (including the immune cell compartment) of the original tumor and its microenvironment for up to 20 days in culture. With the 'mini-tumors' established, we are currently comparing the response to a variety of therapeutic treatments, including those that target the microenvironment, with *in vivo* responses.

#### A High Throughput Screen System for Optimization of Engineered Cardiac Tissue

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Engineered cardiac tissues (ECTs) can be a powerful tool for investigating drug treatments and, potentially, for replacing damaged tissue *in vivo*. The ability to rapidly generate normal and pathological cardiomyocytes through the use of iPS cells only increases the usefulness of these tissues. However, many variables, such as cell density, gel scaffold composition, and growth medium type and supplements need to be assessed experimentally when creating ECTs. To streamline this assessment, we developed a high throughput system (HTS) that allows for relative comparison of the twitch force of centimeter-scale gel-based cardiac tissues. This system is based on principles taken from traction force microscopy and utilizes fluorescent microspheres embedded in a soft polydimethylsiloxane (PDMS) substrate. Recordings of the fluorescent bead movement during tissue pacing are used to determine the maximum distance that the tissue can displace the elastic PDMS substrate. These displacement values are used to determine the effects of the variables on the relative force generation of the ECTs. The HTS allows for rapid sample preparation, data collection, and analysis in a simple and cost effective platform. In this study, a fibrin gel containing iPS-derived cardiomyocytes (iPS-CMs) is formed directly on the surface of the PDMS substrate and allowed to culture for nine days. It was found that the amplitude of bead displacement correlates with direct force measurements and that the twitch force exerted by iPS-CMs is the same in 2 and 4 mg/ml fibrin gels despite the greater macroscopic gel contraction evident in the former case.

#### White Adipose Tissue Engineered Model for Studying Metabolic Behavior *Ex Vivo*

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Obesity is associated with a complex interaction of metabolic disorders. As the prevalence of obesity is increasing there is a growing need for monitoring sustainable, physiologically relevant, three dimensional human adipose tissue models to study detrimental changes in metabolic behavior. Current models of human white adipose tissue use hASCs in 2D or 3D platforms and require lengthy culture times to differentiate into premature adipocytes with multilocular lipid droplets. However, mature adipocytes contain a single unilocular lipid-filled vacuole and can be cultured in silk protein scaffolds *ex vivo*. Silk is a naturally occurring and clinically accepted biocompatible material that has tunable mechanical strength and can be tailored to degrade slowly for long term culture. Cells could be maintained in silk scaffolds for over 3 months, proliferated and maintained triglyceride content suggesting this model tissue system will be useful for future disease models. A human disease model with unilocular white adipocytes is an essential tool for studying the transition from obesity to metabolic diseases such as type II diabetes. In the future this tissue model could also be used for high throughput screening of treatments.

#### Extracellular Matrix Mimicking Scaffolds for Bone Tissue Engineering

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Three-dimensional (3D) open channeled scaffolds of biopolymers are a promising candidate matrix for tissue engineering. Here we report a novel fusion protein hybrid matrix that mimics the bone extracellular matrix, having the ability to promote stem cell adhesion and stimulate osteogenesis. Polymeric based scaffolds were fabricated by 3D printing technology and covered with the fusion protein FN-OCN-CBD (Fibronectin-Osteocalcin-Collagen binding domain). Culture of rat mesenchymal stem cells (rMSCs) showed excellent adhesion, forming focal adhesions arising from the interaction with the FN adhesive domains. Cells were able to proliferate extensively due to the presence of FN and collagen, whereas the presence of OCN in the structure allowed extensive osteogenic differentiation. The hybrid biomimetic matrix significantly improved *in vivo* bone formation in calvarial bone defects, therefore showing the potential of fusion proteins as a promising tool for bone tissue engineering.

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#### Human Tissue Engineered Blood Vessels for *in vitro* Drug Testing

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Rapidly producible, endothelialized human TEBVs that elicit physiological responses to biological and pharmaceutical compounds are a critical development toward an *in vitro* vascular model for drug toxicity testing. We have produced TEBVs 400–800  $\mu\text{m}$  in diameter consisting of human neonatal dermal fibroblasts or human bone marrow-derived mesenchymal stem cells embedded in a dense collagen gel matrix. TEBVs may be produced in less than two hours and subsequently endothelialized with late-outgrowth endothelial progenitor cells from coronary artery disease patients. TEBVs are mechanically strong enough to be immediately introduced into perfusion culture at physiological shear stresses. Endothelialized TEBVs elicited burst pressures of  $236.8 \pm 4.3$  kPa and ultimate tensile stresses of  $191 \pm 36.9$  kPa after 1 week of maturation under perfusion.

TEBV vasoactivity was measured non-invasively over time using a stereoscope and video display. Exposure to  $1 \mu\text{M}$  phenylephrine caused endothelium-independent vasoconstriction, and  $1 \mu\text{M}$  acetylcholine caused endothelium-dependent relaxation. TEBVs released nitric oxide and dilated in response to acetylcholine over 5 weeks of *in vitro* culture. Reversible activation in response to acute inflammatory stimulation with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was detected. Mature TEBVs exposed to 200 U/mL TNF- $\alpha$  for 4.5 hours constricted in response to acetylcholine, indicating endothelial activation and injury. One week after removal of TNF- $\alpha$  from the circuit, vasodilation in response to acetylcholine was restored. TEBVs also demonstrated potential to assess drug-induced vascular injury through dose-dependent vasodilation in response to caffeine and theophylline. Future work will assess TEBV response to statins under healthy and inflammatory conditions.

#### Particulate Oxygen Generating Materials for Preservation of Skeletal Muscle Function During Hind Limb Ischemia in a Rodent Model *In Vivo*

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Ischemic injury can cause irrecoverable loss of skeletal muscle structure and function if untreated. Since oxygen is critical to tissue survival, oxygen delivery therapies are under development to preserve muscle viability and function during ischemia. This issue is especially critical in the context of blunt trauma injuries that result in partial or complete interruption of blood supply to muscle. In this situation, oxygen delivery cannot utilize vasculature. Thus, we examined the effectiveness of different particulate oxygen generator (POG) treatments in a rodent ischemic injury model. Ligation of the iliac artery in female Lewis rats was used to create ischemia, and the contractile response to stimulation of the peroneal nerve (dorsiflexion of the foot) was used to assess muscle function 48 hours prior to ligation (baseline) and 24 hours post ligation (ischemic). Control animals (uninjured) were run in parallel. Experiments were conducted in the absence and presence of POG injection (sodium percarbonate (SPO) and calcium peroxide (CPO)) into the tibialis anterior (TA) muscle (main driver of dorsiflexion). SPO and CPO formulations were tested in the absence and presence of catalase (n=6–9 animals in all groups). Ischemia was verified in ligated animals as a dramatic reduction in contractile force and a significant contrast in T2-weighted MRI and gadolinium (III) contrast-enhanced T1-weighted pulse sequences. Enhancement in both MR images indicates local edema. Of note, injection of SPO + catalase into the TA muscle significantly mitigated edema and improved dorsiflexion during ischemia. Refinement and optimization of POG formulations for more physiological oxygen delivery is underway.

#### Injectable Silk-In-Silk System Loaded with Glucosamine Modulate Mechanical Properties in Bovine Ex vivo Degenerated Intervertebral Disc (IVD) Model

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Hydrogels can be injected *in situ* in nucleus pulposus (NP) of degenerated IVD to mould precisely to the defect spaces and withstand complex loading, thus eliminating the need for invasive surgical procedures. In the present study we demonstrate a silk-in-silk platform with N-acetyl-glucosamine (GlcNAc)-loaded silk hollow spheres embedded in silk hydrogel for *in situ* therapeutic release and enhanced mechanical strength. The rheological properties of the silk-in-silk system demonstrated its injectability withstanding high ionic strength and varied pH conditions prevailing inside IVD. The assembled silk hydrogel provided adequate structural support to the *ex vivo* degenerated disc model in a cyclic compression test at par with the native tissue. Also, controlled spatiotemporal release of GlcNAc from the silk hollow spheres triggered enhanced proteoglycan production from human adipose-derived stem cells (hADSCs) embedded in the composite system. Gene expression analysis showed upregulation of Col I and Aggrecan which was further validated by biochemical and histological studies. The role of MAPK and SMAD pathways in enhancing proteoglycan production was investigated by gene expression and immunohistological analysis. Glucosamine provided exogenously is taken up by the cells and is metabolized to UDP-GlcNAc causing an upregulation of TGF- $\beta$  synthesis. TGF- $\beta$  triggers the GAG production through MAPK and SMAD signalling pathways resulting in enhanced GAG production. This study therefore elucidated the potential of injectable silk-in-silk system for regeneration of degenerated disc tissue by improving its mechanical properties and enhancing proteoglycan production.

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#### Nerve Growth Factor Increases Repair of Crushed Peripheral Nerves

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One of the responses following a traumatic injury to the peripheral nervous system (PNS) is an increased expression of nerve growth factor receptors (NGFR), which helps to stimulate the regeneration of the nerve. In a normal, healthy PNS, NGFRs are rarely found. Following damage to the nerve, NGFRs can be found in high levels around the damaged area. In this study, traumatic injury to the sciatic nerve of rats was performed in order to study the effects of regeneration after a local application of nerve growth factor (NGF). Both a physical crush model and a focal demyelination model were used to simulate the nerve injury. In the crush model, the effect of a crushed extracellular matrix on degeneration and subsequent regeneration was studied. In the focal demyelination model, the effects of local demyelination with an intact extracellular matrix on degeneration and regeneration were investigated. In two additional experimental groups, an intraneural injection of NGF into the damaged sciatic nerve one week after the crush or lysophosphatidylcholine (LPC) injection at the damaged site were performed. The nerves were examined both qualitatively using SEM and immunohistochemistry, and quantitatively using transdermal local electrophysiology. It was found that the addition of NGF increased the rate of nerve regeneration in both models. The models were then combined and LPC was applied to crushed nerves. Adding NGF to LPC-crush nerves regenerated faster than crushed nerves only receiving NGF. Faster regeneration can be achieved through a LPC-NGF combination.

#### Directing Human Mesenchymal Stem Cell Behaviour on Extracellular Matrix Mimetic Supported Lipid Bilayers

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Due to their ease of isolation and multi-potential differentiation capacity, mesenchymal stem cells (MSCs) have been highly attractive and investigated for tissue engineering and regenerative medicine applications. Directing molecular events related to cell-extracellular matrix (ECM) interactions as essential regulators of MSC fate is important for the design of smart interfaces which can enhance biomaterial performance in guiding desired tissue formation. Non-fouling supported lipid bilayers (SLBs) can be employed as cellular interfaces with the opportunity to present biological ligands and modulate their viscoelastic properties. Here, we use SLBs as dynamic supramolecular platforms to direct and understand human MSC (hMSC) behaviour by inserting peptides designed for cell-ECM interactions. Mobile (DOPC) and immobile (DPPC) SLBs were conjugated with RGD ligands at different densities to engineer surfaces with differential ligand separation and affinity. We observed that hMSC adhesion occurred through specific interactions with RGD ligands while non-functional bilayers did not support cell attachment. Furthermore, immunofluorescence analysis for cytoskeletal organization and focal adhesion formation together with live cell imaging revealed a significant dependency of cell response on ligand density as well as mobility indicating that we could specify hMSC behaviour on these surfaces. Currently, we are also studying cellular differentiation events in response to ligand separation and affinity to have a deeper understanding of cellular function on these biomimetic surfaces that will enable us to develop smart biomaterial coatings.

#### Combinatorial Copolymers Reinstating Human Mesenchymal Stem Cell Potency: Study of Structure-Function Relationship

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The potential use of human mesenchymal stem cells (hMSCs) in therapeutic treatments has captured the imaginations of both researchers and clinicians. However, the existing techniques to culture hMSCs *ex vivo* and maintain their potency (e.g. maintain high stemness and differentiation potential) before their use in patients present several problems. Same as hMSCs from aging people, serially-passaged hMSCs to achieve critical cell mass for therapy are typically accompanied by senescence-associated properties as the increased number of cell divisions give rise to complications in cell health and potential cancerous transformation. In our study, we developed a library of synthetic copolymers where certain compositions significantly increased and maintained stemness, reduced senescence-associated activities, and adopted a pro-angiogenic phenotype. Using advanced x-ray scattering techniques and super-resolution imaging, it was found that the phenotype adoption of the hMSCs was driven by altered cell attachment (“forced aggregation”) on the amorphous phase of intermixed PEG and PCL polymer components. Tuning the molar ratio of PEG to PCL and the PEG chain length influenced the PEG hydration-mediated steric hindrance and thereby altered integrin attachment and focal adhesion development. This compositional change also governed the copolymer surface profile that may be reflective of the abluminal surfaces of capillaries that given rise to the enhanced pro-angiogenic properties of the hMSCs. Moreover, forced aggregation of the hMSCs as a result of PEG repellency produced unique, adherent cell masses reminiscent of hanging drops and expressed unexplored cell-cell adhesion proteins, along with certain integrins, yielding a new tool to study fundamental stem cell biology.

#### *In Vivo* Evaluation of the Effects of Mesenchymal Stem Cells and Conditioned Media in a myectomy model of injury

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Incomplete regeneration after traumatic muscle injury with residual functional deficiencies is a common problem in orthopedics and traumatology. Previously we developed a novel experimental muscle injury of the tibialis anterior muscle of the rat by standardizing a 5 mm-diameter myectomy lesion. Using this model we tested the application of hMSCs isolated from the Wharton’s jelly associated to different biocompatible vehicles to induce muscle regeneration.

We tested different treatment combinations (hMSCs and conditioned media) and different vehicles (FloSeal<sup>®</sup>, a hyaluronic acid based hydrogel and fibrin glue). After 15 and 35 days, animals were sacrificed and the anterior tibial muscles were collected and fixed in formaldehyde. The International Standard (ISO 10993-6) for biological evaluation of medical devices was employed for assessment of the local effects after implantation of the different biomaterials used as vehicles in this study. Collagen quantification and immunohistochemistry were also performed post-mortem while kinematics and force measurement for functional assessment. NMR and a cytokine array were used for *in vitro* characterization of the conditioned media. In the groups treated with the conditioned media instead of MSCs a blunted inflammatory response seemed to occur. This finding may be justified by the fact that the conditioned media possesses a variety of growth factors like IL-6, IL-8, IL-10 or TGF- $\beta$  which can be proangiogenic, anti-apoptotic or immunoregulatory. FloSeal and the hydrogel tested were inappropriate as vehicles because of the local effects after implantation. There were no obvious advantages of using hMSCs instead of conditioned media for promoting muscular regeneration.

#### The Effect of Angiotensin-(1-7) on the Recovery of Muscle Function After Injury

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Pathologic fibrosis is a major impediment to the recovery of muscle function after injury, and is augmented in the elderly population.

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous, 7-amino acid peptide hormone of the renin-angiotensin system, which has been shown to reduce fibrosis in the heart, lung and kidney 1–3. The objective of this research was to examine the effect of Ang-(1-7) treatment on the recovery of muscle function in older rats after compression injury.

Compression injury was induced in the hind limbs of 12 month old male Lewis rats as previously described<sup>4</sup>. Ang-(1-7) was delivered at a dose of 24 µg/kg/hr by an osmotic pump implanted immediately after injury. Muscle function was measured via peroneal nerve stimulation as described<sup>4</sup>. The tibialis anterior (TA) muscles were collected at day 14 after injury and processed for histological analyses and quantitative PCR (qPCR) analyses.

Our data showed that treatment with Ang-(1-7) significantly increased muscle function 7 and 14 days after injury. However, we did not find any significant changes in fibrosis with Ang-(1-7) treatment. This suggests that Ang-(1-7) is acting directly on the muscle tissue and/or the local microenvironment in order to promote restoration of muscle function.

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## Mechanophenotype Influences Cellular Organization on Mechanically and Biologically Engineered Surfaces

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Understanding how whole-cell mechanical properties affect cellular behavior and interaction with the microenvironment is essential to directing cellular assembly in regenerating tissues. This study investigated the influence of mechanophenotype on cellular organization across substrates with varying stiffness and protein coatings. Atomic force microscopy was used to mechanically characterize two cell lines with dissimilar, inherent elastic properties. MG-63 (osteosarcoma) cells exhibited a modulus of  $1.3 \pm 0.5$  kPa, and SH-SY5Y (neuroblastoma) cells exhibited  $0.3 \pm 0.1$  kPa. Cells were cultured separately on 0.3, 2, and 12 kPa polyacrylamide gels functionalized with fibronectin, laminin, or collagen-I. Cellular responses were analyzed over four days by imaging daily. We hypothesized that cells would spread and attach more readily on substrates with elastic moduli equal to or greater than that of the cell and with physiologically relevant proteins present. MG-63 and SH-SY5Y cells exhibited two organizational phenotypes: a multi-cell, nodule-like structure and a spread monolayer. MG-63 cells, with a mechanophenotype equivalent to the 2 kPa substrate, formed more nodules on substrates with compliance less than the cell's own elastic modulus. SH-SY5Y cells, with a mechanophenotype equivalent to the softest substrate, showed no organizational transition moving from compliant to stiff substrates. Protein coatings had a modulatory effect on cellular mechanosensing, likely due to varying integrin expression profiles. MG-63 cells spread more on collagen-I, even on soft substrates. SH-SY5Y cells required both specific proteins and stiff substrates to attach and spread. These findings indicate that cellular mechanosensing of the microenvironment is linked to inherent mechanical phenotype.

## Injectable Cross-linked rhCollagen Scaffold Combined with PRP for Soft Tissue Repair

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Platelet-Rich Plasma (PRP) is proposed for treating soft tissue injuries, including tendons, but the clinical outcome is highly variable. We hypothesized that PRP fibrin complex degrades too rapidly to serve its purpose. An injectable gel was developed (Vergenix™-STR) combined with autologous PRP (STR-PRP). Upon injection, the complex forms a collagen fibrin composite at the injury site thus serving as a scaffold for tissue regeneration. Vergenix™-STR-PRP or PRP alone were injected into subcutaneous pockets in rats. Analysis of the injection sites showed a decrease in PDGF content during the first days in the STR-PRP group, followed by gradual increase which last for 30 days. VEGF level was very low at time zero, gradually increased in the first two weeks and reduced to undetectable level at day 30. This was concomitant with collagen/PRP composite degradation. In the PRP alone group, no GFs were measured after the first day and no clots were observed. In a second study, the efficacy of the Vergenix™-STR-PRP was evaluated in rat model for Common Calcaneal tendon (Achilles tendon) tendinopathy. In this model, tendinopathy was induced by collagenase injection followed by treatment with STR-PRP or PRP alone. Rats with developed tendinopathy treated with STR-PRP had significantly lower level of inflammatory mononuclear cells 3 days after treatment as compared with the animals treated with PRP alone and at day 14 the STR-PRP group displayed significantly lower level of immature granulation compared with PRP alone. This study demonstrates the efficiency of collagen-PRP treatment as a platform for tissue regeneration.

## BMP-2 Promotes Mesenchymal Stem Cell Survival in Photopolymerized Alginate Hydrogels for Enhanced Bone Formation

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Mesenchymal stem cell (MSC)-based therapies have significant potential for replacing lost bone volume due to trauma or disease, but ensuring cell survival and functionality post-implantation is a major clinical challenge. Photopolymerizable alginate hydrogels (PAHs) are under investigation for the delivery and localization of therapeutic cells at the defect site, as they can be injected and cross-linked with ultraviolet (UV) light *in situ*. UV light, however, can cause intracellular damage resulting in undesired cell death. The objective of this study was to investigate the viability and osteogenic potential of MSCs in PAHs when co-delivered with a low-dose potent osteoinductive factor, Bone Morphogenetic Protein-2 (BMP-2). We hypothesized that BMP-2 would increase cell survival during photopolymerization, subsequently improving bone formation in an orthotopic bone defect *in vivo*.

*In vitro*, BMP-2 significantly decreased apoptosis and enhanced metabolic activity, which correlated with increased expression of osteogenic markers. Compared to gels without BMP-2, bioluminescence imaging revealed a significant increase in MSC survival using PAHs containing BMP-2 to deploy cells into a critical-sized rat segmental bone defect. Additionally, co-delivery of MSCs and BMP-2 increased tissue mineralization and bone regeneration. Defects containing PAHs carrying both MSCs and BMP-2 demonstrated 100% radiographic union as early as 8 weeks and significantly higher bone volumes at 12 weeks while defects with MSCs alone did not fully bridge.

This study demonstrates that co-delivery of MSCs and low-dose BMP-2 in a photopolymerized alginate carrier increases cell survival

and bone formation. These findings have important implications for improving cell persistence in tissue engineering applications.

#### An Injectable Calcium Phosphate Hydrogel Composite Material Promotes Healing in Critical Sized Femoral Defects in Combination with Recombinant Human BMP-2

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Recombinant human BMP-2 is a potent orthobiologic providing clinical benefit for spinal fusion and traumatic fracture patients. Many of the adverse events and safety concerns associated with rhBMP-2 stem from the rapid release of the growth factor from the collagen sponge carrier. An injectable calcium phosphate (CaP) granule and tyramine-substituted hyaluronic acid hydrogel composite carrier for rhBMP-2 was evaluated in a rat femur critical sized defect model. 5 mm defects were created in adult male rats, stabilized with PEEK internal fixators and filled with ~80 uL of the injectable crosslinking carrier with and without 3.75 ug of rhBMP-2. Additional animals were treated with 0, 0.75 ug or 3.75 ug rhBMP-2 delivered on a collagen sponge. Defects treated with the CaP-hydrogel and rhBMP-2 showed robust torsional mechanical stability that matched or surpassed collagen sponge treated defects by 4 weeks, and by 8 weeks approximated the mechanical stability found in intact limbs. In contrast, with the exception of stiffness at 8 weeks, all mechanical parameters in collagen sponge defects, regardless of rhBMP2 dose, were less than 50% of intact controls. Both in life and *ex vivo* microCT confirmed enhanced healing of defects treated with the CaP-hydrogel and rhBMP-2. Histological analysis revealed the formation of new trabecular bone in intimate contact with the implanted composite material throughout the gap site with trabecular thickening apparent between 4–8 weeks. The porous CaP granules enable rhBMP-2 binding, promoting its gradual release; whereas the crosslinking hydrogel provides injectability and cohesion after implantation, resulting in superior fracture healing.

#### A Novel 3D Bioprinted Angiogenesis System for High-throughput Screening

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Angiogenesis models play important roles in tissue engineering and many other biomedical fields. However, few of these *in vitro* models are sufficient for high-throughput screening with multiple conditions. We report the development of bioprinting technology as an effective way to generate a 3D angiogenesis system for high-throughput screening. A novel high-throughput angiogenesis system was generated by bioprinting human umbilical vein endothelial cells (HUVEC) with bio-ink into each well of a 96-well plate. HUVEC micro tissues with multiple dimensions were printed based on customized designs. The durability, viability and sprouting rate were analyzed upon stimulation of growth factors released by tumor and stem cells. The sizes of printed 3D HUVEC micro tissues range from 200 um to 500 um based on customized design respectively. The micro tissues exhibited a stable dimensions and good viability. In 3D angiogenesis assays, the 3D bioprinted micro tissues started to sprout only 24 hours after bioprinting. The vessel-like sprouting continuously increased and reached the top at D4 followed by a regression. With the stimulation of multiple angiogenesis factors from 3D bioprinted tumor or stem cells micro tissues, the sprouting rate, lumen length and durability were increased significantly. Our novel 3D bioprinted angiogenesis system can be used in high-throughput angiogenesis growth factors detection, angiogenesis and anti-angiogenesis biomolecule screening. It proves a versatile platform for studies in tissue engineering and many other biomedical fields.

#### Bone Marrow-Derived Stem Cells in Response to Intervertebral Disc-Like Matrix Acidity - Implications for Cell-Based Regenerative Therapy

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The intervertebral disc (IVD) is an avascular organ relying on diffusion of essential nutrients through the cartilaginous endplates thereby creating a challenging microenvironment. Within degenerated IVDs, oxygen and glucose concentrations decrease further (<5% oxygen, <5 mM glucose) and matrix acidity (<6.8 pH) increases resulting in adverse microenvironmental conditions. For cell-based IVD regenerative strategies, this challenging microenvironment may severely affect the survival and regenerative potential of injected bone marrow-derived stem cells (BMSCs). The objective of this study was to investigate the response of BMSCs to varying pH environments (blood (pH 7.4), healthy IVD (pH 7.1), mildly degenerated IVD (pH 6.8) and severely degenerated IVD (pH 6.5)) in 3D hydrogel culture. Briefly, BMSCs were encapsulated in 1.5% alginate and ionically crosslinked in 102 mM CaCl<sub>2</sub> solution to form beads (diameter=5 mm) which were cultured in different microenvironmental conditions.

At pH 6.5, increased cell death was observed concomitant with decreased DNA content, minimal GAG and collagen accumulation. At pH 6.8 or higher, beads exhibited increased proliferation, sustained cell viability with increased GAG and collagen accumulation. These findings suggest that a threshold exists at pH 6.8, below which cells cannot survive and accumulate NP-like matrix components (sGAG and collagen). Translation into a multimodal protocol requires the survival of injected stem cells and their ability to function normally amidst the harsh microenvironment. This study has implications for the development of cell-based therapies whereby the stage of degeneration is critical and thereby pH measurements of the central IVD may help identify suitable candidates for successful therapeutic strategies.

#### Development of a 3D-Printed Tissue Engineered Invasion Model of Placental Biology

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Preeclampsia (PE) is a leading cause of maternal and perinatal morbidity and mortality affecting 3 to 5% of pregnancies. However, the pathobiology of PE remains poorly understood and there is a lack of experimental models to study pathogenesis and to test for therapeutics. Current research suggests that impaired trophoblastic invasion and remodeling of maternal spiral arteries contributes significantly to the development of PE. The goal of this work is to create an *in vitro* 3D-printed tissue-engineered invasion model (TEIM) that captures the complex features of the placenta, particularly the vascular network, cellularity, and ECM composition. To this end, we first characterized the vasculature and mechanical properties of an *ex vivo* human placenta using ultrasound imaging and shear wave elastography (SWE). Next, we translated these data to a CAD model and 3D-printed a TEIM of the tissue. Mechanical testing, swelling, degradation, and NMR were performed to characterize the TEIM and confirm similarity to native tissue. Finally, we examined the effect of chemokines, released from the printed vasculature, upon the migratory and invasive behavior of the human cytotrophoblasts. Constructs with heterogeneous mechanical properties, chemokine concentrations, and trophoblast populations (viability=71±5%) fabricated from gelatin-methacrylamide (degree of substitution=63.1±5.8%) hydrogels were 3D-printed. Compressive modulus range of the constructs (45±16 to 1363±30 kPa) encompasses that of placenta.



Our novel approach of utilizing 3D-printing, SWE, and tissue engineering technologies will serve not only as a versatile platform to model various diseases, but also as an efficient technique to develop vascularized, tissue-engineered constructs.

### Extracorporeal Shockwave Treatment Accelerates Peripheral Nerve Regeneration by Altering Schwann cell Phenotype

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The gold standard in peripheral nerve regeneration is the (sensory) autologous nerve graft. This treatment however shows inferior results for motor nerves, as the transplanted sensory Schwann cells and nerve channels differ in behaviour and structure. In this study we show how Extracorporeal Shockwave treatment (ESWT) improves peripheral nerve regeneration by altering Schwann cell phenotype.

*In vivo* effects of ESWT on motor fibers regenerating through a sensory environment were evaluated using Catwalk-analysis, electrophysiology, histology and RT-qPCR. The inferior regeneration of motor axons through a sensory nerve graft was confirmed (compared to a phenotypically matched graft) and treated with ESWT, regeneration was significantly ameliorated.

*In vitro* SCs were treated with ESWT prior to isolation. Cells were evaluated concerning expression of S100, P75, MAG, P0 (FACS) and proliferation (BrdU) in pro-proliferation (PPM)/pro-myelination (PMM) medium. Cultured for 10 weeks in PPM all ESWT SCs showed no change in SC marker expression (P75, S100) or proliferation (BrdU), while marker expression and proliferation of control group steadily decreased. Motor SC cultured in PMM were able to switch to myelination quicker and to a higher extent than sensory SC (MAG, P0), with ESWT both phenotypes showed even stronger myelin-associated protein expression.

Summarizing, significant differences in all parameters were observed only between the motor/sensory control group, and ESWT treated groups showed improved results *in vitro* and *in vivo*. This study indicates that ESWT is able to accelerate peripheral nerve regeneration by altering SC phenotype in a model which reflects the clinical reality after autologous nerve transplantation.

### Nanofunctionalised Hydrogels Mimicking *In Vitro* Cartilage Extracellular Matrix

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Nowadays, no successful method for complete regeneration of osteochondral defects exists. The aim was therefore to develop functionalised hydrogels at nanoscale by Layer-by-Layer (LbL) assembly to promote both cartilage and bone healing. Hydrogels were prepared by an external gelation method in which aqueous sodium alginate solution was poured into a poly(styrene) mould and allowed to gel in the presence of an aqueous solution of cation chloride held at the upper and lower boundaries by porous microcellulose sheets. Successively, hydrogels were immersed in a 2% w/v Type A gelatin solution to obtain a positively charged surface; the samples were modified by LbL technique to generate functional polyelectrolyte (poly(styrene sulfonate)/poly(allyl amine) (PSS/PAH) to obtain 12 nanolayers to incorporate specific transforming growth factors (TGF- $\beta$ ) to promote cartilage healing. XPS spectra showed N1s peak at 399.5 eV and S2p peak at 168 eV, indicating PAH and PSS have been successfully introduced. Furthermore, the surface sulphur/nitrogen atomic ratio (calculated by XPS survey spectra) as function of the number of layers evidenced an alternating regular behaviour showing the PSS and PAH layer deposition. FTIR-ATR showed that the typical absorption bands of PAH and PSS increased with layer number (i.e. SO<sub>3</sub>- stretching vibrations at 1130 cm<sup>-1</sup> for PSS, and

NH scissoring vibrations at 1580 cm<sup>-1</sup> for PAH). Finally, the incorporation of TGF- $\beta$  interfered positively with the processes of cells adhesion and proliferation within the functionalised hydrogels confirming the good biocompatibility of the prepared nanoscale systems.

### *In Vitro* Skin Engineering for Drug Testing: reconstruction of 3D skin equivalents *in vitro* by bioprinting

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There are clear clinical needs for the development of engineered skin models that will better predict efficacy, toxicity and metabolism of candidate drugs or chemical compounds as an alternative to animal testing. However, even though a large variety of 3D human skin models exist, most of engineered skin substitutes do not fully represent the complexity of native skin and they are limited to use of only immortalized cell lines or primary cell cultures of one or two particular cell types [1, 2]. In this work, we present studies for optimizing co-culture/bioprinting conditions and characterization of 3D bioprinted *in vitro* skin constructs (full thickness composed of epidermis, dermis, and hypodermis) with human primary skin cells seeded with hyaluronan-gelatin based polyethylene glycol (PEG) hydrogels. The results demonstrated that bioprinting and 3D co-culture of human primary skin cells did not affect the viability of the cells and the printed skin constructs were well-maintained their original printed tri-layered structures without much cell migration between printed layers. This study proves the feasibility of developing bioprinted multi-skin cell based cellular models *in vitro*, which may be applicable for drug testing.

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### 3D-Bioprinting Induced Shear Stress Strongly Impacts Human MSC Survival and Proliferation Potential

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Shear stress induced cell damage is a major issue in 3D-bioprinting. Using a micro-valve based bioprinter we conducted a study on how shear stress during printing affects cells. We identified three key factors that directly affect the shear stress and investigated their impact on cell viability, proliferation and mesenchymal-marker expression of hMSCs (five independent donors, one to seven days in culture). Experiments with varying printing pressure (0.25–3 bar), varying alginate-hydrogel viscosities (30–300 mPas), and different nozzle diameters (150, 300 and 600  $\mu$ m) were conducted. Using a fluid-dynamical model the shear stress in the nozzle was calculated and correlated with the cell-study results.

Varying the above mentioned parameters we were able to investigate a wide shear stress range (700–11,000 Pa). For shear stresses up to 3,000 Pa the post-printing cell viability was  $97.6 \pm 2.1\%$ . With increasing stress cell survival significantly decreased [ $90.2 \pm 7.6\%$  (3,000–6,000 Pa) and  $73.7 \pm 21.1\%$  (>6000 Pa)]. Furthermore, MSC printed at a shear stress of 2,500 Pa exhibited significantly higher proliferation rates than those printed at shear stresses higher than 3,000 Pa (4,000–10,000 Pa).

The results indicate that shear stress has a strong impact on cell survival and proliferation potential. Interestingly, the measured

effects could mainly be attributed to the level of shear stress regardless of whether the stress was generated by high pressure, by a highly viscous material, or by geometric restrictions. Thus, it is important to reduce the stress level by adjusting the printing parameters in order to achieve high cell survival and proliferation rates.

### Functionalized Electrospun Membrane for Spatial Control of Bone Regeneration

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The potent osteoinductive protein BMP-2 (bone morphogenetic protein-2) is a promising alternative to bone grafting in severe bone loss after trauma or surgery. However, poor dose and delivery control have necessitated usage of supraphysiological BMP-2 doses clinically, leading to inflammation and heterotopic mineralization. While advanced drug-release systems may eventually replace collagen sponge-BMP-2 systems prevalent clinically, determining optimal spatiotemporal bioavailability for regeneration is challenging. Mitigating risks of current BMP-2 therapy by limiting BMP-2 diffusion outside injury sites will improve treatment efficacy and reduce side effects. Hence, we localized heparin microparticles (HMPs), capable of binding large amounts of BMP-2 [1], onto a polycaprolactone (PCL) mesh (HMP-mesh), to spatially retain BMP-2 within tissue injury sites. 5 mg of HMPs were interspersed between layers of PCL during electrospinning using an airbrush. Control meshes were electrospun without HMPs. 6 mm discs from control and HMP-meshes were incubated overnight with BMP-2 (100 ng, 4°C). HMP-meshes bound more BMP-2 (60%) than control meshes (45%; n = 5, p < 0.01). BMP-2-loaded collagen sponges were enclosed within dialysis chambers, and diffusion was permitted only across control or HMP-meshes into 500 µL PBS, recapitulating *in-vivo* BMP-2 delivery. HMP-meshes allowed lower cumulative release of BMP-2 through the mesh than control meshes over 7 days (n = 3, p < 0.05). This study demonstrated the ability of HMP-meshes to bind BMP-2 from solution and limit BMP-2 diffusion from clinically used collagen sponges. Forthcoming studies using supraphysiological BMP-2 doses in a critically sized bone defect model aim to demonstrate the functional efficacy of HMP-meshes for effective BMP-2 retention *in-vivo*.

[1]. Hettiaratchi, *et al.* Biomaterials 35(25):7228–7238.

### Fabrication and Characterization of a Novel Bioactive Poly(ether-ether-ketone) Scaffold for Bone Tissue Engineering

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It is widely accepted that the initial interactions between cells and implant surface are crucial to clinical success. A promising material for bone implants and tissue engineering is polyetheretherketone (PEEK) which has good chemical resistance and mechanical properties similar to those of human bones. However, its hydrophobic and chemically inert surface limits its osteointegration.

Efforts to enhance the latter have thus been undertaken including incorporation of molecules such as hydroxyapatite (HA) or making it porous. The present study aims to demonstrate the biological impact of a process that yields porous PEEK products with high pore density and interconnectivity (US2012323339), and that of its combination with a novel protocol of surface modification through wet-chemistry that enables grafting RGD and OGP10-14 peptidomimetics onto PEEK (US2013172273).

We produced PEEK porous structures by thermally-induced phase separation in combination with a particulate leaching method. Multiple structures with two different pore sizes and porosity higher than

80% were produced, and porosity by itself was shown to influence osteoblast differentiation *in vitro*. Surface functionalization of porous PEEK scaffolds with the RGD/OGP combination was also shown to have an effect on osteoblast proliferation and differentiation according to our *in vitro* results, which led us to perform an *in vivo* assay.

In conclusion, we have developed a process for producing porous PEEK implants as well as a convenient “click” chemistry procedure to modify PEEK surfaces with RGD/OGP peptidomimetics to enhance osteointegration while keeping the bulk properties of PEEK as well as its biocompatibility.

### Mapping the Temporal Impact of 3D Mechanobiology on Mesenchymal Stem Cell Differentiation

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The use of multipotent mesenchymal stem cells (MSC) for regenerative medicine is an important research focus, but much is still unknown about factors that influence their behavior, particularly in 3D systems. Here, we aimed to understand the impact of physical microenvironment on the differentiation of human bone marrow-derived MSC (hBM-MSC) by examining their gene expression over time when cultured in 3D hydrogels. To this end, hBM-MSC were encapsulated in hydrogels composed of hyaluronic acid (HA) and poly(ethylene glycol) diacrylate (PEGDA), and cultured for 3 to 28 days. The stiffness and degradability of these hydrogels were modified by altering the ratio of PEGDA to HA. To test for differentiation, samples were analyzed by quantitative real-time polymerase chain reaction (qPCR) for expression of eight genes encompassing both early and late markers for three classical lineages. These genes were RUNX2, OSX, and OCN for osteogenesis; SOX9, ACAN, and COL2A1 for chondrogenesis; and PPARG with C/EBPα for adipogenesis. Preliminary analysis points to an overall upregulation of the osteogenic differentiation regulator RUNX2, with mRNA levels increasing until a peak at day 14. The expression of PPARG varied with microenvironmental factors, as hydrogels with a lower percentage of PEGDA promoted higher levels of expression. Once complete, this study will succeed in mapping the effects of 3D HA-PEGDA hydrogels on hBM-MSC by linking changes in gene expression with hydrogel physical properties. Moreover, our work may further our understanding of how the *in vitro* microenvironment impacts the behavior of hMSCs and, hopefully, enable their use in reliable therapeutics.

### Reconstructed Glycated Endothelialized and Innervated Skin for Anti-AGE Molecule Screening

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Diabetes is a chronic disease which affects skin. Impaired cells functions and extracellular matrix (ECM) are caused by hyperglycemia through a non-enzymatic glycation reaction between the amine groups of protein and reducing sugars, producing “advanced glycation end products” (AGEs) whose most represented by the Carboxy-Methyl-Lysine. Long term hyperglycemia induces permanent direct alteration, protein cross-link, ECM stiffness and cytoskeleton alteration. Whereas glycation affects both cells and environment, available *in vitro* model include healthy cells cultured in a glycated environment. We developed an innovative tissue-engineered skin combining both a capillary-like and a nerve networks and designed a protocol to induce continuous AGEs formation using glyoxal during 44 days. Progressively alteration of its capillary and nerve networks are observed as epidermal differentiation defect by the lack of loricrin and filaggrin. Addition of aminoguanidine, an anti-glycation compound, prevents all those defects. This tissue-

engineered skin model is the first one to combine a capillary and nerve network and to enable a continuous glycation over a long-term culture period. It is a unique tool to investigate the effects of glycation on skin and to screen new molecules that could prevent AGEs formation.

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### Fabrication of Biomimetic Muscle Flap Composites

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Autologous muscle flap replacement is a routine clinical therapy to treat the volumetric muscle loss (VML), which is limited to sever donor shortage and poor regenerative capacity. Skeletal muscle tissue engineering proposes a promising strategy to address this challenge by fabricating artificial muscle flap analogs. Here we presented a biomimetic method to fabricate muscle flap constructs with cyto-biocompatible hydrogel and nanofibrous polymer film. Photocurable poly(ethylene glycol) (PEG) hydrogel and enzymatically cross-linked gelatin were tested as the muscle body material while polylactic-co-glycolic acid (PLGA) film with the thickness of  $30 \pm 5 \mu\text{m}$  was fabricated by electrospinning. PEG or gelatin solution was cast into the specifically designed PDMS moulds with  $500 \times 500 \mu\text{m}$  groove array. After gelation, electrospun PLGA film was covered on the top surface of PEG or gelatin hydrogel, which was further peeled off from the moulds and wrapped into a cylindrical muscle flap composite. Laser confocal microscope showed the size of both kinds of hydrogel slot is close to the designed value while the swollen ratio for PEG and gelatin hydrogel was  $83\% \pm 3\%$  and  $6\% \pm 2\%$  respectively after cultured with cells in culture media for 7 days. The maximum tensile force of gelatin/PLGA and PEG/PLGA constructs were  $9.465 \pm 0.135 \text{ N}$  and  $9.694 \pm 3.400 \text{ N}$ , which is similar to that of rat gastrocnemius muscle ( $7.170 \pm 0.285 \text{ N}$ ). Structural characterization on the failed samples showed both kinds of hydrogel could tightly bonded with the nanofibrous PLGA film without delamination. It is envisioned that the presented method is a promising way to engineer artificial muscle constructs potentially for VML treatment.

### Development of a Particulate Oxygen Generating Material Platform for Regenerative Medicine Applications: Focus on Utility in Skeletal Muscle Tissue Ischemic Injury

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Oxygen is critical to tissue survival, especially during regenerative stages of cell growth following damage. Tissue trauma can be susceptible to low oxygen environments due to ischemia, which often results in severe physiological changes and tissue death in as little as four hours. In skeletal muscle, prolonged hypoxic conditions result in significant loss of muscle structure and function. Thus, immediate and readily available oxygen is critical during tissue injury, if at the very least, sufficient oxygen to preserve the tissue until a permanent solution is available. Current oxygen treatments involve non-portable devices, such as hyperbaric chambers, or are hemoglobin-based and are not targeted to a specific tissue. We are developing a platform technology for particulate oxygen generators (POGs), with the ultimate goal of preserving skeletal muscle function and homeostasis following blunt trauma that creates partial or complete ablation of vascular supply to the limbs. Under these conditions, oxygen delivery must occur in the ab-

sence of vasculature. We are using peroxide-based oxygen generators derived from sodium percarbonate and calcium peroxide to deliver oxygen to hypoxic tissue. Ongoing studies in rodent models of hind limb ischemia *in vivo* have clearly established proof-of-concept for this approach, and provide a key screen for further refinement and optimization of our technology platform. In addition, dispersion distribution of various POG formulations throughout skeletal muscle *ex vivo* is also being investigated. Future plans include utilization of non-invasive, real-time methods, such as MRI, to further analyze the nature and extent of ischemic injury in the limb *in vivo*.

### Human Demineralized Bone Matrix Granules Enhance Maturation of Osteoclasts in Cell Culture

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Demineralized bone matrix (DBM) is a well know biomaterial used to enhance bone formation. However, little is known about the influence of DBM on osteoclastogenesis and bone resorption. This study is performed in order to verify if the addition of human DBM granules (hDBMg) to the culture medium influences formation and maturation of human osteoclasts (hOCs). Human monocytes (hMo) were isolated (buffy coat) and differentiated to hOCs in the presence of M-CSF and RANKL for 16 days. Therefore the culture was continued in the presence or absence of hDBMg for 9 days. Cell fusions were observed in time-lapse microscope. Hoechst/Actin staining was performed to evaluate osteoclast-like cell morphology. TRAP5b was measured by ELISA. Since it is known that VEGF directly targets osteoclasts<sup>1</sup>, we measured its concentration in the medium in which DBM was incubated (ELISA).

Enhanced number and maturation of hOCs in response to hDBMg was confirmed by more numerous cell fusions, presence of more cells with OC-characteristics actin rings and elevated levels of TRAP5b in the culture with vs w/o hDBMg. VEGF is postulated as one of the mediators of this phenomenon, since we have confirmed substantial release of VEGF from hDBMg into the culture medium.

Therefore we postulate that DBM promotes bone remodeling not only via osteoblast but also via osteoclast stimulation. It may be also a useful tool in a construction of a complex bone niche *in vitro*.

1. Yang Q. *et al.*, *Matrix Biol.*, 27(7):589–9, 2008

### Physical Properties of Enzymatically Cross-Linked Injectable Gelatin Matrices

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Gelatin-hydroxyphenyl propionic acid (Gtn-HPA) is an injectable matrix which can be covalently cross-linked *in vivo* enzymatically by horseradish peroxidase (HRP) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and can serve as an extracellular matrix analog. It has the advantage of providing a 3-D seamless connection between the injected matrix and surrounding healthy tissues, thus facilitating migration of endogenous stem and progenitor cells into the matrix-filled defect. Maintenance of the injected matrix volume is critical. Specific to enzymatically cross-linked systems like Gtn-HPA/HRP/H<sub>2</sub>O<sub>2</sub>, the role of HRP in volume preservation of the matrix was evaluated. Results showed that swelling ratio was dependent on the concentration of HRP, with higher concentrations of HRP resulting in lower swelling ratios consistent with higher cross-linking densities. Also this dependence indicated that the mobility of HRP was limited inside the Gtn-HPA framework even before fully solidification. The ultimate degree of cross-linking density is determined by the concentration of H<sub>2</sub>O<sub>2</sub> and Gtn-HPA, but the concentration of HRP

determines whether this cross-linking density can be achieved. The density of extruded liquid was found higher than theoretical density of the buffer solution by our calculation, which suggested a physicochemical change inside the cross-linked matrix as well as possible leaking of uncross-linked polymer, which supported our hypothesis regarding HRP and cross-linking density. Additionally, the ultrastructure of Gtn-HPA with goat marrow stem cells embedded was directly observed, for the first time, by high resolution electron microscopy without coatings or environmental controls. Extracellular matrix surrounding seeded cells was found remodeled and densified on Day 7.

### Developing Three-dimensional Human Brain Tumor Models for Personalized Drug Screening

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Pediatric cancers remain the leading cause of non-traumatic deaths among children. In an effort to increase survival and decrease the toxicity of current treatment regimens, current research focuses on the aggressive and treatment-resistant cell populations residing within pediatric tumors. Recent evidence indicates that a clonal population of neoplastic cells in the tumors exhibits marked heterogeneity in proliferation and differentiation. In order to target the heterogeneous drug-resistant sub-populations of tumor cells, development of a personalized medicine approach aimed at patient-specific drug screening would be timely. We hypothesize that a three-dimensional (3D) brain tumor model containing patient-specific tumor cells could utilize the self-renewal property of the stem-like cells to generate clonal copies of tumor cells that could be used for personalized drug screening. An *in vitro* 3D human tumor model was generated using silk protein-based scaffolds that were seeded with cells isolated directly from a surgically resected pediatric brain tumor. Initial results indicate key differences between cells in 3D versus 2D cultures. Expression of self-renewal markers was higher in tumor cells cultured in the 3D brain tumor model when compared with conventional 2D culture. Inversely, expression of neural differentiation markers was decreased in 3D systems compared to 2D cultures. The 3D tumor models were maintained for at least one month in culture which would enable development of a platform for high throughput drug screening. The 3D human brain tumor model could serve a useful tool for personalized drug development for pediatric brain tumors.

### The Role of Autophagy on the Differentiation Potential of Adipose Stromal Cell Sheets under Normoxia vs Hypoxia

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It has been demonstrated that the effects of low oxygen tensions in cultures of human adipose stromal cells (hASC) range from increased cell death to changes in proliferation and differentiation potential. Specifically, hypoxia-induced autophagy promotes cell survival as result of Hypoxia Inducible Factor-1 $\alpha$  activation and downstream signalling. In the present project, we intended to verify the existence of a correlation between the levels of hypoxia-induced autophagy and changes in the differentiation potential of hASC sheets. Cell sheets (CS) of hASC were produced and cultured in normoxic and hypoxic conditions (5% O<sub>2</sub>) for 1 to 4 days. At

each time point, samples were collected for western blot and qPCR analysis or further cultured in osteogenic or adipogenic medium. Gene expression analysis of LC3, ULK1, BECLIN1 and ATG5 revealed overexpression of these autophagy players at day 4, independently of the culture oxygen tension. LC3II protein quantification revealed an increase in the autophagic flux at day 4, regardless of the oxygen tension, confirming the qPCR data. This suggests that the state of hyperconfluency of CS, increasing from day 1 to 4, may modulate the autophagic state of cells, masking the effects of low-oxygen tensions. Importantly, CS cultured in hypoxia for 4 days had increased osteogenic differentiation in comparison with day 1 hypoxic samples but decreased adipogenic differentiation, which hints at a possible correlation between higher levels of autophagy and a tendency towards the osteogenic phenotype. This hypothesis is being confirmed using autophagy modulators.

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### Nerve Tissue Engineering using Blends of Polyhydroxyalkanoates for Peripheral Nerve Regeneration

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Polyhydroxyalkanoates blend films with varying ratios of poly(3-hydroxyoctanoate)/poly(3-hydroxybutyrate) (P3HO)/P(3HB) were produced and evaluated to investigate their use as base materials for the manufacture of nerve guidance conduits to assist peripheral nerve repair. Neat films of P(3HO), P(3HB) along with 75:75, 50:50 and 25:75 P(3HO)/P(3HB) blend films were chemically, physically, and biologically characterized. In the surface analysis, the blends exhibited the highest values of roughness compared with the neat films. XRD analysis of the blends showed an increase in the crystallinity with the increase of P(3HB) content. Mechanical analysis showed that 75:25 P(3HO)/P(3HB) and 25:75 P(3HO)/P(3HB) blends presented suitable tensile strength and percentage of strain for their application in peripheral nerve repair.

Live/dead measurement and immunolabelling of NG-108-15 neuronal cells were performed to study cell attachment and differentiation on the evaluated flat substrates using confocal microscopy. Although all of the blends were biocompatible with cultured neuronal cells, the 25:75 P(3HO)/P(3HB) blend showed significantly better support for their growth and differentiation. Aligned electrospun fibres of 25:75 P(3HO)/P(3HB) blend were produced using varying polymer concentrations (5, 10, 15, 20% w/v) under different conditions of voltage (12 kV, 18 kV) and collector speed (1000, 1500 and 2000 rpm). The thickest diameter of electrospun fibres of 25:75 P(3HO)/P(3HB) blend was then chosen as a substrate for cell culture studies using neuronal cells. Live/dead cell tests showed that the growth of cells on the 25:75 electrospun scaffolds was significantly better and cells were more uniformly distributed when compared with 25:75 P(3HO)/P(3HB) blend flat substrate.

### Development of a Three-Dimensional *In Vitro* Model to Study Neovascularization

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One of the greatest challenges currently faced in tissue engineering is the incorporation of vascular networks within tissue-engineered constructs. Knowledge of the major factors that drive vascular ingrowth is key to overcoming this hurdle. The aim of this study was to develop a technique for producing a perfusable, three-dimensional

cell friendly model of vascular structures that could be used to study the factors affecting angiogenesis in more detail. Initially, biodegradable synthetic pseudo-vascular networks were produced via the combination of robocasting and electrospinning techniques. The internal surfaces of the vascular channels were then recellularized with human dermal microvascular endothelial cells (HDMECs) with and without the presence of human dermal fibroblasts (HDFs) on the outer surface of the scaffold. After 7 days in culture, channels that had been reseeded with HDMECs alone, demonstrated irregular cell coverage. However when using a co-culture of HDMECs and HDFs coverage was found to be continuous throughout the internal channel. Using this cell combination, collagen gels loaded with vascular endothelial growth factor were deposited onto the outer surface of the scaffold and cultured for a further 7 days after which endothelial cell outgrowth from the channels into the gel was observed. Furthermore the HDMECs appeared to have formed tubules within the gel. These results show promising steps towards the development of an *in vitro* platform upon which to study angiogenesis.

### The Use of a Novel Clay Hydrogel for VEGF Delivery in an *Ex Vivo* Human Bone Injury Model

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Injectable biomaterials mimic the early stage in fracture healing (hematoma) and afford the sustained release of biomolecules to promote angiogenesis, critical for bone regeneration. The objective of this study was to examine the potential of china clay (Laponite) for VEGF delivery in an *ex vivo* human bone injury model. We have used Laponite together with bone grafts extracted from fresh human femoral heads and engineered as hollow cylinders to resemble a bone injury model. The bone cylinders were perfused with Laponite ± VEGF or Blank cultured *in vitro* (organotypic) or *ex vivo* on chick chorioallantoic membrane as a vascular bed. High resolution computed tomography ( $\mu$ CT) was conducted before and after culture of the bone cylinders to quantify the volume change, followed by histological examination. Viability staining and explant culture showed that the bone tissue remained viable following CAM culture with intimate contact between the CAM and the bone cylinders and the invasion of the chick vasculature into the human tissue and Cathepsin K expression all evidenced by immunocytochemical analysis. Multilevel  $\mu$ CT analysis showed a significant increase in low dense bone (bone deposition) in parallel to a modest decrease in high dense bone (bone resorption) in all treatments. Laponite treatment showed significant bone formation ( $p < 0.05$ ). *Ex vivo* culture of the bone cylinders on the CAM demonstrating significant bone volume change compared to the *in vitro* group ( $p < 0.005$ ). Overall, this *ex vivo* human injury model provides an active bone remodelling process in which Laponite delivery displayed the ability to modulate bone formation

### Development of a 3D *in vitro* Perfused Human Neurovascular Unit-On-Chip

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Currently there is no 3D *in vitro* model that can fully capture the complexity of the human neurovascular unit. Development of such a model will enhance research capabilities and the development of new therapies for central nervous system (CNS) disorders such as Alzheimer's and Parkinson's Disease. Attempts to mimic the 3D *in vivo* environment are still limited to static co-culturing of endothelial cells (EC), pericytes (PC), and neural cells embedded in extracellular matrix (ECM) (1). Although there are interactions between each cell type in the ECM, the vasculature is not fully functional and no true Blood-

Brain Barrier (BBB) is formed. Previously, we have developed a microfluidic platform to generate 3D *in vitro* perfused human microtissues (2). In this study, we have utilized the microfluidic platform to co-culture human EC, PC, and neural cells in a physiological ECM, as a first step in the development of a 3D *in vitro* model of the neurovascular unit incorporating a perfused vascular network with BBB properties. We have optimized the cell culture conditions and the ECM components to stimulate vascular network formation and anastomosis with the microfluidic channels, and to foster interactions between EC, PC, and neural cells in the platform. We are currently determining the role of PC and neural cells in promoting a low permeability vascular barrier.

### Human Skin Cells Modification by a Non-viral Vector System for The Antimicrobial Hcap18/II-37 Expression in a 3D Skin Model

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Skin wounds caused by burns have high global incidence. Inefficient autologous tissue recovery in such injuries increases the susceptibility of patients to infections caused by multiresistant microorganisms, resulting in a high mortality rate [1, 2]. Gene therapy may become an alternative for these patients by using skin biosubstitutes with higher levels of antimicrobial agents than native skin. In this work we have evaluated a strategy of genetic modification of fibroblasts and keratinocytes, in a 3D skin model, in order to over-express the antimicrobial peptide hCAP18/LL-37 [3].

The model was built using a fibrin hydrogel which promotes cell modification and works as 3D scaffold. Fibroblasts and keratinocytes were isolated from human skin biopsies, under prior patient informed consent. Into the fibrin gel, a non-viral system consisting of a polymer/plasmid complex (poliplex) [4] (polyethylenimine/Lentivirus bicistronic vector with tRFP) was used for cell modification. The plasmid used in this complex, allows simultaneous and spatially independent expression of red fluorescent protein (RFP) and the antimicrobial peptide of interest [5].

The plasmid was amplified and encapsulated into the polymer/plasmid complex which showed a particle size of about 200 nm. The complex was lyophilized and incorporated into the hydrogel model. It was determined by the MTT assay, that the concentration of the complex did not generate cytotoxicity on fibroblasts and keratinocytes. Additionally, genetic modification was evidenced by the presence of cells expressing the RFP, which suggests that the hCAP18/LL-37 peptide was also successfully expressed. Subsequent experiments will assess the expression levels of the antimicrobial peptide.

### Accelerated Maturation Of Hesc-cm Within Collagen-based 3D Matrix Upon Niche Cell Supplementation And Mechanical Stretch

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Regenerate injured myocardium with cardiomyocytes derived from stem cells is a promising strategy for myocardial infarction patients, yet maturation of those stem cell derived cardiomyocytes is still one of the main obstacles for clinical application. Facilitated maturation *in vitro* could be achieved by tissue engineering technology, where stem cells further functionalize under physiological-mimicking stimuli within 3D matrix. Here we investigated the parameters with the potential to promote maturation of human embryonic stem cell derived cardiomyocytes (hESC-CM) within 3D collagen matrix.

After harvest cardiospheres differentiated from hESCs between D20 and D30, engineered cardiac tissue (ECT) was fabricated by co-encapsulating digested hESC-CMs with 5% hMSC/hFB in collagen gel and cast into customized PDMS mold, it was allowed to self-assemble into a solid tissue strip with fixed length for 7 days, static or cyclic stretch was applied afterwards. Maturation status of those

ECTs was evaluated by qPCR, immunostaining and mechanical properties.

Niche cell supplementation was found to augment expression of contractile proteins, hypertrophic genes as well as those in charge with calcium handling. Moreover, spreading, modulus and beating metrics were boosted with auxiliary cells as well. Although to a less extent, stretching also significantly impact the maturation with up-regulated expression of Cx45, BNP & SERCA2, better alignment and greater twitch force of ECTs.

The current study demonstrated that maturation of hESC-CM-ECT could be accelerated by the addition of niche cells as low as 5%, while tensional loading could further strengthen the artificial tissue strip into better functional cardiac muscle for regenerative medicine.

### Biodegradable Microspheres for Factor Release and Cultivation of Mesenchymal Stem Cells in a Bioreactor System

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**Introduction:** Expansion of primary human mesenchymal stem cells (hMSCs) in a stirred culture system on biodegradable microcarriers, such as poly(D,L-lactide-co-glycolide) (PLGA) microspheres, offers several advantages due to their properties for factor release, for scaling up 3D cell cultures and for application as injectable scaffold. The objectives of this work were to establish the fabrication of PLGA microspheres as means to deliver CCL25, a potent chemoattractant for hMSCs, and as means to cultivate hMSCs in a spinner flask bioreactor.

**Methodology and Results:** Preparation of PLGA microcarriers using the w/o/w double emulsion and solvent evaporation technique was successfully optimized to achieve a suitable size range, smooth and spherical surface morphology, high encapsulation efficiency and CCL25 release over several weeks. Furthermore, the surface of PLGA microcarriers was coated with poly-L-lysine to yield highly positive zeta potentials and with the matrix molecule fibronectin to achieve efficient adherence of hMSCs similar to commercially available Cytodex™ 3 microcarriers. Cultivation of hMSCs on coated PLGA microcarriers was examined in a spinner flask system over 14 days.

**Conclusions:** Several variables have been identified that enable the successful fabrication of biodegradable microcarriers suitable for factor release and 3D hMSC expansion. Such devices could allow the subsequent injection of expanded hMSCs/factor release particles into the injured tissue site to facilitate regeneration. CCL25-releasing PLGA microspheres could particularly promote recruitment of endogenous stem cells to enhance *in situ* tissue regeneration.

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### Development of an Engineered Intervertebral Disc With Micro-architectures of the Native Disc

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Disc degenerative diseases has been a major musculoskeletal health concern worldwide, causing significant sufferings to the patients and economical burden to the society. While surgical managements involving spinal fusion or replacement prostheses have some efficacy pain management for serious disc degenerative diseases, these treatments often underscore the importance of biological approaches to regenerate the intervertebral disc (IVD). Development of a biological IVD constructs for tissue regeneration has been ongoing for the past decades but most are still inferior to the native disc, thus limiting its advancement towards translational application. In our studies, we developed a strategy to develop an IVD construct that is structurally similar to the macro/micro-architectures of the native

disc. The micro-architectures of the engineered multiple annulus lamellae were first generated with the combination techniques of soft lithography, particulate leaching and tissue culture. Cells encapsulated with hyaluronic acid hydrogel was used to fill up the central of the scaffolds to form the central nucleus pulposus. The assembled IVD constructs were then subjected to differentiation media *in vitro*, and its cellular morphology, cell viability, and biochemical composition were evaluated. The developed IVD construct showed cellular morphologies and biochemical composition that was similar to that of a native disc. Therefore, we had designed and constructed a novel intervertebral disc construct that can potentially become a promising avenue for biological replacement of degenerated IVD disc in future clinical applications as well as a three dimensional tissue model for the pathophysiological study of intervertebral disc.

### Mesenchymal Stem Cell Proliferation on Combined Collagen and Fibronectin Matrix in the Presence of Growth Factors

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**Introduction:** Human bone marrow mesenchymal stem cells (hMSCs) represent an appealing source of adult stem cells for cell therapy and tissue engineering. It has been identified that hMSCs are an attractive cell source for various tissue engineering and play a central role in the repair and regeneration of mesenchymal tissue. In the present study, an attempt was made to determine, hMSCs proliferation in the presence of fibronectin, collagen, TGF- $\beta$ , EGF and IGF by *in vitro* analysis.

**Results:** Proliferation of hMSCs on different concentration of collagen and fibronectin was recorded on Day 0 and 7. The data provides evidence of increased cell proliferation on both substrates. Next, the cell proliferation of hMSCs on different collagen matrix concentrations (2.5, 5, 7.5, and 10  $\mu\text{g/ml}$ ) was recorded on Day 0, Day 3, and Day 7. Cell proliferation seems to reach a plateau when cells are seeded on CL concentration of 7.5  $\mu\text{g/ml}$ . Finally, MSC proliferation on a combined collagen and fibronectin substrate in the presence of different growth factors were examined. Growth factors concentration used were 20 ng/ml each of TGF $\beta$ , EGF, and IGF. Comparing the individual growth factor conditions, TGF $\beta$  demonstrated the highest cell proliferation on Day 3 and 7. On Day 7, in combination, TGF $\beta$  with EGF demonstrated the highest cell proliferation whereas, TGF $\beta$  and IGF showed lowest proliferation of hMSCs. Future studies will include, additional *in vitro* 2D and 3D cell proliferation with growth factors, cell migration, and 3D FACS analysis assays to relate integrin expression with hMSCs differentiation, proliferation, and migration.

### Injectable, Dual-Gelling Hydrogels with Hydrophobicity-Dependent Mineralization for Directing Osteogenesis *In Vitro*

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Reconstruction of complex craniofacial defects remains a challenge as current treatment strategies such as autografts fail to provide adequate healing without invasive surgeries and associated complications. As a minimally invasive alternative, physically and chemically crosslinkable hydrogels based on poly(N-isopropylacrylamide) that allow for injectable administration, rapid *in situ* formation for defect contouring, and degradation for scaffold remodeling were developed. These hydrogels demonstrated biocompatibility *in vivo* and hydrophobicity-dependent mineralization [1].

The objective of this study was to evaluate the efficacy of the hydrogel system to direct osteogenesis of encapsulated mesenchymal stem cells. In a factorial study examining the effects of hydrogel hydrophobicity (10 or 20 wt%), porogen incorporation (0 or 20 wt% gelatin microparticles), cellular predifferentiation stage (osteogenically primed or unprimed), and encapsulation density (15 or 25 million cells/mL), hydrogels and their respective acellular controls were cultured in complete osteogenic media for 0, 7, 14, 21 and 28 days. At each

timepoint, cell viability and osteogenesis were analyzed with Live/Dead confocal microscopy; calcium, DNA, and alkaline phosphatase biochemical assays; and H&E and von Kossa histological staining.

The hydrogels maintained cell viability, and promoted osteogenesis and mineralization over the 28 day period, with significant effects observed with more hydrophobic hydrogels and porogen incorporation. Studies involving the effect of cellular predifferentiation and high seeding densities are ongoing. These preliminary data suggest that injectable, self-mineralizing hydrogels are promising vehicles for cellular delivery in bone tissue engineering.

I. Vo TN, Ekenseair AK, Spicer PP, Watson BM, Tzouanas SN, Roh TT, Mikos AG. J Control Release, <http://dx.doi.org/10.1016/j.jconrel.2014.11.028>.

### Optimization of Lymphatic Endothelial Cell Isolation Towards Autologous Lymphatic Network Replacement

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**Introduction:** Lymphatic vessel deficiencies may result in a disruption of tissue fluid homeostasis, wound healing and immune function. A loss of lymphatic networks is often a sequela of axillary lymph node removal from head, neck and breast cancer patients. We hypothesize that lymphatic network replacement may be possible in conjunction with autologous fat grafting, a rapidly burgeoning reconstructive technique which replaces soft tissue deficits from tumor removal or mastectomy. By isolating large numbers of lymphatic endothelial cells (LECs) from full thickness skin, lymphatic endothelial cells may be delivered in an injectable scaffold, generating new lymphatic channels.

**Methods:** To improve LEC yield above published protocols which scrape the dermis with a scalpel, we compared 3 different enzymatic digestion protocols with variable enzyme concentrations to increase viable cell yield. Dermal samples were incubated for 1 h with Collagenase NB5 (Serva), Liberase TM (Roche) or 0.25% Trypsin (Lonza) at 37°C with shaking.

**Results:** We found that enzymatic digestion of skin samples improved viable cell yield by over 250% when combined with traditional tissue scraping methods. Preliminary results suggest that Collagenase NB5, comprised of Collagenase types I and II with neutral proteases, results in the highest improvement in cell yield producing 600% more cells than Liberase TM or 300% more cells than 0.25% trypsin. Percentage of LECs within the heterogeneous cell population was assessed by CD31, desmoplakin and E-selectin immunofluorescence as well as qPCR for Lyve-1, podoplanin and PECAM.

**Conclusion:** We have validated more efficient methods of LEC isolation for autologous tissue engineering applications.

### Cell-encapsulated Fibrous Scaffolds with Bioactive Glass Addition in Stimulating Osteogenic Responses

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The delivery of cells to the site of defect with controlled phenotypes and microenvironments has become of great importance in tissue engineering. Here we prepared a fibrous core shell like scaffold for the encapsulation of mesenchymal stem cells for bone tissue engineering. The scaffold was obtained by injecting simultaneously alginate and collagen solutions through the core and shell respectively through a concentric nozzle into a calcium chloride containing bath. Bioactive glass nanoparticles (BG) were added to the alginate shell to enhance osteogenic differentiation of the collagen core encapsulated cells while releasing therapeutic ions to the surrounding

tissues. When implanted in subcutaneous rat model, the presence of BG allowed recruitment and penetration of the neighbouring cells into the scaffolds. *In vitro* results showed that the calcium and silicon released from the BG induced osteogenic differentiation of the encapsulated cells, confirmed by significantly enhanced osteogenic protein and gene expressions compared to the pristine core shell scaffolds. Conclusively, the addition of BG nanoparticles enhanced the osteogenic responses of the encapsulated cells while delivering signalling to the surrounding tissues, showing its potential for bone tissue engineering.

### Controlling Single Cell Geometry in 3D

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The fact that cell shape can regulate cell growth and fate is well recognized. However, the majority of these studies have been limited to two-dimensional surfaces. To understand how cell geometry in a three-dimensional (3D) context affects function, the capability of regulating cell shape in well-defined 3D extracellular environments is essential.

In this study, a novel culture system was developed to modulate single cell geometry in such 3D microenvironments. The approach exploits single cell patterning with *in situ* matrix gelation, thus allowing independent control of cell geometry and extracellular matrix. With this approach, a biomimetic 3D environment (i.e. a “basket” enveloping individual chondrocytes *in vivo*) was created for the generation of differentiated chondrocyte phenotype either from de-differentiated chondrocytes or from mesenchyme stem cells. A multitude of interconnected parameters, including cell shape, cell volume and cell-matrix interaction have been investigated in parallel. It was found that a balance between cell spreading area and spherical shape is essential to generate a differentiated phenotype. The approach described here can be readily applied to other types of pluripotent cell and biomaterials, offering a versatile platform in the search for niches towards either self-renewal or targeted differentiation.

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### Mature Chondrocytes Possess “Reserved Stemness” for Efficient Articular Cartilage Regeneration

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**Objectives:** Articular cartilage is not a physiologically self-renewing tissue. Injury of cartilage often progresses from the articular surface to the subchondral bone, leading to pathogenesis of tissue degenerative diseases, such as osteoarthritis (OA). Autologous chondrocyte-based therapies to treat cartilage defects have been developed and used for more than 20 years; however, the challenge of chondrocyte expansion *in vitro* remains. A promising cell source, cartilage stem/progenitor cells (CSPCs), has attracted recent attention. As their origin and identity is still unclear, the application potential of CSPCs is under active investigation.

**Methodology & Results:** Here we have captured the emergence of a group of stem/progenitor cells that were derived from adult human chondrocytes, highlighted by dynamic changes in expression of the mature chondrocyte marker, COL2, and mesenchymal stem cell (MSC) marker, CD146. These cells are termed chondrocyte derived stem cells (CDSCs). The stemness and differentiation status

of CDSCs were determined by physical, biochemical and cell density cues during culture. A low-density, low-glucose 2-dimensional culture condition (2DLL) was critical for the emergence and proliferation enhancement of CDSCs. CDSCs showed similar phenotype as bone marrow mesenchymal stem cells, but exhibited greater chondrogenic potential. Moreover, the 2DLL cultured CDSCs proved efficient in cartilage formation both *in vitro* and *in vivo*, and in repairing large knee cartilage defects (6–13 cm<sup>2</sup>) in 15 patients.

**Significance:** These findings suggest a facile phenotype conversion between chondrocytes and CDSCs, and provide conditions that promote the conversion. These insights expand our understanding of cartilage biology and may enhance the success of chondrocyte-based therapies.

#### Angiogenic Potential of Diaphragm Derived Acellular Matrix Obtained using Detergent Enzymatic Treatment

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Skeletal muscle tissue engineering aims at recreating a healthy functional tissue to treat muscle malformations and diseases. Most of the myopathies display an impairment of the extracellular matrix (ECM), therefore it is necessary to mimic normal tissue composition and architecture to restore functionality, particularly in the diaphragm, an essential muscle for life sustainability. Acellular matrices derived from decellularized skeletal muscle represent a well-known category of ECM-derived scaffolds that adhere to these criteria. Beside the non-immunogenic properties of these scaffolds, already determined *in vivo*, another essential feature for successful engraftment of transplanted organs is blood perfusion. Our aim was to evaluate the angiogenic properties of acellular scaffold derived from mouse diaphragmatic muscle. We decellularized samples via detergent enzymatic treatment, a protocol able to provide nuclear depletion whilst maintaining tissue architecture. A comparison between fresh and decellularized tissue through ELISA test revealed how VEGF, a powerful chemoattractant for blood vessels, was still present after treatment. Chorioallantoic membrane assay confirmed this data as our scaffold attracted a number of vessels comparable to a control loaded with VEGF. Moreover, 15 days after orthotopic patch implantation in a healthy recipient, we found CD31+ cells, small vessels and capillaries vWf+ within the applied patch and in the host diaphragm, especially in the area close to the graft. Further experiments will investigate perfusion and functionality of vessels inside the patch and will analyse which mechanisms and angiogenic pathways are activated after patch transplantation.

#### Engineering Tissues with Zonal Characteristics Mimicking Native Articular Cartilage using Decellularized Cartilage Grafts Seeded with Infrapatellar Fat Pad Derived Stem Cells

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Engineering cartilaginous tissues with collagen structure and mechanical functionality equivalent to the native articular cartilage remains a major challenge. The objective of this study was to explore if re-populating decellularized articular cartilage grafts with adult stem cells can lead to the development of cartilage constructs with structure, composition and depth-dependent mechanical properties mimicking the native tissue. Porcine articular cartilage derived from skeletally immature and mature animals were decellularized using a protocol that significantly reduced the tissue DNA (~88%) and

GAG (~100%) content, while maintaining the collagen content and architecture. These decellularized cartilage grafts were then seeded with human infrapatellar fat pad derived stem cells (FPSCs) and maintained in chondrogenic conditions for 4 weeks. Decellularized grafts supported stem cell attachment, viability and proliferation, with cells seeded in skeletally mature grafts displaying significantly higher levels of proliferation and collagen synthesis compared to those seeded in skeletally immature grafts. FPSCs were also found to align and orient their synthesized matrix in a pattern mimicking the underlying collagen architecture of the decellularized cartilage grafts. The impact of this was that cartilage tissues engineered using skeletally mature cartilage grafts repopulated with human FPSCs demonstrated a collagen structure and content, depth-dependent mechanical properties and a straining softening behavior characteristic of native mature articular cartilage. To the best of our knowledge this is the first time such collagen structure and mechanical functionality have been recapitulated in engineered cartilaginous tissues.

#### Human Primary Osteoblast Behaviour on Bioinert Ceramics with Nano- and Micro-Topography

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**Introduction:** Initial tissue response after surgery determines implant osseointegration. There is evidence that cells respond to micro- and nano-topography on various types of materials. However, the behaviour of human osteoblasts (hObs) on micro and nano-rough bioinert ceramics is still unclear. Here we investigated the effect of micro-roughness or combination of micro- and nano-roughness of zirconia toughened alumina BIOLOX<sup>®</sup> delta (ZTA) on the behaviour of hObs *in vitro*.

**Methods:** Nano-roughness was introduced on as-sintered and micro-rough (Ra=0.13 and 0.77 µm) ZTA discs by selective chemical etching. Controls included as-sintered and micro-rough samples without etching. hObs from femoral heads of patients undergoing hip replacement, ethical approval KEK 2010-0444 were seeded (10000 cells/cm<sup>2</sup>) and samples were cultured in osteogenic medium for 30 days. Analyses included cell attachment, proliferation, alkaline phosphatase (ALP) activity and alizarin red staining.

**Results:** A superior cell attachment was observed on small micro-rough (Ra=0.13 µm) samples, while it was lower on large micro-rough (Ra=0.77 µm) and as-sintered samples. Initial hOb attachment was reduced on nano-rough samples. At later time points, some cell detachment occurred on micro-rough samples, but not on etched samples (except for etched Ra=0.77 µm). Moreover, ALP activity was significantly higher for chemically-etched micro-rough samples compared to only micro-rough samples. Alizarin red staining was similar among groups.

**Conclusions:** Selective chemical etching is a simple technique to produce nano-roughness that effectively reduces hOb detachment on micro-rough ZTA and improves ALP activity *in vitro*.

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#### Transplantation of Adult Human Muscle Progenitor Cells into Rats with Compartment Syndrome Injury

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Compartment syndrome (CS) is a serious complication arising from a variety of skeletal muscle injuries. Cell therapy is being developed as an approach to enhance recovery after muscle injury, however a reliable source of human muscle progenitor cells (hMPCs) for clinical applications is still debated. The objective of this research was to characterize different sources of hMPCs *in vitro* and to test their ability to survive and engraft into host muscle *in vivo* in rat model of CS injury.



hMPCs from three different sources and aged donors were used in this study. Immunofluorescence staining and qRT-PCR were used to examine myogenic marker expression before and after differentiation. Cells were injected into the tibialis anterior muscle of adult male nude rats 4 days after injury. Human specific Lamin A+C and Dystrophin antibodies were used to track the location and engraftment of the hMPCs in host muscle 14 and 28 days after injury.

The three hMPCs showed varied expression of myogenic markers and proliferation ability. All were able to differentiate into myotubes *in vitro* as evidenced by staining for myosin heavy chain and qRT-PCR assays. All injected hMPCs were detected *in vivo* with varied cell migration and fusion with host cells to form chimeric myofibers as detected by human dystrophin staining.

hMPCs of different sources can be expanded while keeping their myogenic differentiation potential *in vitro*. hMPCs can survive, integrate and differentiate into myofibers after transplantation into CS injured muscle. More studies are needed to improve the engraftment potential of these cells.

#### Adipose-Derived Stem Cells Promote Elastin Production by Smooth Muscle Cells *In Vitro* and Slow Aneurysm Enlargement in a Computational Model of Aneurysm Growth and Remodeling

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Stem cell therapy is a potential treatment for abdominal aortic aneurysm (AAA) since stem cells secrete factors that stimulate smooth muscle cells (SMC) to make new elastin (1–5). To test this concept *in vitro*, human adipose-derived stem cells (hASCs) were co-cultured with human SMCs in fibrin gels. Constructs were subjected to 10 days of cyclic uniaxial stretch (10% at 1 Hz) and then imaged with an Olympus multiphoton microscope to visualize elastin via its intrinsic autofluorescence. The average intensity of images was calculated from five representative images of each sample and total elastin was also measured using a ninhydrin assay (6). hASC/SMC co-cultures produced elastin as measured by both techniques. Interestingly, elastin production was also seen in SMC-only constructs that were stimulated with hASC conditioned media, suggesting a paracrine mechanism of action.

To address the question “how much SMC elastin production is necessary to halt AAA progression?” we implemented a computational model of AAA growth and remodeling (7) which captures aspects of AAA progression such as dilation and wall thinning. We modified the published model to “turn on” elastin production (from 2–16x natural degradation rate) after 30%, 40%, and 50% dilation of the vessel. Allowing elastin production universally reduced the aneurysm enlargement rate in a manner that was inversely proportional to the elastin production rate. Intervention at 30% growth of the vessel even caused the aneurysm to begin to contract (i.e. residual tension begins to pull the artery back towards its original size) with most elastin production rates.

#### Anatomically-Shaped Tissue-Engineered Cartilage with Tunable and Inducible Anti-Inflammatory Capabilities

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**Objectives:** Functional replacement of entire articular surfaces is a potential strategy for treatment of the cartilage degeneration that occurs in osteoarthritis (OA). However, the inflammatory environment within an OA joint may inhibit chondrogenesis and induce degradation of native and engineered cartilage, particularly through the action of interleukin-1 (IL-1). The goal of this study was to engineer an anatomically-shaped, functional cartilage construct capable of tunable and inducible expression of IL-1 receptor antagonist (IL-1Ra) in adipose-derived stem cells (ASCs) via scaffold-mediated lentiviral gene delivery.

**Methods:** Large (22 mm diameter) hemispherical scaffolds were fabricated from 3D woven poly( $\epsilon$ -caprolactone) (PCL) fibers using a closed mold thermoforming process. Doxycycline-inducible eGFP or IL-1Ra lentivirus was immobilized to the PCL to transduce ASCs upon seeding. EGFP or IL-1Ra-expressing constructs were treated with 0 or 0.1 ng/mL IL-1 during 28 days of chondrogenesis.

**Results:** Constructs showed even tissue distribution and maintained their hemispherical shape throughout culture. IL-1Ra-expressing constructs produced 900 ng/mL of IL-1Ra, while eGFP-expressing constructs produced less than 0.30 ng/mL of IL-1Ra. IL-1 treatment did not affect glycosaminoglycan and collagen content in either eGFP or IL-1Ra-expressing constructs, suggesting that ASCs have a low sensitivity to IL-1 while undergoing chondrogenic induction. However, IL-1 treatment significantly increased MMP activity in the conditioned media of eGFP-expressing constructs but not in IL-1Ra-expressing constructs.

**Significance:** We engineered an anatomically-shaped and sized, inflammation-resistant cartilage hemisphere that shows promise for total articular resurfacing in an arthritic environment. Tunable production of IL-1Ra from an IL-1-resistant cartilage tissue may have therapeutic effects throughout the joint.

#### Establishing Three-dimensional Co-culture System of Hepatocytes for Drug Metabolism Evaluation

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Liver metabolism plays a central role in drug development in the context of absorption, distribution, metabolism, excretion and toxicity (ADME/Tox). A vigorous *in vitro* model of liver that could recapitulate hepatic phenotype and functionality *in vivo* would exclusively improve the efficiency of metabolism evaluation *in vitro*. Thereafter, much effort recently has been focused on improving reproducibility and standardization of primary hepatocyte cultures with a paradigm shift to 3D culture. This is important because increasing literatures have demonstrated indispensable role of 3D microenvironment in supporting viability and function of hepatocytes *in vitro*. In the present study, an improved 3D co-culture system of hepatocytes was established in which rat primary hepatocytes were co-cultured with two types of predominant hepatic stromal cells on silk porous scaffolds. Silk scaffolds with incorporated extracellular matrix provided a suitable microenvironment for maintaining the viability, morphology and gene expression of the primary hepatocyte *in vitro*. The presence of stromal cells promoted primary hepatocyte to generate cellular aggregates with well-organized 3D architecture after 3 days of co-culture *in vitro*. These aggregates exhibited proper morphology similar to liver tissue *in vivo*. Consistent with their phenotypic appearance, well-maintained functionality of hepatocytes was also observed in the co-cultures, where mRNA expression of multiple CYPs enzymes increased significantly compared to the monolayer cultures. Additionally, this 3D multicellular culture model displayed a physiologically relevant response to model drugs treatment. Thus, this culture system would potentially provide a robust tool for drug metabolism evaluation *in vitro*.

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#### KNEE OA Treated with ÜB Matrix: A Clinical Study

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The treatment of knee osteoarthritis (OA) is often frustrating. Knee OA is a very debilitating condition that adversely affects the quality of life (QoL) of many patients. Several factors have been shown to augment OA, yet the entire pathophysiology remains unknown. knee OA is associated with pain, stiffness and debility. Current treatment approaches do not deter the progression of OA or alter the inflammatory environment of the knee in osteoarthritis. Urinary bladder matrix basement membrane (UBM) has been shown to modulate the immune response locally after injury and induce site

specific remodeling in a variety of tissue systems. This retrospective study is the first to our knowledge to evaluate the effect of intra-articular injection of MatriStem Micromatrix in humans with symptomatic knee OA. A single intra-articular dose of MicroMatrix revealed no side effects or adverse reactions. Significant improvements in pain and functional level were reported one year post treatment. The present study supports the continued use of MicroMatrix for treatment of pain and debility associated with knee osteoarthritis. References 1) Badylak S.F., Gilbert T.W., Immune Response to biologic scaffold materials, *Semin Immunol* 2008; 20 (2): 109–116 2) Rose W., Wood J. D., Simmons-Byrd A., Spievack A.R., Effects of a xenographic urinary injectable bioscaffold on lameness in dogs with osteoarthritis of the coxofemoral joint (hip): a randomized, clinical trial, *Inern J Appl Res VetMed* 2009; 7: 13–22.

### Session: Poster Session 4, Sunrise

**Date and Time: Thursday, September 10, 2015,  
7:00 AM - 8:00 AM**

#### Effects of Pro-inflammatory Cytokine Pretreatment on MSC Potency, Survival, and Migration

S. Kim, B. Milo, J. Frank;

NIH/CC, Bethesda, MD.

Mesenchymal stem cell (MSC) therapy shows great promise in many inflammatory diseases and GVHD by modulating host inflammatory immune reactions. However, the therapeutic potency of MSC in relation to survival and homing of the cells need to be elucidated to improve cell therapy.

Recently, pro-inflammatory activation of MSC with interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and/or interleukin (IL)-1 $\beta$  was reported to improve the efficacy of MSC therapy in various inflammatory disease models. Also, prediction of the MSC potency *in vivo* by measuring TNF-specific gene (TSG)-6 levels of the therapeutic cells was reported.

We used these recent studies to correlate changes in physical parameters and surface markers to cell homing, and to identify pretreatment regimen for optimal potency and survival of MSC in cell therapy. MSC were pretreated with IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , and combination of IFN- $\gamma$  + TNF- $\alpha$  for 24 h. IFN- $\gamma$  + TNF- $\alpha$  combination group resulted in a cell size increase, upregulated cell adhesion surface markers (CD49e, CD49f, CD162, CD11a, CD54) and chemotaxis receptors (cMet, CXCR4), and increased HLA-DR. The combination group showed the greatest immune suppression and potency with upregulation in IDO, CD274, and TSG-6 compared to IFN- $\gamma$  or TNF- $\alpha$  alone. However, pretreatment with TNF- $\alpha$  resulted in the greatest expression of matrix metalloprotein. Migration of cells in wound-healing assay showed the same trend in TNF- $\alpha$ , but not in the combination group. Finally, effects of pro-inflammatory cytokine pretreatment in cell proliferation, xeno/allo-graft blood compatibility, and survival under oxidative stress condition were investigated to identify pretreatment group to maximize therapeutic effect.

#### Wnt-loaded Nanoparticles for Specific Enhancement of Mesenchymal Stem Cell-Mediated Bone Fracture Healing

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With more than 3.5 million osteoporotic bone fractures each year at an EU cost of ~€37 billion/pa (>2 million and ~\$19 billion/pa, respectively, for the US), there is a strong demand for therapies that enhance bone regeneration. Wnt signalling has been implicated in the regulation of mesenchymal stem cells (MSCs) and osteoprogenitors, responsible for bone healing, although Wnt signalling can result in both positive and negative effects on osteogenesis. We hypothesize that timed delivery of Wnt growth factor, specifically to MSCs, can enhance bone regeneration. To ensure spatiotemporal delivery we

entrapped Wnt in nanoparticles, such as liposomes. The size and concentration of nanoparticles was measured by dynamic light scattering, nanoparticle tracking analysis and TEM, and composition was confirmed using gas chromatography. The degree of association of Wnt and various compositions of liposomes was assessed using single molecule spectroscopy (TIRFM and TCCD). We tested their activity *in vitro* and measured the uptake of nanoparticles by MSCs using FACS. The liposome preparations were 100 ± 20 nm in size and of concentration ~10<sup>14</sup> particles/ml. We found a high degree of association between Wnt and liposomes (10x higher vs. control protein, n=3). Encapsulation in DOPC lipid-based nanoparticles enhanced Wnt protein activity, even in a PEGylated to 5 mol% formulation (200 ± 5% p<0.001 and 131 ± 3% p<0.001, n=3, respectively). Critically, more than 80% of particles were taken up by MSCs within 1 h. Forthcoming *in vivo* data will provide evidence as to whether liposomes could be exploited for safe and efficient selective targeting of Wnt to stem cells for bone regeneration.

#### Multilayered Tissue Cartridge using Electrospun Membrane

S. Hong, H. Cho, C. Hwang;

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Fabrication of vascularized thick tissue is one of the major challenges in tissue engineering and regenerative medicine. In this study, electrospun polymer web based multilayered tissue cartridge was tried for quick generation of vascularized thick tissue. Tissue cartridges were prepared by seeding cells on electrospun nanofibrous web on a frame. Mouse fibroblast and endothelial cell were seeded on the electrospun web, and stacked with predetermined order and were punched together to have multilayered disc. Multilayered membranes were cultured *in vitro* or implanted *in vivo* into subcutaneous pocket of nude mice. Assembled tissue cartridges had 3 or 11-layered constructs. Histological staining showed that the cells were distributed along the edge of the constructs *in vivo*. Tissue cartridges with ratio of endothelial cell layer: fibroblast layer = 1:2 showed red blood cells in the middle section of the constructs. High endothelial cell content showed better vascularization. The membrane bonded frame was easy to handle and to be multilayered. However, nanofibrous membrane did not successfully generated vascularized tissues with multilayered configuration, which is a common issue in cell distribution and proliferation in thick scaffold. This frame based tissue cartridge has high potential for automated tissue stacking and thick tissue formation with proper vascularization strategy. Further inspiration may facilitate the successful formation of blood vessels in the thick tissue constructs.

#### Hierarchical Aligned Fibrillar Fibrin Hydrogel Designed for Spinal Cord Injury Repair

X. Wang, S. Yao, X. Liu;

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Biomaterial-based strategies for nerve regeneration have attracted extensive attentions in recent years. In this study, a hierarchical aligned fibrillar fibrin hydrogel was prepared through electrospinning technique and the concurrent molecular self-assembly process at the moment of collection. The aligned fibrin hydrogel that simulated the elasticity and oriented structures of nerve tissue simultaneously provided biomimetically biophysical cues to direct *in vivo* or *in vitro* cells thus constructing nerve-like microtissues rapidly. *In vitro* studies showed that human umbilical cord mesenchymal stem cells could “feel” the cues afforded by aligned fibrin gels and extend along the long axis of the fibers soon after cell attachment and undergo strong neural differentiation after two weeks cell culture in regular growth medium without any soluble growth factors. More interestingly, the rat dorsal root ganglion neurons also could follow the fiber structure in a highly oriented longitudinal manner with three-dimensionally neurite elongation to total 3.92 mm just in 3 days basic culture. In rat T9 dorsal hemisection spinal cord injury, the host neural cells with  $\beta$ -tubulin III-positive migrated into the aligned fibrin hydrogels very quickly. And the axon reconstruction

and locomotor performance was improved starting from 2 weeks after the surgery in the aligned fibrin hydrogel group. Our study suggested that the hierarchical aligned fibrillar fibrin hydrogels will not only have great promising for directing stem cells towards nerve lineage, but also may have great potential applications in spinal cord regeneration.

#### Human Mesenchymal Stem Cells Neuroprotective Effect in a Compartmentalized Neuronal Membrane System

L. de Bartolo<sup>1</sup>, A. Piscioneri<sup>1</sup>, S. Morelli<sup>1</sup>, M. Mele<sup>2</sup>, M. Canonaco<sup>2</sup>, E. Drioli<sup>1</sup>;

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In this work, we developed a compartmentalized membrane system using hippocampal neurons and human Mesenchymal Stem Cells (hMSCs) in order to investigate and clarify hMSC-mediated neuroprotective effects. To elucidate such interaction we use an *in vitro* oxygen-glucose deprivation (OGD) model that mimics the *in vivo* central nervous system insult. Cells were cultured in a membrane system with a sandwich configuration in which the hippocampal cells are seeded on a fluorocarbon membrane, and are separated by the hMSCs through a semipermeable polyethersulfone (PES) membrane that ensures the transport of molecules and paracrine factors but prevents cell-to-cell contact. This system was used to simulate a cerebral ischemia damage by inducing OGD for 120 min. The core contribution of the work highlights for the first time the neuroprotective effect of hMSCs on hippocampal cells in a membrane system. The promising results show that hMSCs secretome factors protect hippocampal cells from OGD insult as evidenced by the sustainment of specific structural and functional cell features together with the development of a highly branched neural network after the damage. Moreover, neuronal cells treated with hMSCs before OGD insult were able to maintain BDNF production and O<sub>2</sub> consumption, and were protected from apoptosis, expressing none of the apoptotic markers that were encountered in neuronal samples similarly insulted, but not treated with hMSCs.

Our compartmentalized membrane system represents a very useful and reliable system for the study of the molecular mechanisms based on such mediated neuro-protection, and for the identification of the factors mainly involved in this mechanism.

#### Nanoparticle-mediated Reprogramming Glial Cells into Non-glial Cells

X. Li<sup>1</sup>, Y. Cheng<sup>1</sup>, K. Kozielski<sup>2</sup>, H. Liu<sup>1</sup>, J. J. Green<sup>2</sup>, H. Mao<sup>1</sup>;

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Central nervous system diseases and injuries are accompanied by reactive gliosis and scarring. Gliosis is a common pathological process that involves the activation of glial cells, including astrocytes, NG2 cells, and microglia, to proliferate and become hypertrophic and dense barrier structures, which are difficult to reverse with existing treatment approaches. Recently, through viral vector-mediated transfection, many studies have demonstrated trans-differentiation across different cell lineages [1], including reprogramming glial cells into neurons through transcription factors, such as SOX2, Neurogenin 2, Mash1 and NeuroD1 [2–4]. Although viral vector-mediated transduction methods have been shown to effectively deliver these transcription factors to astroglial cells to direct reprogramming *in vitro* and *in vivo*, from the clinical translation perspective, non-viral transfection methods are preferred due to the concern of viral vectors regaining reproductive capability or tumor formation through insertional mutagenesis. In this study, we have developed transfection protocols using nanoparticles compacting plasmid DNA with polycations including poly( $\beta$ -amino esters) (PBAEs). First we prescreened a series of PBAE carriers and identified the top carrier with high gene expression efficiency and low

cytotoxicity using EGFP as a reporter gene. Using the optimized transfection protocol, we successfully delivered SOX2 into human astrocytes. SOX2-expressing astrocytes generated a larger number of nestin+ and neuronal progenitors and Tuj1+ pre-mature neurons compared with non-transfected cells *in vitro*. Current study has been delivering specific transcription factors by nanoparticles *in vivo* to genetically engineer glial cells at the lesion site into non-glial cells to improve neural regeneration after brain injury.

#### Effect Of Beta-tricalcium Phosphate Ceramics With Rhbmp-2 for Bone Formation in Canine Alveolar Bone Defect Model

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**Introduction:** The present study focuses on the beta tricalcium phosphate ( $\beta$ -TCP) with rh-BMP2 for bone formation during early period in a canine alveolar model.

**Method and materials:** Artificial mandibular bone defects (8-mm diameter, 5-mm length) were made in the mandible of each dog using a trephine bur with physiological saline cooling under anesthesia at the sites of the tooth extractions.

In total, 5 beagle dogs were randomly assigned to 5 groups.

The implant materials, including beta-tricalcium phosphate and rhBMP-2 (5, 15, 45, 90  $\mu$ g), were filled into left or right mandible defects. 4 weeks after implantation, mandible region are processed for histological analysis including HE and MT staining. Newly formed bones are also evaluated with micro-CT as well.

**Results:** Micro-computer tomography-based evaluation at 4 weeks after implantation showed that advanced bone formation with rhBMP-2 compared with vehicle control. In particular, low doses of rhBMP-2 (15, 45  $\mu$ g) exhibited greater amounts of new bone compared with high doses of rhBMP-2 (90  $\mu$ g). Our prior study demonstrated that 90  $\mu$ g of rhBMP-2 generated higher bone volume at 8 weeks compared with same dose at 4 weeks.

**Conclusion:** In early bone formation period, low doses of rhBMP-2 with  $\beta$ -TCP developed new bone formation compared with high dose of rhBMP-2. However, additional studies will be needed to explore the effects of rhBMP-2 in long term period.

#### An On-Demand Nanoparticle Delivery System for Precise Temporal Presentation of Bioagents

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Natural tissue generation/repair processes are intricately controlled on specific spatial and temporal scales, which motivates the development of tissue engineering devices with precisely controlled delivery of instructive cues. However, the majority of non-invasively activated on-demand delivery systems demonstrate considerable leakiness. We proposed that a more controlled system could be achieved by conjugating bioagents to nanoparticles that remain physically entrapped within hydrogels; ultrasound can then reversibly stimulate their release from self-healing alginate hydrogels.

To test this, PEGylated gold-nanoparticles ('PEG-AuNPs'; hydrodynamic radii  $\sim$  30, 60, 100 nm) were incorporated in calcium-cross-linked alginate hydrogels or microbeads and treated with ultrasound on days 1 or 5. A dramatic increase in the release rate of PEGylated AuNPs was observed (diffusion vs. US-stimulated; day 1  $\sim$  200-fold; day 5  $\sim$  5000-fold). Next, bone morphogenetic protein-2 (BMP-2) was

covalently conjugated to AuNPs via thiol groups in cysteine residues. These were incubated with mouse-MSCs and their osteogenic potential tested using alkaline phosphatase. BMP-2-AuNPs at 100 ng BMP-2/ml enhanced osteogenesis vs. controls (DMEM, osteogenic media, 10 ng/ml unconjugated-BMP-2;  $p < 0.01$ ) and were equally as effective as the maximum dose of unconjugated-BMP-2 tested (500 ng/ml;  $p = 1.0$ ). Finally, we confirmed the ability of these nanoparticles to be entrapped in alginate, released using ultrasound and to maintain their bioactivity (US-released BMP-2-AuNPs vs controls,  $p < 0.015$ ).

This approach to on-demand delivery of bioactive factors allows for more precise control over delivery of bioagents and can be used to enhance temporal profiles of bioagent delivery, while potentially reducing cost and side effects. Furthermore, the favorable physicochemical properties of nanoparticle-based bioagents can be exploited.

#### Placental Mesenchymal Stromal Cells Rescue Ambulation in Ovine Myelomeningocele

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Myelomeningocele (MMC) - commonly known as spina bifida - is a congenital birth defect that causes life-long paralysis, incontinence, musculoskeletal deformities, and severe cognitive disabilities. The recent landmark Management of Myelomeningocele Study (MOMS) demonstrated for the first time in humans that in utero surgical repair of the MMC defect improves lower limb motor function, suggesting a capacity for improved neurologic outcomes in this disorder. However, functional recovery is incomplete and 58% of treated children were unable to walk independently at 30 months of age. In the present study, we demonstrate that using early gestation human placenta-derived mesenchymal stromal cells (PMSCs) to augment in utero repair of MMC results in significant and consistent improvement in neurologic function at birth in the rigorous fetal ovine model of MMC. *In vitro*, human PMSCs express characteristic MSC markers and trilineage differentiation potential. Protein array assays and the enzyme-linked immunosorbent assays (ELISA) show that PMSCs secrete a variety of immunomodulatory and angiogenic cytokines. Compared to adult bone marrow MSCs (BM-MSCs), PMSCs secrete significantly higher levels of brain derived neurotrophic factor (BDNF) and hepatocyte growth factor (HGF), both of which have known neuroprotective capabilities. *In vivo* functional and histopathologic analysis demonstrate that human PMSCs mediate a significant, clinically relevant improvement in motor function in MMC lambs, and increase the preservation of large neurons within the spinal cord. These preclinical results in the well-established fetal ovine model of MMC provide promising early support for translating in utero stem cell therapy for MMC into clinical application for patients.

#### Airbrushed Polymer Films and Nanofibers Embedded with Clay Nanotubes for Tissue Engineering and Drug Delivery Applications

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Halloysite nanotubes (HNTs) are naturally formed alumina silicate clays, which display tubular features. The clay nanotubes can be loaded with a variety of materials for sustained and extended releases and exhibit high levels of biocompatibility. This study showed the multifunctional applications of HNTs embedded in airbrushed polymer scaffolds. Airbrushed PLGA, PCL, PMMA, and chitosan thin films were studied and compared to films that were embedded with HNTs. Scaffold surfaces were monitored with a scanning and transmission electron microscopes. Picosirius red staining and NucBlue assays were used to monitor NIH 3T3 fibroblast adhesion, proliferation, and collagen production on the scaffold surfaces. To show the nanomaterial versatility, HNTs were loaded with methotrexate (MTX) and nitrofurantoin (NF), and embedded within the scaffolds. MTX-loaded scaffolds were monitored with XTT proliferation assays to study the effects on osteosarcoma. NF-loaded scaffolds were examined

against *E. coli* and *S. aureus* through agar diffusion and liquid broth assays. Results showed that NIH 3T3 cellular adhesion, proliferation, and collagen production increased with the addition of HNTs. Additionally, MTX-loaded HNT scaffolds reduced the proliferation of osteosarcoma, and the NF-loaded HNT scaffolds displayed antibacterial activity against gram-negative and gram-positive bacterial strains. SEM images displayed the dispersion of HNTs in the scaffolds, and showed that HNT-nanofibers can be fabricated by adjusting the airbrush needle and tip size. It is suggested that these airbrushed films and nanofibers have potential in tissue engineering, medical device coatings, and drug delivery systems.

#### Engineered Porous Scaffold for Periprosthetic Infection Prevention

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Periprosthetic infection (PPI) is a devastating consequence of implant insertion involving 1% to 5% of procedures. PPI prevention should involve either increasing the rate of new bone formation and the release of antibiotics such as vancomycin. Three-dimensional porous scaffold using HA and  $\beta$ -TCP, with high porosity and interconnectivity (above 50%, pore size 100–300  $\mu$ m) was functionalized with an engineered layer compound of: Pectin (Pec) from citrus peel loaded with Vancomycin (Vca), cross-linked on the surface of scaffold, by Ca<sup>2+</sup> ion released from the ceramic. High solubility of Pec could be allow a high single burst of drug released, therefore more stable polyelectrolyte (PE) in physiological solution was made using Chitosan from white mushrooms. *In vitro* test was performed and have shown that the engineered coating allow a controlled release along 1 week in a therapeutic windows (50 mg/day considering a *in situ* administration). The Agar germ test confirmed that the entrapped antibiotic is able to inhibit the growth and proliferation of *Staphylococcus epidermidis*, and after 1 week of release the system still decreases bacterial growth on the scaffold surface (CFU/scaffold was much lower than control without coating). Since fibroblast from gingiva proliferate faster than osteoblast, the antiadhesive properties of PEI coating was studied in order to avoid fibroblast infiltration in the early stage of implantation, and promote osteoblast proliferation exposing phosphate phase during linear degradation of PEI. A biomimetic bone substitute developed could be an ideal system to struggle against PPI, by inhibiting bacterial growth and stimulating bone formation.

#### Sustained Release of Pro-Angiogenic Peptides from PLGA Nanoparticles for Tissue Regeneration

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Peptide mimetic sequences that recapitulate biological functions of full proteins provide a promising alternative in tissue engineering and regenerative medicine applications due to their inherent ease in synthesis and chemical modification and decreased susceptibility to degradation *in vivo*. Sustained peptide delivery is a key requirement to avoid rapid clearance *in vivo* as well as rapid diffusion from implantable tissue engineered constructs.

The purpose of the study was to promote sustained release of an angiogenic peptide, QK, previously shown to mimic the receptor binding interface of vascular endothelial growth factor using poly(lactic-co-glycolic acid) PLGA nanoparticles (NPs) formed using a water-in-oil-in-water double emulsion solvent evaporation protocol. Alterations in QK release kinetics were achieved through alterations in PLGA copolymer molecular weight (MW) as well as the ratio of lactide to glycolic acid (L-G) in the NPs formulation.

QK loaded NPs allow for sustained release over several weeks and result in a triphasic release profile with an initial burst, 10–25  $\mu$ g of QK per 1 mg of NPs, followed by a lag phase, and first order release. NP formulations with decreasing copolymer MW and fixed L-G ratio

resulted in increases in cumulative QK release. Increases in G content at constant molecular weight also increased QK release. Current efforts are aimed at using this approach for controlled peptide delivery within implantable scaffolds for tissue regeneration.

### Scalable, Modular Bioreactor Systems for Automated Tissue Engineering

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Despite impressive recent achievements progress in tissue engineering lags behind society's high expectations. One reason is that tissue engineering is still a cumbersome process often involving many tedious manual steps. During setup researchers have to manage a wealth of tubes, connectors, containers, pumps, sensors, incubators etc. constituting the bioreactor system while during tissue cultivation they have to spend precious time on tasks like medium exchange or monitoring. Consequently such an approach lacks scalability and reproducibility and represents a huge road block for research efficiency. The few existing bioreactors adopting a scalable approach are typically highly specialized devices for tissue production in the contexts of certain applications. However, no convincing automatic bioreactor systems exist that are flexible enough for research and development.

To address this challenge we have developed a programmable modular bioreactor platform covering a wide range of applications. The chosen approach allows researchers to easily modify the cultivation process without sacrificing automation, scalability and ease of use. Automation helps users to focus on their original medical or biological objective instead of 'babysitting' the process. No specific bioreactor expertise is necessary for its operation, hence the entry barrier to tissue engineering projects is significantly reduced. The presentation sheds light on how better reproducibility and decreased effort per experiment enable systematic scanning for the optimal culture conditions. Further, a clearly defined open interface is proposed empowering users to develop their own compatible system components while benefiting from the application independent basis.

### 3-D Bioprinting of Complex Structure for Engineering Muscle-Tendon Unit

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Three-dimensional (3-D) integrated organ printing (IOP) technology seeks to fabricate tissue constructs that can mimic the structural and functional properties of native tissues. This technology is particularly useful for complex tissues such as those in the musculoskeletal system, which possess regional differences in cell types and mechanical properties. Here, we present the use of our IOP system for the processing and deposition of four different components for the fabrication of a single integrated muscle-tendon unit construct. Thermoplastic polyurethane (PU) was co-printed with C2C12 cell-laden hydrogel-based bio-ink for elasticity on the muscle side, while poly( $\epsilon$ -caprolactone) (PCL) was co-printed with 3T3 cell-laden hydrogel-based bio-ink for stiffness on the tendon side. The construct was elastic on the PU-C2C12 muscle side ( $E=0.39 \pm 0.05$  MPa), stiff on the PCL-3T3 tendon side ( $E=46.67 \pm 2.67$  MPa) and intermediate in the interface region ( $E=1.03 \pm 0.14$  MPa). These constructs exhibit >80% cell viability at 1 and 7 days after printing, initial tissue development and differentiation. This study demonstrates the versatility of the IOP system to create integrated tissue constructs with regional biological and mechanical characteristics for muscle-tendon unit engineering.

### Blood Clot Formed on Rough Titanium Surface Induces Early Cell Recruitment

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The initial contact of blood with biomaterials and subsequent recruitment of inflammatory and mesenchymal stem cells are among the first phases of bone regeneration. The aim of this study was to investigate the migratory potential of rat bone marrow mesenchymal stromal cells (rBMSCs) under the influence of the blood clot formed on implant surfaces. Cell attachment and morphology on the blood clot was observed using scanning electron microscopy and cell proliferation was assessed by the MTT and CyQuant cell proliferation assays. Cytokines released from the blood clot formed on various titanium surface were detected using the rat cytokine antibody array C2 series. Scratch wound assay and transwell migration assay were performed to determine the effect of blood-implant conditioned medium on cell migration and movement. No significant difference was found in cell attachment and morphology on the blood clot formed on smooth and rough surfaces. Increased proliferation of rBMSCs was observed in blood clot on rough surfaces. The profiles of cytokine secretion showed a significant increase of CINC-2 $\alpha$ , IL-2, L-selectin, MCP-1, prolactin AA, and VEGF levels in the elution of blood clot formed on rough titanium surfaces. Significantly enhanced cell migration and wound healing ability were found when rBMSCs were stimulated by the extract from the blood clot formed on rough surfaces. The present study has demonstrated that the rough titanium surface can influence the blood clot formation and properties, which are in favor of cell recruitment for wound healing and tissue regeneration.

### Protective Effect of Telomerase-based 16-mer Peptide Vaccine (gv1001) in Ischemia-reperfusion Injury Rat Model Superficial Inferior Epigastric Island Skin Flap Survivability

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Ischemia-reperfusion injury (IRI) is a constant threat in free flaps and replantation procedures. Telomerase-based 16-mer peptide vaccine in addition to its anti-cancer potential properties, proposed to have anti-inflammatory effect. In this study, we have proved hypothesis that telomerase-based 16-mer peptide vaccine is able to reduce negative effects caused by IRI in rat flap model. For evaluation of telomerase-based 16-mer peptide vaccine, 5x5 cm<sup>2</sup> superficial epigastric artery based island skin flap were dissected in 39 8 weeks old Sprague-dawley rats weighing 180–230 g. Experimental groups were divided into three groups (i.e., Group I; non-ischemia-reperfusion (IR), Group II; IR with saline treated and Group III; IR with 10 mg telomerase-based 16-mer peptide vaccine treated). These drugs were administered intramuscularly directly before and after ischemia. Tissue were analyzed for neutrophils infiltration, cytokine release level, MDA quantity and SOD activity, respectively. Flap survivability was analyzed at 7 days after surgery. Mean flap survival areas in group II and III were calculated to be 34.69%  $\pm$  16.44% and 58.88%  $\pm$  11.44%, respectively. Flap survival area was significantly higher in group III compared to group II ( $p < 0.05$ ). Neutrophil infiltration grade wasn't significantly different between group II and group III ( $p < 0.05$ ). However, MDA quantity and SOD activity were slightly decreased in Group III. Also cytokine release level of group III was revealed significantly lower than group II, ( $p < 0.05$ ). It meant that telomerase-based 16-mer peptide vaccine was able to inhibit skin necrosis in IRI via cytokine (i.e., IL-1, IL-6 and TNF- $\alpha$ ) regulation.

### A Novel Bioreactor Chamber Combining Perfusion And Electrical Stimulation for the Culture, Non-invasive Monitor and Contractility Test of Engineered 3d Cardiac Constructs

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engineering laboratory, IRCCS Galeazzi Orthopaedic Institute, Milan, ITALY.

Tissue Engineering bioreactors represent key device to recreate biomimetic environments to guide tissue development and maturation *in vitro*. In particular, to engineer cardiac constructs, there is a need for platforms that allow to apply stimuli for tissue development and to monitor tissue maturation. In this perspective, we developed a new culture chamber fitting in an oscillating perfusion bioreactor able to culture 3D constructs by coupling bidirectional interstitial perfusion and electrical stimulation, as well as allowing direct optical monitoring and contractility testing during culture. The chamber design was optimized through finite element modeling, in terms of both fluid dynamics and electric fields to ensure a uniform perfusion speed and a linear decay of the electric potential throughout the scaffold.

The chamber, mainly realized in PDMS, was integrated with a silicone tube for culture medium oxygenation and equipped with gold-coated electrodes to supply typical rectangular biphasic waves for cardiac tissue culture and two glass windows for high definition imaging. Two round arrays of pillars hold the scaffold ensuring interstitial perfusion, while allow releasing it for testing, when electrodes pace it, evaluating contractile performance without interrupting the culture. We verified that human bone marrow stromal cells and cardiac cells seeded on collagen scaffolds, subjected to a combination of bidirectional perfusion (speed 100  $\mu\text{m/s}$ ) and electrical stimulation (5 V/cm, 2 ms duration, 1 Hz), maintained the viability over time. The bioreactor supports up to 18 culture chambers in parallel, which independently allow for applied *in vivo*-like multiple stimuli and direct optical construct maturation testing during whole culture.

#### **In Vitro Evaluation of Micrometastasis of Pancreatic Carcinoma Cells**

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Cancer metastasis involves complex events including invasion to the stromal tissue, transendothelial migration (TEM), circulation of cancer cells in the bloodstream, and extravasation to the secondary site. Although *in vivo* and *in vitro* model for observing cancer metastasis have been developed, it has not been clearly elucidated cancer cell-environments and cancer-cancer cell interactions. In this study, we developed a spatio-temporally controlled *in vitro* model mimicking interaction between cancer cell-microenvironment for metastasis of cancer cells. To mimic the *in vivo*-like microenvironments, a capillary level flow was employed to construct endothelial cells barriers in a microfluidic device. Using the microfluidic device, we successfully observed micrometastasis procedures including the invasion to extracellular matrix (ECM), TEM, colonization, and floating carcinoma cells. During TEM, the most round carcinoma cells aggregated in groups, crossed the endothelial barrier, and showed intravasation as a group. After intravasation, these groups of pancreatic carcinoma cells were identified that the carcinoma cells formed colonization in flowing channel, and floated groups in the in flowing medium. In conclusion, we successfully observed the micrometastasis of pancreatic carcinoma cells by using this *in vitro* model.

**Acknowledgment:** "This study was supported by grant (2015-7213) from the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea."

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#### **PLGA Capsulated Porous Silicon Particles for Sustained Intravitreal Delivery of Daunorubicin**

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**Objective:** We aimed to develop a better controlled vitreous release system using PLGA capsulizing Daunorubicin (DNR) loaded porous silicon particles.

**Methods:** Fresh etched porous silicon film was sonicated to produce pSi microparticles. pSi particles were oxidized at 800°C and further silanized for daunorubicin loading. DNR was absorbed into the pSi particles. The DNR loaded pSi particles were divided into two groups: group 1 with PLGA (poly (lactic-co-glycolic acid) coating and group 2 without. For PLGA coating, DNR loaded pSi particles were allocated into 10% PLGA dichloromethane solution and vortex for 20 min. The mixture was dispersed into 2% PVA (polyvinyl alcohol) aqueous solution for evaporation of dichloromethane. PLGA coated particles were characterized under a light microscopy for PLGA capsulation. The particles with or without PLGA were allocated each into three closed vial with 1.5 mL of DPBS and incubated under 37°C on a mini labroller. At designated sampling time points, 1 mL supernatant was collected and the same amount of DPBS was added back into the vial.

**Results:** The DNR loading into pSi particles was determined to be 32.99  $\mu\text{g/mg}$ . Light microscopy showed 80% pSi particles were capsulated by PLGA and the non-capsulated pSi particles had a mean diagonal size of 75  $\mu\text{m}$  (median 68.4  $\mu\text{m}$ ). DNR release from pSi without PLGA capsulation demonstrated a predicted peak concentration of 7200 ng/mL while only 1200 ng/mL for PLGA capsulated pSi. The DNR release profile from pSi particles was typical first-order kinetics while a sustained release mode was achieved through PLGA capsulation.

#### **The Cardiac Regeneration Potential of an Anisotropic Silk Fibroin/Gelatin Scaffold in Combination with Chemically-induced Porcine Mesenchymal Stem Cells**

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The heart has often been a central subject across different disciplines, and this is also the case in physiology. Its role in the human body is of utmost significance - as evidenced by the fact that the lack of cardiac electrical activity is considered to be one of the criterions to pronounce a person clinically dead. Given its importance, its vulnerability is increased by its limited regenerative capability. Dead heart tissue is often replaced by scar tissue, which decreases the heart's efficiency. Different strategies are currently being explored, such as cell-based strategies, gene-based therapies, and tissue engineering regeneration. However, due to the limited clinical success of cellular strategies and the higher risks involved in genetic therapies, the possibility of using cardiac tissue engineering to surgically restore heart function remains to be noteworthy of investigation. A majority of the proposed cardiac tissue engineering solutions are based on isotropic scaffolds which do not effectively mimic the natural tissue anisotropy. On the other hand, a number of proposed anisotropic scaffolds are tedious to synthesize. Current research points to a need to develop a simple fabrication process to develop an anisotropic platform. To address this, we made silk fibroin/gelatin scaffolds using ice templating. Bone marrow-derived porcine mesenchymal stem cells which are pre-exposed to 5-azacytidine and ascorbic acid are then seeded into these scaffolds. The *in-vitro* cardiac regeneration potential of the proposed scaffold will be compared with isotropic scaffolds using the same material using immunofluorescence and gene expression analysis.

#### **Multiplexed Microbioreactor Arrays for Rapid Optimisation of Pluripotent Stem Cell Maintenance and Differentiation**

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Deploying human pluripotent stem cells (hPSCs) in regenerative medicine relies on effective control of their undifferentiated expansion and differentiation into desired lineages. To efficiently produce sufficient, defined cell populations, we must be able to direct stem cell fate choices. This is substantially hindered by undefined culture components, signal crosstalk between multiple exogenous and endogenous factors, and spatiotemporal variations in microenvironmental composition inherent to conventional culture formats. We have developed credit card sized, scalable, valveless, continuous-flow microbio-reactor arrays (MBAs) that both provide a combinatorial set of exogenous factor compositions, and allow controlled accumulation of paracrine factors. These arrays have been used to survey up to 8100 individual, perfused cellular microenvironments in parallel. Through screens of pluripotency maintenance and differentiation of hPSCs into primitive streak, cardiac and kidney cells, as examples, we demonstrate the unique ability of this platform to separate, visualise, identify and modulate paracrine effects that are not otherwise readily accessible with standard culture formats. Culture conditions optimized with the arrays have been shown to readily translate to conventional static culture protocols, leading to improved cardiac and kidney differentiation. With our latest generation 8100-chamber MBA we have assessed the impacts and interplay of developmental factors on proliferation of hPSC-derived cardiomyocytes, exemplifying the utility of the device for patient-specific early drug stratification. These multiplexed MBA platforms decipher factor interplay and signalling hierarchies that control stem cell fate, and are applicable as a universal microenvironmental screening platform for bioprocess optimisation, media formulation design, quality control for cellular therapies and cell-based drug screening.

#### The Physical Properties and *In Vivo* Bioactivities of the Silkworm-cocoon-derived Membrane

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The purpose of this study was to evaluate silk membrane (SM) for application in guided bone regeneration (GBR). Silk cocoons are primarily composed of the proteins fibroin and sericin. We have developed a simple and green processing technique to obtain the thin membrane from cocoons for GBR. SM are classified by their origin from the cocoon layers e.g., inner, middle, and outer. They were compared with commercially available GBR membranes such as collagen membrane (CM) and polytetrafluoroethylene membrane (PM). For the comparison, scanning electron microscopy, FT-IR analysis, tensile strength evaluation, and the animal study were done. The physical properties of SM were comparable to the commercially available GBR membranes. The  $\mu$ -CT analysis showed significantly higher bone volume in the middle of SM group ( $P < 0.05$ ). On the histological exam, there was no foreign body reaction in SM group and showed significantly higher new bone formation. In conclusion, the SM group could be used for GBR technique.

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#### The Use of Star Poly(D,L Lactic Acid) Polymers for Controlled Drug Release

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An important credential for well controlled drug delivery systems is the exhibition of a defined, consistent drug release performance, often with zero order behaviour. Biodegradable drug delivery systems provide the potential to greatly reduce the need for multiple drug administration treatments, reduce the amount of drug required, increase the efficacy of drug action and tissue targeting and provide safe, alternative administration techniques for drugs commonly delivered per os. For many drug formulations however, achieving reliable delivery profiles from common drug delivery systems proves challenging due to issues with drug solubility and very commonly the effects of burst release behaviour are observed, leading to a quick loss of active material, shorter therapeutic performance and more importantly, potential localised cytotoxic effects. In attempts to overcome these common issues observed with poorly soluble drugs, we have assessed the use of poly (D,L lactic acid) (PDLLA) polymers, synthesised with star architectures and processed in the form of solvent cast substrates and emulsion-formed nanoparticles for the delivery of the HMG-CoA reductase inhibiting drug, simvastatin. The system shows excellent solubility of simvastatin and results have shown slower degradation rates and more controlled, consistent drug release profiles for star polymers relative to linear polymers of comparable molecular weights. *In vitro* cell culture of these materials with human osteoblast cells has shown excellent biocompatibility and the preservation of drug activity over time with results showing the potential of statins as a therapeutic agent for degenerative bone disorders.

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#### Kidney Regeneration with PLGA/Mg(OH)<sub>2</sub> Scaffold

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Poly(lactic-co-glycolic acid) (PLGA) is a useful biomedical material, but acidic by-products during *in vivo* degradation are serious concern, because it is an inflammatory reaction source. In this work, magnesium hydroxide, Mg(OH)<sub>2</sub>, was adapted to neutralize the acidic environment, and manufactured Mg(OH)<sub>2</sub> incorporated PLGA scaffold (PLGA/Mg(OH)<sub>2</sub>) expecting reduced inflammation and provide host stem cell recruitment. The PLGA/Mg(OH)<sub>2</sub> scaffold (5 × 2 × 2 mm<sup>3</sup>) was implanted into partial nephrectomized area, and *in vivo* histological, immunohistochemical, renal cell differentiation of the recruited host stem cell, pro/anti-inflammatory factors and fibrosis related gene and protein expression levels were evaluated. The PLGA/Mg(OH)<sub>2</sub> scaffold showed low inflammatory response, which means that this scaffold have effective neutralizing ability of acidic environment. These benefits result stimulated host cell recruiting, which was revealed by regenerated glomerular number and renal differentiation related gene expression. The glomerular number per unit area (magnification, X100) of the PLGA/Mg(OH)<sub>2</sub> scaffold was 14.67 ± 1.53, and PLGA scaffold was 10.00 ± 2.00 at week 12 (control, 9.33 ± 1.15). The expression levels of stem cell and renal differentiation markers in the PLGA/Mg(OH)<sub>2</sub> scaffold were higher than that of the PLGA scaffold for 12 weeks experiment. Therefore, incorporated Mg(OH)<sub>2</sub> into the PLGA scaffold resulted the reduced inflammatory responsibility and the enhanced renal reconstructivity, so, the PLGA/Mg(OH)<sub>2</sub> scaffold could be one of the promising scaffolds for renal regeneration. (NRF-2014M3A9D3033887)

#### Fibrin Gels as Degradable Matrices for Adipose Stem Cells Encapsulation in Peripheral Nerve Repair

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Fibrin is one of the main proteins found in the developing matrix at the site of a nerve injury. Its formation regulates the re-organization and regeneration of the tissue. However, fibrin may inhibit Schwann cell differentiation and nerve remyelination in the long term, therefore it is usually considered only as a "transient component" of the

extracellular matrix. Fibrin-based gels have been used in tissue engineering to deliver growth factors and cells for specific applications. Hereby, we demonstrate the suitability of fast-degrading fibrin gels to encapsulate adipose-derived stem cells (ASC). The latter were found to be able to differentiate in Schwann cells and prevent neuron apoptosis. Cell viability and metabolic activity were monitored for up to 5 days, by which the gels started to degrade. Very few dead cells were observed after 24 hours of culture in the gels, with an increase of encapsulated cells mortality along the gel degradation. However, once released from the gel, ASC maintained their proliferative activity and were still able to grow in their surrounding environment. *In vitro* experiments using DRG explants also demonstrated that fibrin gels do not hinder the formation and sprouting of neurites, nor the migration of endogenous satellite cells from the tissue towards the gels. These preliminary results pose high promises for these hybrid gels to be used as transplantation matrices in peripheral nerve repair and support tissue regeneration.

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#### Epithelial-Myofibroblast Co-Culture: Exploring Cross Talk at the Epithelial-Mesenchymal Barrier

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A functional syncytium of specialised smooth muscle like fibroblastic cells (myofibroblasts) reside subjacent to the intestinal epithelium. Myofibroblasts have been shown to be significant in the differentiation and regulation of the epithelial layer. This ultimately affects the barrier properties of epithelia and furthermore is not limited to the intestinal epithelium. 2, 4. Nanofibre scaffolds, produced by electrospinning, which mimic the basement membrane and extracellular matrix structures found *in vivo* represent a growing area of interest for recreating anatomically relevant matrices for cell culture. Recent literature suggests that subtle environmental cues can alter the functionality of the Caco-2 model, 3, 6, 7 and could drive the development of more physiologically relevant cell based *in vitro* tools.

The effect of co-culturing myofibroblasts with routinely used intestinal epithelial cell lines (Caco-2 and HT-29) were investigated alongside Calu-3 (lung airway epithelial cells) and the barrier properties in terms of electrical resistance and molecular flux across the epithelial barrier were studied.

Results demonstrate the nanofibre scaffold as a suitable substrate for cell culture. Monocultures of the epithelial cell lines demonstrated lower resistance values when cultured on nanofibre substrates than their Transwell<sup>®</sup> equivalents suggesting a potentially more permeable model. It was also found that the different cell types responded differently to the myofibroblast co-culture, Caco-2 demonstrating reduced and Calu-3 showing enhanced barrier integrity with respect to trans-epithelial electrical resistance. The effect on the barrier integrity was further investigated by establishing the permeability of a panel of drugs and fluorescent probes in all of the developed systems.

#### Assessment of Matrix Organization and Cell Metabolic Changes During the Early Stages of Osteoarthritis using High-Resolution Multiphoton Imaging

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**Objectives:** Multiphoton microscopy is a noninvasive and sensitive means of imaging tissue explants and living animals. The objective of this study was to assess changes in the matrix organi-

zation and the cellular metabolic state during the onset of osteoarthritis (OA) using a combination of second harmonic generation (SHG) and two-photon excited fluorescence (TPEF) imaging relying on entirely endogenous sources of contrast.

**Methods:** Knee cryo-sections from mice were acquired 1–10 weeks post OA-inducing surgery (destabilization of medial meniscus, DMM) and corresponding sham surgery of the other knee. They were imaged using SHG and TPEF. 3D stacks of SHG images revealed the organization of collagen fibers, and the corresponding TPEF images revealed information regarding the cellular content of two metabolic co-enzymes, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). A novel algorithm was used to characterize the 3D organization of collagen fibers, while cellular metabolic status via the redox ratio, defined as the ratio of FAD to the sum of NADH and FAD fluorescence.

**Results:** 3D collagen fiber orientation analysis revealed that the directional variance of the fibers within the superficial cartilage zone increased over time for both knees; however, that of the DMM knee was significantly higher than that of the sham-operated knee ( $p < 0.05$ ). TPEF results showed a significant difference in the redox ratio between DMM and sham treatment groups as OA progressed ( $p < 0.05$ ).

**Significance:** Multiphoton microscopy provides sensitive assessments of OA progression, which may provide key insights for improving OA diagnosis and treatment.

#### Recapitulating Cranial Osteogenesis with Neural Crest Cells in 3-d Microenvironments

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Tissues comprise cells and intra- and inter-cellular components that are highly organized both at the organismal level and in the developmental time. Models and biomaterials recapitulating the complexity of native tissues and enabling precise control over the microenvironment are becoming essential for pre-clinical tests of therapeutics. Neural crest cells (NCC) can differentiate into osteogenic cells that, in turn, generate cranial skeletal tissues in the vertebrate head. Cranial bones, which cover the skull, face and jaws, thus, have distinct developmental origins from the rest of bones in the body. We described a strategy to develop an *in vitro* platform to study the developmental biology of cranial osteogenesis. Here, we directly osteo-differentiated NCCs in *in vitro* 3-D bioengineered microenvironment. Cells were encapsulated in gelatin-based hydrogel and cultured up to 3 weeks. This platform allows to successful differentiation of NCCs, which start to express osteogenic markers in the developmental sequence of intramembraneous ossification. We observed a decrease in the expression of early osteogenic marker Runx2 as opposed to increased levels of mature osteocyte and osteoblast markers such as osterix, osteocalcin, osteopontin and bone sialoprotein. We have analyzed the ossification of the secreted matrix with alkaline phosphatase and quantified the newly secreted hydroxyapatite. The FESEM analysis of the bioengineered constructs revealed the native-like osteocytes, osteoblasts and cranial bone tissue morphology with canaliculi-like inter cellular connections. This platform provides a broadly applicable model to potentially study diseases involving primarily embryonic disorders, where direct diagnosis and adequate animal disease models are limited.

#### Skin-on-Chip: Integrating Skin-Tissue and Microsystems Engineering

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**Problem:** All skin disease has an underlying immune component e.g. skin-cancer, fibrosis, allergy. Animal models or simple petri-dish tissue cultures are inadequate for studying these complex human interactions. Microsystems engineering offers dynamic control of the tissue microenvironment, enabling better *in-vitro* models.

**Objectives:** We aim to create a microsystem suitable for studying interactions between immune cells migrating into and out of human skin tissue.

**Methodology:** A device has been designed with two channels separated by a microporous membrane. The membrane supports tissue culture on both sides to mimic the blood vessel-skin tissue interface through which immune cells migrate. Using a numerical model we designed the microfluidics system to mimic blood flow. Novel waferscale microfabrication and laser machining were employed to create interchangeable membranes and microfluidics device. For preliminary device validation we performed microbead flow experiments. Biocompatibility was validated by culturing skin-equivalent and endothelium on the membrane (primary keratinocytes, fibroblasts, HUVECs). The morphology was compared to native skin (histology). To validate that cells may migrate through the membrane, a simple chemotactic assay was performed using THP-1 monocytes.

**Results:** We show 1) measured flow resembles designed flow; 2) the cultured tissues closely resemble native tissue; and 3) immune cells migrate through the membrane.

**Significance:** The realized microsystem is suitable for immunological assays involving skin tissue. Future steps will incorporate immune cells into the skin equivalent, and integrate biomarker readouts through *in-vitro* microscopy. This approach enables detailed, systematic analysis of skin biology and, using patient derived material, patient specific skin disease.

#### Culture and Characterisation of Naive Bone Marrow-derived Cells Encapsulated in Fibrin

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**Objectives:** Bone marrow-derived mesenchymal stem cells (BMSCs) exhibit diverse phenotypic changes during monolayer expansion, leading to efforts focused on developing 'one-step' intraoperative therapies using mononuclear cells. In order to better understand the biology and regenerative potential of mononuclear cells, including the naive BMSC population, we require suitable culture systems.

**Methods:** Freshly isolated, human bone marrow-derived mononuclear cells (hBM-MNCs) were cultured in fibrin gels for 2 weeks with/without FGF2. Viability was assessed by Live/Dead staining at day 7 and 14. Cells were characterised by cell-surface marker profiling (CD45, CD73, CD34, CD146, NG2, PDGFr $\beta$ ). Cell cycle analysis was performed using an 5-ethynyl-2'-deoxyuridine incorporation assay. CFU efficiency of retrieved cells was determined at day 7 and 14. Immunohistochemistry for Ki67 protein and histology was performed.

**Results:** Mononuclear cells, comprising mixed cell populations, were cultured for up to 14 days. Discrete populations including MSCs, pericytes and haematopoietic stem cells were maintained in relative quiescence for the first 7 days of culture. The CFU efficiency of enzymatically retrieved MSCs was significantly higher at day 14 compared to day 0; and was FGF-2 dependant.

**Significance:** Fibrin gels provide a short term 3D culture system for hBM-MNCs. Discrete cell populations, could be maintained in relative quiescence for 7 days. This system may provide a novel platform with which to study primitive mononuclear cell populations and aid the development of both intraoperative and MSC homing therapies.

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#### Effects of Epicatechin, a Crosslinking Agent, on Human Dental Pulp Cells Cultured in Collagen Scaffold

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**Aim:** The purpose of this study was to investigate the biologic effects of epicatechin (ECN), a crosslinking agent, on human dental pulp cells (hDPCs) cultured in collagen scaffold. Furthermore, the role of extracellular signal-regulated kinase (ERK) was investigated as a mediator of the differentiation.

**Methods:** To evaluate the effects of ECN on proliferation of hDPCs, cell number counting was performed by using optical and fluorescent microscope. Alkaline phosphatase (ALP) activity, alizarin red staining, real-time polymerase chain reaction (PCR), and western blotting was performed to assess odontogenic differentiation. The compressive strength and setting time of collagen scaffold containing ECN was measured. Differential scanning calorimetry (DSC) was performed to analyze the structural change of collagen in the presence/absence of ECN.

**Results:** ECN per se increased ALP activity, mineralized nodule formation, and mRNA expression of dentin sialophosphoprotein (DSPP), a specific odontogenic-related marker. Furthermore, ECN up-regulated the expression of DSPP in hDPCs cultured in collagen scaffold. ECN activated extracellular signal-regulated kinase (ERK) and the treatment ERK inhibitor (U0126) blocked the expression of DSPP. The number of cells cultured in the ECN-treated collagen scaffolds significantly increased compared to the cells in control group. Compressive strength of collagen containing ECN was increased and setting time was shortened in dose-dependent manner.

**Conclusion:** Our results revealed that ECN promoted proliferation and differentiation of hDPCs. Further, the differentiation was regulated via the ERK signaling pathway. Change of the mechanical properties and structure are related to the cell fate. Collectively, ECN treatment is desirable for dentin-pulp complex regeneration.

#### Reconstruction of Large Mandibular Defects using Tissue-Engineered Autologous Free Tissue Flaps Generated from *In Vivo* Bioreactors in an Ovine Model

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The reconstruction of large bony defects in the mandible presents many challenges to surgeons, such as sourcing appropriate tissue for transfer and successfully restoring facial aesthetics. In order to circumvent donor site morbidity and also produce tissue with geometries that conform to the mandible, we have designed a strategy for producing tissue-engineered flaps at an ectopic *in vivo* site distal to the mandibular defect. Bioreactors, or chambers with custom-designed geometry, can be implanted with an open face against the periosteum of the rib. After several weeks, progenitor cells from the periosteum migrate into the bioreactors and generate tissue to fill their dimensions. This tissue is vascularized and can be transferred as a free flap to the mandibular defect. In an ovine model, we compared the use of morcellized autograft (AG) to synthetic ceramic graft (SG) as scaffold material for the bioreactors by analyzing chamber contents by micro-computed tomography (bone volume/total volume, trabecular number, trabecular separation, trabecular thickness), histomorphometry, and RT-qPCR for osteogenic markers after 9 weeks of bioreactor implantation. Tissues generated with SG were transferred into a large mandibular defect as either a vascularized flap (n=3) or avascular graft (n=3) for reconstruction. We demonstrated that AG resulted in significantly greater bone volume/tissue volume within bioreactors compared to SG by microcomputed tomography analysis after 9 weeks of implantation. From a clinical standpoint, sheep tolerated mandibular reconstruction using these tissues as either vascularized flaps or

avascular grafts. These data validate this strategy in a clinically-relevant large animal model.

### DNA Minicircle Vector Technology: A New Solution to Overcome Plasmid Size Restrictions on Magnetic Particle Based Gene Delivery to Neural Stem Cells

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Safe, efficient and sustained gene transfer to neural stem cells (NSCs)-a key transplant population- is a major goal for regenerative neurology and for *ex-vivo* gene therapy. Current approaches used to genetically engineer these 'hard-to-transfect' cells rely heavily on viral methods, which have significant drawbacks for clinical translation (safety and production issues). However, non-viral methods such as electroporation and lipofection demonstrate low transfection and high toxicity, especially in primary cells. Here, we utilise magnetofection (magnetic nanoparticles deployed with oscillating magnetic fields) to safely, efficiently and reliably transfect primary murine NSC monolayers. Using reporter gene delivery, we observe an inverse relationship between transfection efficiency and plasmid size. This presents a major challenge for therapeutic biomolecule delivery which inevitably results in increased plasmid size. To address this critical issue, we present a novel solution using minicircle DNA technology. Minicircles are small vectors devoid of a bacterial backbone offering significant clinical benefits (safety, efficient gene transfer and long-term biomolecule expression). Notably, we report >40% transfection efficiency with minicircle reporter gene delivery, the highest non-viral transfection levels reported to date in primary NSCs. Further, we engineered minicircle constructs to express Brain-derived Neurotrophic Factor (BDNF), a neuro-therapeutic biomolecule. For the first time, we demonstrate 1) safe therapeutic gene transfer to NSCs; 2) high therapeutic transfection efficiency (>30%); and 3) sustained BDNF secretion. Therefore, fusion of minicircle and magnetofection technologies offers an attractive gene transfer methodology of wide benefit to the neural tissue engineering community.

### The Effectiveness of using a Bio-3D Printer in the Development of a Scaffold-free Regenerative Vascular Graft

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Recently the investigation of regenerative medicine has become more extensive. Nonetheless, small caliber synthetic vascular grafts and/or functionally biological grafts are not clinically available due to the inherent complexity of these technologies, although large artificial conduit grafts consisting of polyester or polytetrafluoroethylene are clinically used.

We used a "Bio-3D Printer" (Regenova<sup>®</sup>, Cyfuse Biomedical K.K., Japan) to assemble multicellular spheroids (MCS) for constructing scaffold-free tubular tissue. The "Bio-3D Printer" skewers MCS into needle-array according to a three-dimensional structure pre-designed on a computer system. In this system, the MCS were aspirated by a robotically controlled fine suction nozzle from the 96-well plate and inserted into the needle-array made of multiple medical-grade stainless needles.

The reasons for the superiority of constructing a vascular graft using a Bio-3D printer are: 1) the structure does not contain external foreign matter, which may imply good resistance to infection, and is constructed entirely from viable cells, 2) this formation of biomaterials is designable and the diameter size can be modified, which is suitable for the target for the recipient, 3) multiple structures can be joined together

to achieve the required length, and 4) the structures generated using a Bio-3D printer have excellent reproducibility and can be produced rapidly beyond conventional bioengineering techniques.

The scaffold-free vascular grafts made of MCS using a Bio-3D printer were successfully implanted in rat models in an acute study. Currently, a large animal study is underway. Further studies are promising for the clinical application of this novel technology.

### Light-based Bioprinting of Native Silk

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Silk is undergoing a renaissance, in particular garnering researcher's interest in the fields of biomaterials and tissue regeneration [1]. Yet despite the impressive diversity of silk applications, the manufacturing processes are yet to be fully exploited and the potential of the materials to be fully explored. Currently, most silk bioprinting techniques involve a complex multi-stage process to prepare silk solutions, prior to any structuring [2]. In this research, we have opted to investigate the potential of native silk, extracted straight from the silk gland, which has not undergone such complex processing and is as close to the gold standard of natural silk as possible. We present a direct route for silk bioprinting using photo-absorbents where microfabrication techniques including direct laser writing and digital micromirror device based micro-stereolithography were used for the fabrication of various microstructures down to a few microns in scale. We show that silk structures can be bioprinted from native silk solution down to 0.3 wt% by combining with various photo-absorbents. The partial structural conversion (i.e. solidification) of the silk solution was confirmed spectroscopy and microscopy. The results of this study indicate that complex silk structures for various biomedical and tissue engineering application can be controlled through light-based solidification.

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### Optimization of the Autologous Rat Model for Adipose Stem Cell *In Vivo* Research

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**Introduction:** There is an increasing demand for the animal models for preclinical testing of stem cell therapies. Being the "classical" laboratory animal, rat is a model for many human diseases which allows for relatively low-cost experiments. Our aim of present study was the optimization of the rat model for testing autologous ASC applications for tissue regeneration.

**Materials and Methods:** Laboratory WAG rats (inbred strain) were used throughout experiment. The autologous rat model consisted of animals having resected, under general anesthesia, the cervical/interscapular fat deposit and left for 4 months upon final examination. The control groups consisted from: (1) sham-operated rats, (2) rats used for ASC examination parallelly of fat collection in experimental group, and (3) untreated rats examined after 4 months. In all groups fat volume in interscapular/cervical, perirenal, gonadal and inguinal fat deposits, the quantity and quality of ASCs, CFU-F

numbers, and cell surface markers CD11b, CD45, CD29, and CD90 were examined.

**Results and conclusions:** Autologous adipose tissue collection resulted in isolation of  $5 \times 10^6$  ASCs per animal. After 4 months, animals of experimental group did not differ in quantity and quality of ASCs from controls. Fat content in cervical/intrascapular area was reduced when compared to controls, being partially compensated by higher density of ASCs. It may be concluded, that our model is suitable for experiments based on the autologous applications of ASCs in rats.

Experiment was approved by the Local Ethical Committee.

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### Osteogenic Differentiation of Dental Pulp Stem Cells and Laser Irradiation

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Dental Pulp Stem cells (DPSCs), has been used on bone tissue engineering studies. Several studies have shown that low level laser irradiation has beneficial effects on bone regeneration. The aim of this study was to examine the “*in vitro*” effects of low level laser irradiation at different power densities and irradiation time during DPSCs osteogenic differentiation.

DPSCs were obtained from deciduous teeth of 10 Cleft Lip and Palate patients. Appropriate osteogenic medium was used during 21 days and the strains DPSC were exposed to a 660 nm and 20 mW laser every day in three groups of power density and irradiation time (group 1 : 5 J/cm<sup>2</sup> for 5 seconds, group 2: 10 J/cm<sup>2</sup> for 10 seconds, Group 3: 20 J/cm<sup>2</sup> for 20 seconds). Control group did not receive irradiation. Cell differentiation was evaluated by alizarin red and the quantification of calcium deposition was measured by enzyme-linked immunosorbent assay (ELISA) at day 21.

All 10 DPSC strain have positive reaction for mesenchymal stem cell antigens (CD29, CD90, CD105, CD73, CD166), and negative reaction for hematopoietic (CD45 e CD34) and endothelial cell markers (CD31). After induction to osteogenic differentiation under appropriate cell culture conditions all 10 DPSC strains produced calcium deposition. Laser therapy significantly stimulated DPSCs differentiation independently of laser power density. The optimal laser power density was 20 J/cm<sup>2</sup> to increase the osteogenic differentiation.

In conclusion the use of low level laser irradiation during osteogenic DPSC differentiation increase the osteogenic potential of DPSC.

### 3D Printing of Microspheres for Tissue Engineering Scaffolds

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Microspheres have tremendous potential as a scaffold material for tissue engineering applications due to their capability of encapsulation and controlled release of factors that assist tissue regeneration in the desired fashion. Gradient scaffolds consisting of multiple types of microspheres can release different factors at different sites of the scaffold. Current microsphere scaffold production methods, however, cannot address the need for internal architectures to meet specific requirements in different scaffold regions, e.g., for mechanical properties or porosity. We combined 3D printing and microspheres to create scaffolds with defined internal architectures and tailored placing of materials, intended for bone/cartilage interfaces.

Poly(lactic-co-glycolic acid) microspheres were mixed with alginate to create a highly viscous suspension, which was manually expressed through a syringe needle to test feasibility. Subsequently, scaffolds were fabricated using a RepRap printer equipped with a syringe extruder.

The matrix of the printed material dried and hardened quickly through evaporation of water. This allowed to print a porous “green” body, which was then further stabilized by sintering. The amount of alginate played an important role on the suspension’s viscosity and drying time to fabricate a stable construct without sagging in unsupported areas.

This is the first demonstration of direct 3D printing of microsphere based scaffolds. This adds a high degree of freedom for the fabrication of such scaffolds with local definitions for mechanical properties, porosity, focal placement of phases, and controlled release of encapsulated factors.

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### Cold Atmospheric Plasma (CAP) Modified 3D Printed Scaffolds for Human Mesenchymal Stem Cell (hMSC) Differentiation

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3D printing is a new fabrication method for tissue engineering. The significant advantage of this new fabrication method is that it can precisely control scaffold architectures at the nanoscale. However, scaffolds not only need 3D biocompatible structures, but they require mimicking of the extracellular matrix properties of the tissue they intend to replace, such as stimulating bone tissue formation [1]. In order to achieve this, the objective of the present *in vitro* study was to use cold atmospheric plasma (CAP) to modify a 3D printed scaffold’s surface roughness and chemical composition [2]. Under the optimized CAP conditions, the water contact angles on a poly-lactic-acid (PLA) scaffold dramatically dropped from  $70 \pm 2^\circ$  to  $24 \pm 2^\circ$  after CAP treatment. In addition, surface roughness also changed after CAP treatment via atomic force microscopy (AFM) measurements. The roughness (Rq) of the untreated scaffold, CAP treated scaffolds for 1, 3, and 5 min were 1.168 nm, 10.45 nm, 22.87 nm, and 27.60 nm, respectively. This study then showed that both hydrophilicity and nanoscale roughness changes of these scaffolds played an important role in enhancing cell attachment and bone cell functions. Those promising results suggest that CAP surface modification may have potential applications previously not thought of for enhancing bone tissue engineering applications.

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### Strontium Incorporated Nano-scale Pores on Titanium Implant Surface for Rapid Osseointegration

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More and more evidences indicate that angiogenesis process in bone and osteogenesis are coupled. Blood vessels play an important role in promoting bone healing because that they are not only transport essential oxygen and nutrients, but also can deliver osteogenic cells and even secrete osteoinductive growth factors to the region of bone defect. Therefore, we propose here a concept that the combined angiogenic/osteogenic ability of dental implant surface would be favorable for rapid osseointegration.

We fabricated a multi-functional titanium surface using a modified micro-arc oxidation (MAO) technique with a high-conductivity solution and low operating voltage. The new TiO<sub>2</sub> layer displayed uniform hierarchical micro/nano-topography and homogeneously distributed nano-scale pores, unlike those different sized micro-scale holes generated by traditional MAO technique. The nano-pores

structure not only directly promoted cells adhesion and differentiation, but also could deliver growth factors to enhance its biofunctionalization. Moreover, after strontium ions incorporation, MAO-Sr coating showed obvious angiogenic and osteogenic effects on canine BMSCs via MAPK/Erk and PI3K/Akt signaling pathways. In details, the MAO-Sr coating facilitated their osteogenic differentiation and promoted angiogenic growth factors secretion to recruit endothelial cells and form blood vessels. On the large animal implantation models, the MAO-Sr coating significantly enhanced fast bone formation within initial six weeks. The *in vivo* performance of the MAO-Sr coated dental implants on rapid osseointegration was comparable to that of commercially available ITI implants. These results therefore suggest a novel implant coating to achieve rapid osseointegration by enhancing both of angiogenesis and osteogenesis.

### Behavior of CMPCs in Unidirectional Constrained and Stress-free 3D Hydrogels

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Cardiomyocyte progenitor cells (CMPCs) are a candidate cell source for cardiac regeneration therapy. Although, like other stem cells, after cell transplantation into the heart, cell retention and differentiation capacity of CMPCs is low, probably due to the mechanical loads imposed by the heart. A possible solution to overcome this problem is to combine cells and biomaterials, since encapsulation of CMPCs may stabilize survival and proliferation. By combining CMPCs and hydrogels more insight into the behavior of CMPCs upon encapsulation in a biomaterial and/or after injection into the beating host tissue can be gained. Therefore, we cultured CMPCs in unidirectional constrained and stress-free 3D collagen/Matrigel hydrogels. Our data suggest that CMPCs in a 3D environment are viable and keep their cardiac progenitor profile for at least 9 days. Moreover, the 3D environment induced the cardiomyocyte differentiation potential of CMPCs and increased their expression of matrix producing and remodeling genes. Constraint of 3D hydrogels causes CMPCs to become readily mechanosensitive. They respond to the constraint by orienting in the direction of the constraint. Furthermore, constraint appears crucial for cardiac marker expression. In summary, our data demonstrate that culturing CMPCs in a 3D environment since encapsulation of CMPCs enhance the differentiation and remodeling capacity of the cells, which may improve efficiency of cardiac stem cell therapy.

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### Jet-sprayed Hybrid Nanofibrillar Matrices with Controlled Deposition and Delivery of Nanoparticles

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**Objectives:** Combination of biodegradable nanoparticles (NP) with nanofibrillar (NF) matrices to localize and deliver active biomolecules is a sensible approach to modulate cell environments along tissue formation. Since jet-spraying creates nanofibers by air-flow diffraction of polymer solutions, we hypothesized that NP could be permanently deposited onto NF surfaces during their formation by co-projection. Alternatively, we postulated that electrostatic inter-

actions would allow NP post-adsorption and controlled release from highly porous NF matrices.

**Methodology:** Poly(lactic-acid) (PLA) fluorescent NP were synthesized by nano-precipitation and co-projected (paintbrush) during jet-spraying of poly(lactic-co-glycolic-acid) (PLGA) NF. Alternatively, NP aqueous solutions were incubated with jet-sprayed PLA matrices, either blank or poly-lysine (PLL) pre-coated. NP and NF structure, distribution, efficiency of NF/NP association and NP release were determined (DLS, SEM and fluorescence quantitation).

**Results:** NP co-projection during jet-spraying efficiently deposited fluorescent NP (254 nm, zeta potential - 67 mV) on fiber surfaces as droplets-like structures. The adjustable amount of NP fully covered NF (305 µg/mg of NF), while matrices remained highly porous and interconnected.

Incubation of negatively charged NP with jet-sprayed PLA NF matrices either blank (negative surface) or pre-coated with PLL (positive surface) resulted in complete and homogeneous fiber covering, although in lower amount than co-projection (38 and 83 µg/mg for blank and PLL-coated NF). However, NP release could be modulated (up to 4 days), unlike co-projection, where NP remained bound to the NF.

**Significance:** Controlled NP association and release from NF matrices allows providing defined functionality to inert structures. Ongoing experiments with protein-loaded NP will further confirm their safety and resulting bioactivity.

### Multi-functional Materials and Materials Processing for Nerve Guidance Scaffolds

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Approximately 1.2 million Americans are disabled by spinal cord injuries (SCI). Approximately 500,000 surgeries are performed each year to repair peripheral nerve injuries (PNI). While physical rehabilitation can improve functional recovery, in some cases, the lack of spontaneous recovery requires interventional technology. The purpose of this work is to develop an acellular therapy to bridge the nerve gap. The approach consists of multifunctional nerve growth scaffolds (NGS) providing physical cues to guide axons toward distal targets while simultaneously eluting growth factors to stimulate growth. Progressing toward clinical relevance, the focus now turns to material selection, engineering, and NGS design. In this work, the integration of FDA approved materials into NGS is discussed, e.g. synthetic polymers and hydrogels. Details describing mechanical properties, degradation rates, cell attachment, and integration into various NGS designs will be presented. Specific attention to porosity control and the effect on stiffness and strength is a focus. Optimizing these properties enable thin-wall NGS designs to maximize open channel volume. Drug delivery of brain derived neurotrophic factor (BDNF) from pH-triggered polyelectrolyte functionalized hydrogel NGS will also be discussed. The goal is to achieve a 50 ng BDNF/ml per day dose response for two weeks to stimulate axon growth. Though not the emphasis of this paper, SCI and PNI *in vivo* efficacy test data will also be presented.

### 3-D Printing Fabrication for Periodontal Complex Regeneration; Bone-PDL-Cementum Regeneration Platform Developments

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**Introduction:** The physiological periodontal functionalities to support tooth-structures are provided from spatiotemporal organization of fibrous connective tissues, periodontal ligament (PDL)<sup>1</sup>. Recently, we have investigated the freeze-casting fabrication method to manage pore angulations in gelatin scaffolds with structural similarity to healthy PDL<sup>2</sup>. However, due to poor physical/mechanical

properties under masticatory/occlusal loadings, mechanically-improved architectures were required. We here develop supportive scaffolds, engineered periodontal unit (EPU) with the spatial compartmentalization for periodontal complex; cementum, PDL, and bone.

**Experimental Methods:** Based on micro-CT image-dataset of extracted teeth, multi-interfacial scaffolds were designed for cementum, PDL, and bone using CAD. After a 3-D printer manufactured wax molds, poly-ε-caprolactone was casted for semi-porous PCL scaffolds. They were embedded into gelatin and freeze-casting fabrication was utilized to create directional pores. Pore angulations were analyzed using micro-CT and mechanical evaluations were performed. *In-vitro* and *in-vivo* experiments are performed to evaluate PDL alignments and tissue formations.

**Results:** In micro-CT, gelatin structures in PDL interfaces had longitudinal pore architectures with submicron-scaled diameters ( $54.21 \pm 26.93 \mu\text{m}$ ), a high porosity ( $88.03 \pm 4.73\%$ ), and pore angulations, which is similar to healthy PDL. Moreover, EPU and gelatin-embedded EPU had significantly improved mechanical properties. *In-vitro* study demonstrated specific-directional cell orientations and organized cell collectiveness in cementum and bone regions.

**Conclusion:** EPU provides spatiotemporal compartmentalization for periodontal complex neogenesis and PDL regeneration platform for specific, functioning fibrous orientations.

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## Free Volume: The Key Parameter for Molecular Transport in Tissue Engineering Materials

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Mass transport has been identified as one of the most crucial pending challenges that hinders progress in tissue engineering. Free volume is a key parameter in physical, nanomechanical and diffusional properties of polymers, biomembranes and other biomaterials. Positron annihilation lifetime spectroscopy (PALS) is a unique technique for measuring the free volume void sizes and distributions inside soft matter. Several studies have shown the potential of PALS in biophysics and polymer research. In this presentation we are introducing the concept of free volume in biomaterials and discuss the results of PALS in combination with other techniques in biopolymer-based scaffolds. In addition to contribute to a better understanding of the molecular transport in scaffolds for tissue engineering, the approach used here can serve as a framework for rational design of these materials.

## Tenocytic Differentiation of Adult Stem Cells Cultured in a Mechanostimulator

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Cyclical mechanical stimulation for up to two weeks in a customized bioreactor has induced tenocytic differentiation of rat adult stem cells seeded on a decellularized human umbilical vein (HUV). Mechanostimulation (0.5 cycle/min - 2 cycles/min at 2% strain for 30–120 min/day) induced increased cellularity, improved mechanical properties, and tendon-like extracellular matrix appearance. A 0.5 cycle/min frequency and 30 min/day stimulation resulted in the highest cell proliferation after 1 week. Histology showed an increase

in fibril quantity and diameter with alignment in the direction of stretching. In addition, tendon ECM-related genes such as collagen, biglycan, and elastin were upregulated compared to static controls. Overall, long term culture of adult stem cells with mechanostimulation produced increased tenocytic differentiation in cell/HUV constructs denoting the importance of extended periods of mechanostimulation and possibly the need for extensive ECM remodeling before the cells can reach the tenocytic phenotype. When the mechanostimulation profile has been modified using 3–12 short 10 minute periods of stimulation in a day instead of a continuous 30–120 min/day at 2% strain using 1 cycle/minute the tendon ECM-related profile was altered. Adding a tenocytic cellular extract to the cell-seeded decellularized HUV had a strongly mitogenic effect after 2 weeks of culture (150% improvement compared to dynamically cultured constructs without extract). The constructs with extract had higher ultimate tensile strength (37%) compared to dynamically cultured constructs without extract. Dynamic culture with the addition of tenocytic extract generate great promise as a strategy for tendon regeneration in adult stem cell seeded decellularized HUVs.

## Targeted Delivery of Liquid Micro-Volumes into the Lung

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The ability to deliver drugs to specific sites in the lung could radically improve therapeutic outcomes of a variety of lung diseases, including cystic fibrosis, severe bronchopneumonia, chronic obstructive pulmonary disease, and lung cancer. Unfortunately, conventional methods for pulmonary drug administration (e.g., intravenous, oral, and inhaled administration) lack the ability for precise, localized delivery of exact doses of drugs to target regions in the lung. Here, we describe a novel approach to deliver soluble reagents in the form of microliter-volume liquid plugs to targeted branches of the pulmonary airway tree (e.g., upper airways, small airways, or the most distal alveoli). In mathematical modeling and experimental studies, we developed a method for introducing micro-volume liquid plugs into an upper airway and advancing these plugs to a specific location in the airway tree by air ventilation. Plug motion induced by the airflow results in the deposition of liquid film onto the lung epithelium in target airways. With the specific conditions (i.e., plug volume, ventilation regime) determined by our mathematical model, we confirmed delivery of a liquid plug into a target airway in both the conducting and respiratory zones of the rat lung using three different *in vivo* imaging modalities. We propose that instillation of micro-volume liquid plugs into a ventilated pulmonary airway could be an effective strategy to deliver exact doses of drugs to targeted pathologic regions of the lung, especially those inaccessible by bronchoscopy, to increase *in situ* efficacy of the drug, improve pharmacodynamics, and minimize systemic side effects.

## Silver Nanoparticles Embedded in a Cryogel Matrix and Their Potential Application in Wound Healing

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Bacterial burden in wounds is one of major obstacles that prevent wound closure and regeneration. The antimicrobial activity of silver nanoparticles is well known. However, some bacterial strains are able to develop resistance. One way to solve this problem is to increase the antibacterial action of nanoparticles. For this purpose, we decided to study the application of microwaves on silver

nanoparticles. To increase the availability of this effect, nanoparticles were incorporated into a cryogel matrix, which large pores. Initial cytotoxicity studies were also conducted.

To prepare cryogel, acrylamide and N,N'-Methylenebisacrylamide were mixed in 4:1 ratio (w/w) in distilled water to obtain 6.3% concentration (w/v). After addition of silver nanoparticles, TEMED and ammonium persulfate, the mixture was placed into a cryobath at  $-20^{\circ}\text{C}$  for 5 hours and then into freezer at  $-20^{\circ}\text{C}$  for 12 hours. These materials were assessed with SEM. Cryogel, nanoparticles, and cryogel with nanoparticles were tested for cytotoxicity using MTT assay on fibroblast cell culture.

Allowing polymerization reaction to occur at freezing conditions leads to the formation of medium to large pores in the polymer matrix. Ice crystals that form during freezing melt at room temperature and leave empty spaces, pores. Morphological assessment showed that nanoparticles were attached to pore walls of the cryogel. This way, the contact area between nanoparticles and wound exudate was increased. Cytotoxicity studies showed no toxicity either from cryogel or nanoparticles or both of them together. The obtained results demonstrate a great potential of cryogel with incorporated silver nanoparticles as a wound dressing.

### Regeneration of The Anterior Cruciate Ligament using a Silk-fiber Based Scaffold - Histological Results

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**Purpose:** The regeneration of ligament tissue relies on scaffold devices that provide mechanical stability while providing the optimal environment for the tissue to grow. It was the aim of this study to show if a novel silk-fiber based scaffold is able to initiate ACL regeneration under *in-vivo* conditions.

**Methods:** 31 mountain sheep were divided into 2 experimental groups. After removing the natural ACL, in 14 animals, an ACL reconstruction was performed using the scaffold alone (SA), in 17 animals the scaffold was seeded with autologous adipose derived stem cells (CS). Unrestricted weight bearing was allowed immediately. After 6 months and 12 months respectively, half of the animals were sacrificed and their ACLs were removed for histological workup.

**Results:** After 6 months, ligament tissue regeneration was seen in both groups. The CS-group demonstrated higher cellularity and more tissue than the SA-group. The percentage of silk-fibers was lower in the CS-group. After 12 months, the differences in cellularity as well as in tissue regeneration were not significant anymore. Also the percentage of silk-fibers showed further degradation in both groups without significant differences between CS and SA.

**Conclusion:** The ACL regeneration under *in vivo* conditions using a silk-fiber based scaffold seems to benefit from additional cell seeding within the first 6 months. After 12 months, this advantage was not seen anymore.

### In Vivo Potential of Injectable Microspheres for Regeneration of Tissues of the Dentin-Pulp Complex

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**Objective:** One of the most important challenges in dental Tissue Engineering is the development of a suitable material for the generation of tissues for dental therapeutic needs. Several pathologies may affect the functionality of the dentin-pulp complex and the current endodontic treatments are based on the use of inert filling biomaterials that do not promote tissue regeneration. The objective

of this work is to evaluate the *in vivo* potential of fibrous (NF-MS) and smooth (SS-MS) nano-structured injectable microspheres based on poly (L-lactic acid) to regenerate tissues of the dentin-pulp complex.

**Methods:** Extracted human teeth were collected and prepared by sectioning the crowns and preparing the root canals. Then, 0.3 mg of NF-MS or 3.0 mg of SS-MS were used to fill the canals. After this, shaped roots were grafted at the inter-scapular area of nine 6-week-old Fox 1 nu/nu immunodeficient athymic mice. Histological and immunohistochemical analysis were performed after 7, 14 and 28 days of implantation.

**Results:** NF-MS and SS-MS did not show any signals of chronic inflammation and were able to induce the synthesis of proteoglycans, especially after 28 days. Moreover, injectable microspheres revealed strong positive DMP-1 expression after 14 days in SS-MS. Similarly, collagen type I expression was positive in all samples with higher expression in SS-MS.

**Conclusions:** Our results suggest that injectable microspheres could promote *in vivo* regeneration of tissues of the dentin-pulp complex.

### Effects of Thermosensitive Hydrogel Containing Ferulic Acid for Corneal Wound Healing

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The cornea is an avascular tissue on the ocular surface of the eye, and its integrity is vital to normal vision. Oxidative damage to cornea can be induced by ultraviolet B light and alkaline chemical burn which may cause vision loss or blindness. Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) (FA) belongs to the polyphenolic compounds and possesses an excellent antioxidant property. In the present study, we are going to investigate the effects of FA in corneal epithelial cells under oxidative stress and then evaluate the possible therapeutic effects of thermosensitive FA-loaded hydrogel on corneal wound healing. The results of crystal violet assay demonstrated that 200  $\mu\text{M}$  of FA might be the threshold dose to treat rabbit corneal epithelial (RCE) cells without cytotoxicity. The results of chemiluminescence assay suggested that 25  $\mu\text{M}$  FA might enough to terminate the free radical reaction caused by 200  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>. In the post-treatment model, the results of mRNA gene expression showed that the inflammation-level (TNF- $\alpha$ , IL-1 $\alpha$ , TGF- $\beta$  and MMP-9) was significantly decreased. The results of annexin v and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining showed that the post-treatment of FA on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress RCE cells could decrease the apoptosis. After the alkali burn in rabbit cornea, the area of positive fluorescein staining was significantly decreased in the post-treatment of FA-loaded hydrogel group. The results of study suggest that the FA-loaded hydrogel may have potential applications in corneal wound healing in the near future.

### Promotion of the Osteogenic Differentiation Potential of Human Inferior Turbinate-derived Mesenchymal Stem Cells by Mechanical Memory

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Human turbinate-derived mesenchymal stem cells (hTMSCs) can be potentially used as a source of adult stem cells for therapeutic application due to their easy availability, cultivation, and high proliferative ability. We evaluated the capacity of hTMSCs for osteogenic differentiation. We hypothesized that different surfaces of the

nano-pore surface plate affect the morphology, proliferation, and osteogenic differentiation of hTMSCs.

In this study, we used human turbinate-derived MSCs after the second passage. hTMSCs were seeded in flat surface plates and nano-pore surface plates. The seeded hTMSCs were assessed using a scanning electron microscope (SEM) and focal actin staining to determine the morphology. The proliferation of hTMSCs was evaluated by measuring the cell metabolic activity using a Cell Counting Kit-8 (CCK-8). The media used for osteo-differentiation was changed every 2–3 days. Osteogenic differentiation of hTMSCs was identified using alkaline phosphatase (ALP), alizarin red S staining, and vonkossa staining. Real-time quantitation of mRNA of COL1A1, osteocalcin, BMP-2, osterix, and bone sialoprotein were also performed. There was no significant difference in the proliferation of seeded hTMSCs on a flat surface plate and those on a nano-pore surface plate. However, high osteogenic differentiation was observed using alkaline phosphatase (ALP), alizarin red S staining, von Kossa staining, and real-time PCR of seeded hTMSCs on the surface of a nano-pore plate. These findings suggest that hTMSCs are apparently redirected toward the osteogenic phenotype in an *in vitro* culture under specific conditions using bone-formation stimulating factors. Further, the osteogenic potentials observed were superior to other tissues originating from MSCs.

#### Controlled Release Kinetics of Vitamin-Loaded Poly(glycerol sebacate)

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The *in vitro* degradation profile of poly(glycerol sebacate) (PGS) was studied using thermoset PGS and a PGS-coated PET textile mesh. In addition, the *in vitro* release kinetics of PGS were determined using PGS loaded with vitamin B<sub>12</sub> at 1% and 3% w/w (thermoset) and 3% w/w (PET coating). Infrared microscopy shows even distribution of the vitamin within the PGS matrix at both concentrations. After an initial bolus response in the first four days the degradation profile exhibits surface erosion characteristics, both as a thermoset and as a coating, illustrated by the linear mass loss and decrease in mechanical strength over time. The vitamin-loaded samples also experienced a bolus release in the first few days, followed by a linear vitamin release over time signifying zero-order release kinetics. The percentage of vitamin released was independent of loading concentration, and a linear vitamin release was seen in the PGS thermoset and coating. As a comparison, the *in vitro* degradation characteristics were investigated of a textile mesh coated with PLGA and as opposed to bulk eroding polymers like PLGA, the surface erosion degradation mechanism of PGS makes it a more favorable candidate for controlled release.

#### Effects of Age-Related Changes in ECM upon Host Macrophage Responses

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Macrophage polarization has been demonstrated to be a critical factor in the successful remodeling of extracellular matrix-based biomaterials (ECM) and degradation products of ECM have been shown to promote a unique M2-like phenotype. The objective of the present study was to investigate the contributions of source animal age in determining macrophage responses to decellularized scaffold materials.

Porcine small intestine submucosa was produced from 12 week, 26 week and 52 week-old animals. DNA, collagen, GAG, and growth factor content were assessed. Bone marrow derived macrophages from 8 week-old mice were exposed to M1 (IFN- $\gamma$ /LPS) or M2 (IL-4)-polarizing cytokines or ECM degradation products for 24 h. Additionally, cells were exposed to ECM for 24 h then challenged with M1/M2 stimulus. Macrophage phenotype was assessed using immunolabeling, qRT-PCR, phagocytosis and nitric oxide assays.

DAPI staining and gel electrophoresis showed effective decellularization of tissues. Increases in collagen and decreases in GAG and growth factor content were observed with increasing source

animal age. In all assays, ECM exposure produced an M2-like phenotype with a trend towards increased M1 phenotypes with increasing ECM age. ECM harvested from older animals promoted significant increases pro-inflammatory phenotypes following M1 stimulus and young ECM promoted significant increases in anti-inflammatory phenotypes following M2 stimulus.

These results show a compromised ability of ECM to support the polarization of macrophages to anti-inflammatory phenotype with age. Ongoing studies suggest a link between the observed differences in macrophage response to ECM and the accumulation of advanced glycation end products (AGEs) within tissues over time.

#### Strong Protein Hydrogel from Bombyx mori Silk Fibroin

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Hydrogels, which are well-known to be similar to the macromolecule-based extracellular matrix, show great promise for applications in biomedical field. Silk proteins, due to their excellent biocompatibility, biodegradability, and minimal allergenic and immunological response, have attracted much attention in the area of biomaterials in recent years. However, the use of silk protein-based hydrogel is severely limited because of its long gelation time and poor mechanical properties. Here, we report a facile way to produce strong silk protein hydrogel simply by mixing regenerated *Bombyx mori* silk fibroin (RSF) solution and surfactant. For instance, when RSF content is 12.5%, surfactant sodium dodecyl sulfonate (SDS) concentration is 20 mmol/L, the compressive modulus (compression = 50%) and tensile modulus of the hydrogel is 1.39 MPa and 1.53 MPa respectively, which is close to some natural tissues in the body, such as cartilages, tendons and ligaments. We find other anionic surfactant (for example, sodium dodecyl benzene sulfonate, SDBS) and non-ionic surfactant (for example, polyethylene glycol octylphenol ether, Triton X-100) also produce similar strong RSF hydrogels, but cationic surfactant cannot. The strength of the RSF hydrogel increases significantly with the increase in protein content, while does not much relate to the surfactant concentration. Such a strong RSF hydrogel may have great potential to be used as a multi-functional biomaterial, for instance, a magnetic hydrogel by carrying Fe<sub>3</sub>O<sub>4</sub> nanoparticles.

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#### Craniofacial Osseous Reconstruction using *In-Situ* Tissue Engineering; Superior Outcomes with Minimal Morbidity

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Osseous defects of the craniofacial skeleton regardless of etiology prove to be not only deforming but functional limiting for the affected patient. While many reconstructive surgeons have turned to osseous or osteocutaneous microvascular free tissue transfer, the current state of regenerative medicine in our practice for reconstruction of osseous continuity defects of the maxillofacial skeleton relies upon the tissue engineering. Using a triad of potential cells (marrow derived stem cells), a graft scaffold (crushed cancellous cadaveric bone particles) and a signaling molecule (recombinant bone morphogenetic protein 2) we are able to reconstruct significant continuity defects of the the maxilla and mandible, the functional units of the craniofacial skeleton. This technique not only provide a reconstruction that is superior to the free tissue transfer in mandibular reconstruction but carries with it significantly less morbidity and a much lower cost of care. In a retrospective review of nearly 100 consecutive cases of maxillomandibular reconstruction using *in-situ* tissue engineering we discuss our surgical techniques, surgical successes and complications. Through this case series our group hopes to demonstrate that a equivalent to superior functional outcome can be had in reconstruction of the maxillofacial skeleton without the morbidity of

autogenous bone harvest or microvascular osseous or osteocutaneous free tissue transfer. Understanding of this technique will have direct implications on the reconstruction of the following: Benign and Malignant tumor defects, Avulsive traumatic defects, reconstruction of bone damaged by radiation, ridge augmentation for dental Implants, reconstruction of congenital defects, and cleft lip and palate grafting.

### Magnetic Iron Oxide Nanoparticles for MRI Tracking of Adipose Tissue-Derived Mesenchymal Stem Cells

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Magnetic resonance imaging (MRI) is an ideal detection technique for cell tracking experiments to evaluate the fate of transplanted stem cells *in vivo* and to develop successful cell therapies. Different compositions of magnetic iron oxide nanoparticles (e.g. crystal size, arrangement of crystals in matrix material) for MRI applications exist. This study aims at comparing two nanoparticle types differing in composition\* regarding both MRI properties and effects on stem cell characteristics.

For labeling of adipose tissue-derived mesenchymal stem cells (ASC), bionized nanoferrite (BNF-Starch) and superparamagnetic nanomag<sup>®</sup>-D-spio particles (SPIO), size: 100 nm, were used at different labeling concentrations. R2\* mapping of labeled cells was performed using a 7.1 Tesla MRI system. A linear correlation between R2\* times and labeling concentration for BNF (R<sup>2</sup>=0.989) and SPIO (R<sup>2</sup>=0.856) was found, showing a steeper slope for BNF. As number of proliferating cells in S+G2/M-phase increased with increasing labeling concentrations, the differentiation potential (adipogenic: deposition of lipid droplets, osteogenic: extracellular matrix calcification, chondrogenic: expression of collagen type II) of ASC was decreased in a dose-dependent manner. Generally, BNF exerted greater impact on cell functions than SPIO.

In conclusion, improved MRI properties of BNF were accompanied by a higher degree of altered cell functions compared to SPIO. Regarding the role of mesenchymal stem cells in regenerative processes, experiments prior to cell tracking are needed to balance between nanoparticles' MRI properties and their effects on stem cell characteristics.

**Reference:** \*Bordelon DE *et al.*, J Appl Phys. 2011;109:1249041-8.

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### From Cd362 To Cyndacel: The Rapid Development of a Novel Stromal Cell Therapy

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Orbsen is developing highly purified, antibody-selected CD362/Syndecan-2 positive mesenchymal stromal cells (MSCs) isolated either from human bone marrow (Cyndacel-M™), human umbilical cord (Cyndacel-C™) or human adipose tissue (Cyndacel-A™). Notably, antibodies to CD362 can also be used to purify MSC from rat, rabbit and horse bone marrow for the first time. Murine CD362+ MSC are perivascular, express gp38, PDGFRa and nestin and can be identified in bone marrow, lymph nodes, spleen, thymus and skeletal muscle. We demonstrate that may represent a functional component of the immuno-modulatory MSC. CD362 is a cell surface heparan sulfate proteoglycan that is stimulated by hypoxia and inflammatory cytokines. Notably, CD362 protein suppresses cytokine-mediated NFkB activation and IL-6 secretion. Anti-CD362 antibodies suppress T helper 17 cells activation, proliferation and IL-17 secretion. As part of the REDDSTAR and MERLIN EU FP7 consortia, we have tested

Cyndacel-M™ and Cyndacel-C™ in preclinical models of inflammatory disease. Specifically, we report that a single low dose of intravenous Cyndacel-M™ was effective in reducing inflammation and sclerosis and improving kidney function up to 8 weeks post-administration in a chronic diabetic (db/db) model of nephropathy. Intravenous Cyndacel-M™ alleviates thermal and mechanical pain in a SD rat model of diabetic neuropathy. Topical Cyndacel-M™ improves wound healing in a NZ rabbit model of non-healing diabetic ulcers. Intravenous Cyndacel-C™ improves liver function in a murine CCL4 model of liver inflammation and lung oxygenation in a SD rat model of acute lung injury. Orbsen will test the clinical safety of Cyndacel-M™ and Cyndacel-C™ in clinical trials starting in 2015.

### Preparation and Culture of Collagen Microspheres as a Scaffold for Liver Tissue Engineering

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**Introduction:** Vascularization is a continuing challenge to liver tissue engineering. We previously implemented a microfluidic coculture model by integrating 3D hepatocyte tissues and endothelial capillary-like structures. Now, we focus on providing a hydrogel scaffold of collagen microspheres interspersed with hepatocytes, which facilitates vascularization of tissue-engineered constructs. The aims of this study are to fabricate collagen microspheres with a diameter of 20 μm, which is equivalent to the size of hepatocytes, and to demonstrate the potential of the microspheres for the 3D culture of hepatocytes and endothelial cells.

**Methods:** Collagen microspheres were fabricated using the water-in-oil emulsion technique. After being filtered and washed, microspheres were mixed with rat primary hepatocytes or rat microvascular endothelial cells (rMVECs), and seeded into a cell culture dish or a microfluidic device. Growth and morphogenesis of the cells cultured with microspheres were monitored by phase-contrast microscopy, while fine structures and viability were examined by fluorescent microscopy.

**Results and Discussion:** We could fabricate the desired size of collagen microspheres by adjusting the rotating speed and surfactant concentration. The fabricated collagen microspheres were then cultured with hepatocytes and rMVECs. The viability of both hepatocytes and rMVECs cultured with collagen microspheres was not significantly different from those cultured without microspheres, suggesting that the microspheres had no cytotoxicity. In addition, hepatocytes attached on the surface of microspheres and formed 3D structures. These results demonstrated the potential of collagen microspheres in further applications to promote endothelial morphogenesis beside hepatocytes in 3D culture.

### Label-Free Continuous Monitoring of *In Vitro* Chondrogenesis

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There are a few types of tissues that can be constructed *in vitro* upto the level of similarity to natural counterpart. Being one of those kind of tissue, cartilage can be engineered *in vitro*, and tissue-engineered cartilage might bring tremendous benefit for elderly osteoarthritic patients. During *in vitro* chondrogenesis, the developing cartilage tissue exhibited changing electrical tissue properties such as conductivity and anisotropy. Based on this phenomena, we propose a new method to monitor the tissue formation by measuring the electrical conductivity and anisotropy during *in vitro* chondrogenesis. Using tensor probe with 17 electrodes and bioimpedance spectroscopy device, glycosaminoglycans (GAGs) and collagen, the ECM components, that comprising cartilage tissue, and also various mixtures of them were clearly distinguished and showed the possibility of providing diagnostic information of the *in vitro*-grown tissue



structure and compositions. During 6 weeks of *in vitro* chondrogenic culture, conductivity values and the anisotropy ratios measured at the bottom and top surfaces of tissue samples showed time-dependent changes. These results indicate that measurement of the electrical tissue property can provide a way of continuously monitoring individual tissue during the entire culture period without any adverse effect. This label-free monitoring technology might significantly increase the productivity of cartilage tissue engineering.

#### Novel Nanofibrillar Composite Matrices of Multiple Polymers and Improved Properties for Peri-implantitis

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**Objectives:** Peri-implantitis induces dental implant loss in about 20% of the patients, due to slow tissue healing and scar tissue formation of lower quality and higher sensitivity to bacteria. Current fast resorbable guided tissue regeneration membranes lack efficient cellular colonization and sufficient mechanical support over time for tissue maturation. Hence, we hypothesized that easily cellularized nanofibrillar matrices composed of multiple biodegradable polymers could enhance peri-implant tissue quality by providing distinct mechanical properties around the implant over time.

**Methodology:** Using the jet-spraying approach (polycaprolactone (PCL) matrices supporting extensive cell colonization), we developed PCL and poly(lactic-co-glycolic acid) (PLGA) nanofibrillar composites. After having determined PLGA nanofibers spraying parameters (factorial design), we evaluated the composites structural (SEM), mechanical (tensile tests), shrinkage and degradation properties in PBS at 37°C.

**Results:** Jet-sprayed PLGA nanofibrillar matrices were homogeneous, controlled (median fiber diameters: 0.24 to 1.43 µm) and highly porous (93 to 98%). PLGA/PCL composite matrices revealed intimately entangled nanofibers from each polymer that conserved their respective structures and diameters. The adjustable ratio of each polymer modulated composites properties. Young's modulus and ultimate tensile strength was increased as compared to PCL and PLGA matrices alone (up to 2 and 4 fold respectively). Shrinkage of the composite matrices could be selectively reduced by increasing PCL ratio while degradation was inversely and selectively related to PLGA ratio.

**Significance:** These results present a simple and efficient approach to create composite nanofibrillar matrices of controlled and improved properties. Ongoing experiments will further determine cell colonization and selective mechanical properties modulation over time.

#### Microstereolithography-based Fabrication of Anatomically Shaped beta-Tricalcium Phosphate Scaffolds for Bone Tissue Engineering

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Porous ceramic scaffolds with shapes matching the bone defects may result in more efficient grafting and healing than the ones with simple geometries. Computer-assisted microstereolithography (MSTL) can fabricate complex three-dimensional (3-D) structures with greater accuracy than other solid free-form fabrication technologies. However, thermal contraction mismatch between the scaffold material and mold results in shape distortion during sintering. We have developed a novel gelcasting indirect MSTL technology to overcome this problem, and successfully fabricated two scaffolds according to CT images of rabbit femur. Negative resin molds with outer 3-D dimensions conforming to the femur and an

internal structure consisting of stacked meshes with uniform interconnecting struts, 0.5 mm in diameter, were fabricated by MSTL. The second mold type was designed for cortical bone formation. A ceramic slurry of beta-tricalcium phosphate (β-TCP) with room temperature vulcanization silicone as binder was cast into the molds. After complete gelation, the composite was sintered at 1500°C for 5 h. Both gross anatomical shape and the interpenetrating internal network were preserved after sintering. Even cortical structure could be introduced into the customized scaffolds, which resulted in enhanced strength. Biocompatibility was confirmed by vital staining of rabbit bone marrow mesenchymal stromal cells cultured on the customized scaffolds for 5 days. This fabrication method could be useful for constructing bone substitutes specifically designed according to local anatomical defects.

#### The Development of a Non-Invasive Monitoring System for Real-Time Monitoring and Prediction of Cell Behavior *in Vitro*

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Live cell imaging systems have become a requisite analytical tool in the wide ranging field of regenerative medicine. However, directly linking real-time changes in the environment to the dynamic behaviour of cells still remains challenging. Hence, a combined use of visual data with online monitoring of environmental parameters such as oxygen and lactate could provide detailed information on the behaviour of cell populations and the role of the environment in cell fate.

In this study we developed a Live cell Monitoring System (LiMSy) by incorporating a temperature- and CO<sub>2</sub>-controlled perfusion chamber and oxygen sensors on a microscope stage. Cell cultures were monitored in real-time by combining image analysis with bio-response measurements and validated data-based modeling. Human periosteum-derived cells (hPDCs) were seeded in 12-well plates (5700 cells/cm<sup>2</sup>) in growth media containing different serum levels and monitored for 7 days. Already after 1 day of culture, the system was able to detect significant differences in growth curves between the cultures and could provide an early warning to the user. In a second set of experiments hPDCs were monitored during harvesting with TrypleE and EDTA and the optimal time to stop the harvesting reaction could be accurately predicted (R<sup>2</sup> = 0.98 ± 0.01). Next, cells were treated with EtOH (0.25–2 mM) and subsequently cultured for 5 days. Changes in cell shape were evaluated and could be directly correlated to cell death. Taken together, we showed that the newly developed LiMSy is a promising tool for non-invasively monitoring and predicting of cell behaviour in real-time.

#### Monocalcium Phosphate Induces greater Hydroxyapatite Mineral Formation than Tricalcium Phosphate on Dental Composites Containing ε-poly-L-lysine

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Remineralising, antimicrobial dental composites are under development for enhanced treatment of dental caries, the most prevalent disease globally. Composites enable minimal tissue removal and have superior aesthetics and load-bearing properties compared to other restorative materials. Longevity can, however, be reduced by polymerisation shrinkage and subsequent microbial microleakage,

recurrent caries and eventual failure. Current research focuses on utilising antimicrobial and remineralising agents in order to overcome these issues<sup>1-3</sup>. Calcium phosphates can be released to form new mineral between the tooth and restoration, to prevent microleakage and enhance mechanical properties of the interface, whilst antimicrobials can prevent post-operative infection. Mono/tricalcium phosphates (MCP/TCP) have previously been combined with chlorhexidine to promote formation of the stable calcium phosphate phase hydroxyapatite (HA)<sup>1</sup>. In the present study, the individual and combined effects of MCP/TCP were investigated for the first time. Antimicrobial poly-peptide  $\epsilon$ -poly-L-lysine ( $\epsilon$ PL) was also evaluated. Composites were stored in simulated body fluid (SBF) for one week and analysed by SEM, Raman, biaxial flexural, shear bond and water sorption tests. MCP induced greater mass and volume increase in H<sub>2</sub>O and SBF than TCP. MCP content may therefore be carefully regulated to compensate for shrinkage. Minimal HA/brushite deposits formed on TCP- $\epsilon$ PL composites. MCP- $\epsilon$ PL composites had the greatest HA precipitation, indicating that TCP is not required for speciation of HA. TCP omission resulted in improved strength and shear bond to human dentine. Highly soluble  $\epsilon$ PL enhanced mineral release and precipitation. MCP- $\epsilon$ PL composites have great potential to prevent recurrent caries and restoration failure by enhancing the tooth-restoration interface.

### Integrated Micro/Nanofibrous PLGA-Collagen Scaffold: an Optimized Method for Plastic Compression of Collagen into PLGA Microfibers

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Fabrication of an ideal scaffold mimicking the native extracellular matrix components, structure and function has remained a challenge in tissue engineering. The basic structural element in ECM is collagen type I with a fibrillar nano-scale structure. Collagen nanofibers can be fabricated using electrospinning or plastic compression (PC) of collagen hydrogel. PC is a simple and fast method, which has appeared better than electrospinning, since fluoroalcoholic solvents used in collagen electrospinning denature collagen to gelatin. The collagen layer obtained from PC contains small inter-fiber pores limiting cell infiltration and is mechanically weak. Performing collagen PC onto another polymeric substrate leads to a two-layered construct with improved mechanical properties. However, the top collagen layer has still limited cell infiltration and is prone to post-implantation separation.

We demonstrate a fabrication procedure to distribute collagen nanofibers in-between poly(lactic-co-glycolic acid) (PLGA) microfibers. An electrospun layer of PLGA was placed between two layers of collagen hydrogel. Varying parameters included collagen concentration in the hydrogel and immediate or postponed compression of the construct. Morphology and infiltration of nanofibers was studied using scanning electron microscopy. We observed that high concentrated collagen and immediate compression led to dense collagen layers covering the PLGA. Decreasing the collagen concentration along with postponed compression led to more infiltration of collagen around PLGA microfibers. Herein, a new method to fabricate integrated hybrid micro/nanofibrous constructs with appropriate structure for cell infiltration and improved mechanical properties compared to compressed collagen is introduced.

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### 3D Cell Printing of Composite Tissue for Ear Regeneration

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Human's ear is a very complex composite tissue composed of cartilage and fat. To date, many effects have been attempted to reconstruct the outer ear using artificial prosthesis and curved rib cartilage; however, these approaches still have severe adverse effects such as infection, erosions, and dislodgements. In this study, we applied three-dimensional (3D) cell printing technology to create 3D composite structure with ear shape. 3D cell printing technology has been demonstrated as a promising method for combining biologically relevant biomaterials and living cells into a 3D structure [3]. Auricular chondrocyte laden cartilage decellularized extracellular matrix (cdECM) hydrogel and human adipose derived stem cell (ASC) laden adipose decellularized extracellular matrix (adECM) hydrogel were separately printed into the poly( $\epsilon$ -caprolactone) (PCL) framework with consideration of histological and geometrical characteristics of native ear tissue. *In vitro* and *vivo* test using 3D cell printed ear, we demonstrated that cartilage and fat tissues in the 3D cell printed ear were successfully regenerated similar to auricular cartilage and fat of native ear.

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### Large Animal Model of Sepsis for Dialysis-like Therapeutic Evaluation

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Sepsis is a life threatening bloodstream infection, affecting 18 million people worldwide. With sepsis mortality rates at 30-50% and antibiotic-resistant microorganisms contributing to a rise in sepsis incident rates, there is a critical need for alternative therapies to treat sepsis. We have developed an extracorporeal system to remove live and dead pathogens from the bloodstream without the need to first identify the pathogenic agent, mimicking the function of the spleen. This system utilizes a genetically engineered opsonin, mannose-binding lectin (MBL), which has broad-spectrum affinity to multiple strains of bacteria, fungi and yeast. Using magnetic nanobeads coated with the engineered MBL, pathogens and toxins have been cleansed from infected blood *in vitro*, using magnets to pull the opsonin-bound pathogen and toxins from the blood. Using this system, pathogen and toxin removal from blood has been demonstrated *in vivo*, in small animal models of gram-negative, gram-positive and endotoxin induced bacteremia, in which the cleansed blood was returned to the animal. We have developed a large animal model of gram-positive bacteremia and gram-negative sepsis using swine, with conscious infusion of pathogens. We optimized the blood-cleansing system for use in these large animal models of sepsis by immobilizing the engineered opsonin on hollow fibers. Following pathogen infusion and 8 hours of extracorporeal opsonin therapy at 200 ml/min, we observed rapid removal of live pathogen from the blood of infected swine, by decreasing the time to blood culture negative.

### A Topology Optimized Model Based on the Level-Set Method Incorporating Angiogenesis for Porous Bone Scaffolds

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In a well-designed porous scaffold, there is a need for a convenient balance between biological compatibility and mechanical functionality such that the porosity permits tissue regeneration and angiogenesis and solid structure carries the load in the best possible way.

In the proposed computational model, different parameters such as mechanical strength, fluid permeability and angiogenesis are considered together in order to yield an optimal design for the scaffold. The relationship between these parameters are governed by relevant physical equations and mathematical sensitivity analysis helps to reform the structure in a way so that all the parameters are enhanced. COMSOL Multiphysics provides the opportunity to build a model where all these equations are coupled together and studied at the same time to reach an optimally performing tissue. Using level-set method, we start with an initial geometry, which satisfies the physical constraints and at each time step, and this geometry is improved based on sensitivity analysis results.

Solution converged to an optimized structure, which can be manufactured using current available additive manufacturing techniques. Finite element method integrated to the level-set based topology optimization is proven to be among the most computationally efficient and generic design tool for solving non-intuitive tissue engineering problems. Hence, the proposed design framework, when implemented with corresponding physical models, is equally applicable to other hard and soft tissue designs.

### Effect of Different Cell Sheet Matrix Microenvironment on Vascular Network Formation

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Engineering a three-dimensional vascularized tissue for craniofacial bone reconstruction remains extremely challenging. Cell sheet engineering technique, creating an intact extracellular matrix (ECM), brings a promising potential for robust vascularization. However, the effect of different cell sheet on endothelial network formation is not well understood. This study was to investigate the effect of different cell sheet and different seeding density of endothelial cells on vascular network formation. We cultured human bone marrow mesenchymal stem cells (hBMSCs) under two culture conditions to obtain undifferentiated and osteogenic differentiated hBMSCs sheets. We then seeded human umbilical vein endothelial cells (HUVECs) on the two different hBMSCs sheets with different seeding densities. Our results showed that the osteogenic differentiated cell sheet (ODCS) significantly facilitated the migration and alignment of HUVECs and promoted the network formation, while the matrix structure in the ODSC was realigned due to the tubulogenesis process. Quantitative analysis showed that the network number on the ODSC was higher than that on the undifferentiated hBMSCs sheet. Results further showed that the optimal seeding density of HUVECs was  $5 \times 10^4$  cell/cm<sup>2</sup> based on the number and morphology of the formed networks. These results together indicated that the ODSC promoted vascular network formation and that the vascularized ODSC provides a promising potential for craniofacial bone reconstruction.

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### Development of a Seeded Scaffold in the Great Omentum Feasibility of an *in vivo* Bioreactor for Esophageal Reconstruction

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**Objectives:** The purpose of this study was to determine the feasibility of an artificial esophagus using the omentum as an *in vivo* bioreactor.

**Methods:** The artificial esophagus was constructed using 3D printing and electrospinning technique, composed of an outer and inner layer of poly( $\epsilon$ -caprolactone). After constructing multiple ring shaped skeleton with 3D printing technique, depositing the inner and outer layer of the PCL scaffold by electrospinning was created. Eight rats were anesthetized using inhaled anesthesia. The artificial scaffolds were seeded into the omentum. The grafts were harvested 2 weeks later and circumferential defects of the cervical esophagus were created and reconstructed with the artificial esophagus. Result Groups of rats were sacrificed after the 1st and 2nd weeks. No gross evidence of a fistula, perforation, abscess formation, seroma accumulation, or surrounding soft-tissue necrosis was observed after the 1st and 2nd weeks. The artificial esophagus constructs produced complete healing of the circumferential defects by the 2nd week. The fusion of the PCL scaffold and the regenerative tissue remained intact. Histologic and immunostaining analysis of the construct confirmed the presence of a multilayer organised tissue composed of differentiated cells and mature fibroblasts without evidence of inflammation or necrosis.

**Conclusion:** The omentum permitted *in vivo* maturation of seeded scaffolds with the development of a dense vascularisation that is anticipated to prevent fibrosis and loss of contractility. However, further studies on circumferential defect reconstruction in a rat model are still required.

### Biomimetic Scaffold for Guided Interfacial Tissue Regeneration in Regenerative Dentistry

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**Objective:** Guided tissue regeneration (GTR) is a clinical procedure used when tissues have to be separated for functional regeneration. The major challenge in GTR for organized interfacial tissues like periodontal ligament (PDL) and pulp dentin complex (PDC) is the lack of a differentially permissive scaffold. In this study we engineered a biodegradable scaffold for GTR that provides spatial control, supports cell adhesion and tissue growth on both sides, with differential cell penetration.

**Methodology:** 12% or 20% (w/v) poly (lactic-co-glycolic) acid (PLGA) was dissolved in dimethylsulfoxide (DMSO). A combination of diffusion induced phase separation (DIPS) and tape-casting was used to make porous scaffold sheets. Channel/pore structures obtained were analyzed through SEM. Cell biomaterial interaction was tested using Dental Pulp Stem Cells (DPSC). Cell penetration into the scaffold was visualized by Laser Scanning Confocal Microscopy (LSCM) and quantified using MATLAB.

**Results:** We engineered porous scaffolds with directional continuous channels that closely resembled the directionality of the PDL and PDC. The tapered channels gradually reduced in diameter from one side to the other. Altering PLGA concentrations changed channel/pore morphologies and caused differential DPSC response to the scaffold. The pore structures can be tailored by adjusting processing parameters such as polymer/solvent concentrations, which can then dictate the depth of cell penetration.

**Significance:** The preformed directional channels provide a biologically relevant blueprint for spatial control of penetrating DPSC into permissive scaffold and will eventually regenerate the organized interfacial tissue. Our engineered scaffolds can be a promising adjunct for GTR in regenerative dentistry.

### Osteogenic Differentiation of Mesenchymal Stem Cells is Affected by Alendronate

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**Objectives:** Various conditions like osteoporosis and Paget's disease show altered bone homeostasis skewed towards increased

resorption warranting use of anti-resorptive drugs like Alendronate (nitrogen-containing bisphosphonate). However, a prominent side effect of alendronate is medication induced osteonecrosis of jaw bone (previously termed BRONJ). Although *in-vitro* and *in-vivo* studies have shown that Alendronate affects bone cells, gingival fibroblasts and local vascularity, literature evaluating the influence of alendronate on pluripotent stem cells is distinctly lacking. Thus, our *in-vitro* study was aimed at evaluating the effects of alendronate on the proliferation, osteogenic differentiation and mineralization potential of human mesenchymal stem cells (MSCs).

**Methods:** MSCs were cultured *in-vitro* with alendronate at 0.25  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$ , 2  $\mu\text{M}$  and 3  $\mu\text{M}$  in culture media for 10 days and proliferation was assessed using an Alamar Blue assay. Live-Dead assay was used to determine a suitable concentration for the differentiation assay. Osteogenic differentiation was conducted in osteogenic media over 21 days and Alizarin Red staining was undertaken to confirm mineralization and quantify the staining.

**Results:** Alamar Blue assay showed that Alendronate was cytotoxic at 2  $\mu\text{M}$  and 3  $\mu\text{M}$ . Optimal alendronate concentration for differentiation of MSCs without causing cell death was 1  $\mu\text{M}$ , as confirmed by the Live-Dead assay. Subsequently, differentiation assay and Alizarin red staining showed mineralization occurred at 21 days in differentiation media. Quantification showed that Alendronate significantly affected the functional aspect of differentiated MSCs.

**Significance:** These findings suggest direct effects of alendronate on MSCs could have a significant role in inhibiting optimal bone remodelling and mineralization thereby ensuing MRONJ.

#### Novel Contrast Agents for Contrast-Enhanced CT to Visualize in 3D the Blood Vessel Network and Fat Cell Distribution in Bone Marrow

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A detailed visualization of the complex 3D blood vessel and adipose tissue network in bone and bone marrow might be necessary to link alteration in these tissues with impaired bone healing and regeneration. For this purpose, we propose contrast-enhanced computed tomography (CE-CT). We have compared phosphotungstic acid (PTA) with two novel contrast agents for their non-invasive character and their potential to visualize in 3D blood vessels and adipose tissue. Both novel contrast agents are metal-substituted polyoxotungstates and are further referred to as Hf-POT and Zr-POT (Hf- and Zr-substituted).

To investigate whether the staining provoked tissue shrinkage, we scanned, stained and rescanned femurs of 7 week old mice. Using image registration, it was shown that PTA does induce shrinkage of bone after 24 hours of staining in a 3.5% PTA/PBS solution. Both novel contrast agents however did not induce shrinkage using the same concentration and staining time.

We also investigated the potential to perform immunological staining after CE-CT imaging. Therefore, we first scanned tibias of 4 weeks old mice using CE-CT and processed subsequently for CD31 immunostaining. PTA staining did not allow CD31 staining, while both novel contrast agents showed excellent CD31 tracing.

Finally, we scanned tibias of old (30 weeks - OLD), young (7 weeks - YNG) and diabetic (30 weeks - diet-induced obese model, DIO) mice. For the DIO mice, the bone marrow compartment contained more adipose tissue compared to the YNG and OLD mice. YNG mice showed a higher content of blood vessels compared to the other groups.

#### Delivery of Drugs into Cells with PHBV Nanoparticles

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The field of polymeric nanoparticles has been expanding because they are able to penetrate sites not accessible to larger particles and they have large surface area-to-volume ratios that increase drug release rates. The main objective of this study was to prepare nano sized polymeric spheres to deliver compounds through the cell membrane and preferably into the nucleus. This would improve the biostability of macromolecular drugs (growth factors and polynucleotides), and increase their efficacy. Nanospheres of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) were tested for their penetration into Saos-2 human osteosarcoma cells and L929 cells. The nuclear targeting agent, Nuclear Localization Signal (NLS) with the peptide sequence reported by Misra and Sahoo (2010) was used.

**Experimental:** PHBV nanospheres with diameters around 260 nm were loaded with Nile Red using oil-in-water (o/w) technique. For surface activation the PHBV nanospheres were treated with EDC and NHS, and then conjugated with NLS. These nanoparticles were tested with Saos 2 cells. Cell nuclei and cytoskeleton were stained after fixation with FITC-Phalloidin and Draq-5, respectively. CLSM showed nano sized particles accumulating near the nucleus, with some of them within the nucleus as the z-stack of the image has shown. This shows that nuclear transfer of nanoparticles using the targeting agent NLS was successful. High magnification microscopy techniques such as TEM and biological tests will be used to further prove the penetration.

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#### Using Human Derived Adipose Stem Cells and Optimized 3D Bioprinted Scaffolds for Osteochondral Tissue Engineering

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Osteoarthritis (OA) is a degenerative joint disease that limits mobility of the affected joint due to the degradation of articular cartilage. The limited regenerative capacity of cartilage tissue presents significant challenges to repair or reverse the effects of damaged tissue. The most common approach to treat advanced stages of OA is joint arthroplasty, which replaces the joint tissue with orthopedic metals and plastic materials. This highly invasive surgery lasts between 15–20 years, and represents a significant challenge for young adults undergoing this procedure. The most promising future to treat osteochondral injuries relies on tissue engineering techniques.

Our goal is to develop a less invasive alternative to treat patients suffering from osteoarthritis by generating a full osteochondral scaffold using human adipose derived stem cell (ASC) and 3D bioprinting techniques. Our novel scaffold is fabricated using biodegradable polycaprolactone (PCL) with either tricalcium phosphate (TCP) or decellularized cartilage extracellular matrix (dECM) to induce site-specific osteogenesis and chondrogenesis respectively. Osteogenesis in PCL-TCP scaffolds was assessed by alizarin red staining and calcium accretion assay. Chondrogenesis in PCL-ECM scaffolds was assessed by qPCR, alcian blue, safranin-O and collagen II immunostaining. Our results show that our scaffold design can successfully induce site-specific hASC differentiation, and we generate a full osteochondral scaffold using a single adult stem cell source. This technique would be less invasive as none of the healthy remaining cartilage tissue would be removed, while still minimizing tissue rejection via use of an abundant and accessible source of autologous stem cells.

#### Genetically Modified Human Muscle Precursor Cells Overexpressing PGC-1 $\alpha$ Support Early Myofiber Formation in Bioengineered Muscle Tissue

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Muscle precursor cells (MPCs) are quiescent muscle cells capable of muscle fiber reconstruction. Therefore, autologous MPC transplantation is envisioned for the treatment of muscle diseases, many occurring in the aged population. However, density of MPCs, proliferation and differentiation potential gradually decline with age. The goal of this research was to explore the possibility of genetically modifying human MPCs to overexpress the peroxisome proliferator-activated receptor gamma co-activator (PGC-1 $\alpha$ ) to enhance skeletal muscle formation and quality. hMPCs were harvested from *M. rectus abdominis* of patients undergoing abdominal surgery. After genetically modifying the cells to overexpress PGC-1 $\alpha$  (or GFP control), viability, proliferation and myogenic phenotype were evaluated *in vitro*. The expanded cells were suspended in collagen carrier and *s.c.* injected on the back of nude mice. One, two and four weeks later the bioengineered skeletal muscle tissues were harvested and assessed by histology, WB and RTPCR. We were able to confirm the sustained myogenic phenotype of the genetically modified hMPCs. Viability and proliferation potential were not significantly different compared to native cells *in vitro*. Fiber formation capacity and contractility were enhanced in PGC-1 $\alpha$  modified hMPCs *in vitro*. Subcutaneously injected cell-collagen suspensions were harvested after 1, 2 and 4 weeks and histological analysis confirmed the earlier myotube formation in PGC-1 $\alpha$  modified samples. Increased contractile protein levels were detected by WB. By genetically modifying hMPCs to overexpress PGC-1 $\alpha$  we were able to enhance the early myotube formation *in vitro* and *in vivo*, thereby developing a novel strategy for improving skeletal muscle tissue engineering.

### Comparison of the Use of Two Different Silkworm-cocoon-derived Membrane for the Guided Bone Regeneration

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The purpose of this study was to evaluate silk membrane for application in guided bone regeneration (GBR). Silk cocoons can be separated by several layers and the middle was come from the cocoon middle layer (middle group). It was compared with not-separated cocoon (total group). For the comparison, cell culture with alkaline phosphatase assay and MTT assay were done. The animal study was done using the calvarial defect model of New Zealand white rabbits (n=12). The results of alkaline phosphatase assay demonstrated higher value in the middle group. The  $\mu$ -CT analysis showed significantly higher bone volume in the middle group (P<0.05). In histological exam, the middle group showed significantly higher new bone formation at 8 weeks postoperatively than that of the total group. In conclusion, the middle group could be better for GBR technique than the total group.

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### 3D Printed Polyethylene Terephthalate (PET) Scaffold for Bone Tissue Engineering

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Despite the advances in surgery, the treatment of large bone defects still represent significant medical problem. In many cases, performance of surgeons is limited by deficiency of transplantable autologous bone grafts. Moreover the harvesting procedure represents additional burden for patient and also increased risk of morbidity and mortality. The tissue engineering provides new approaches using appropriate biomaterials and autologous cells as well as technology of 3D printing to fabricate biological substitutes of tissues or organs including bones. In the present study we used FDM Felix 3 printer to develop polyethylene terephthalate (PET) scaffolds with different internal porosity. These scaffolds were tested for cytotoxicity and biocompatibility with human adipose tissue-derived stem cell which underwent osteogenic differentiation *in vitro*. Finally, we demonstrated the ability to print bone scaffold according to patient's computed tomography scans. Obtained results prove that PET scaffolds were non-toxic and biocompatible with used stem cells and after further testing they should be used in tissue engineering and regenerative medicine of bones.

### Release Kinetics of Collagen Barrier Membranes Supplemented with the Secretome of Activated Platelets and Hypoxia-Mimetic Agents

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Strategies using platelet concentrates and hypoxia-mimetic agents (HMA) can stimulate soft and hard tissue regeneration when healing is compromised. We follow a novel strategy where collagen barrier membranes are used as carriers for the secretome of activated platelets (PSEC) and HMA. Here, we analyzed the release of unwashed-PSEC, washed-PSEC, and HMA from the membranes. The mitogenic and pro-angiogenic effects were assessed in bioassay with human oral cells. Growth factor release was investigated based on PDGF-BB in supernatants taken from the collagen barrier membranes with immunoassays. The release of hypoxia-mimetic agents was assessed by photometric measurements.

Our data show that PSEC loaded collagen barrier membranes stimulate proliferation. The highest PDGF-BB levels were found in supernatants taken from the loaded collagen barrier membranes after one hour. The effect on proliferation and the PDGF-BB levels decreased rapidly as observed in supernatants taken after 3, 6, 24, 48 hours. Supernatants from unwashed-PSEC-loaded collagen barrier membranes induced a stronger mitogenic response than supernatants from washed-PSEC-loaded collagen barrier membranes.

Collagen barrier membranes loaded with HMAs increased the pro-angiogenic capacity of oral cells. The highest HMA levels were detected in supernatants of loaded collagen barrier membranes after one hour. The effects on the pro-angiogenic capacity and the levels of HMA decreased in supernatants taken after 3, 6, 24, 48 hours.

In summary, collagen barrier membranes release PSEC and HMA in a burst-like release kinetic. Whether these mitogenic and pro-angiogenic effects of PSEC or HMA-loaded collagen barrier membranes stimulate oral tissue regeneration will be assessed in preclinical studies.

### Fabrication of Multi-well PDMS Chips for Spheroid Cultures and Implantable Fibrin Constructs with High Cell Density Regions through Low-cost Rapid Prototyping Techniques

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Three-dimensional cell spheroids represent key models in basic research as well as the building blocks for bioprinting. Here, we exploited laser ablation and replica molding to fabricate polydimethylsiloxane (PDMS) multi-well chips for spheroid generation and culture. The chips fit standard 12-multiplates and allowed to culture 228 spheroids (average diameter 1–1.5 mm) in each multi-plate. Cell seeding and medium refresh operation time were significantly reduced by using the multi-well chips. PDMS optical transparency allowed for spheroid monitoring during culture. Articular chondrocytes (ACs) spheroids generated in multi-well chips showed superior glycosaminoglycan and collagen-II deposition compared to standard spheroids, demonstrating the platform validity.

Generating constructs with controlled architecture is a key step to develop innovative 3D models. Exploiting a similar prototyping approach, we used clinical-grade fibrin to biofabricate implantable multi-well constructs allowing the precise distribution of multiple cell types. After 7 days *in vitro*, high cell density regions, successfully generated in AC-seeded multi-well constructs as shown by histology and fluorescent cell staining, improved the expression of SOX9, ACAN, COL2A1, COMP. After 5 weeks *in vivo*, the cell distribution pattern was maintained in multi-well constructs and the glycosaminoglycan content was significantly increased respect to pre-implantation samples.

In conclusion, we generated functional 3D spheroids using multi-well PDMS chips and we improved cell differentiation using constructs with a controlled architecture. Remarkably, our approach exploits tools accessible to most research labs, representing a cost-effective method for the prototyping of cell culture platforms and 3D constructs useful for *in vitro* and *in vivo* studies in several research areas.

#### Electrospun Curcumin-gelatin Blended Nanofibrous Membrane: A Novel Medicated Biomaterial for Drug Delivery and Accelerated Wound Healing

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Acute wound occurs as a result of trauma as well as surgery, which is on the rise every year. The traditional surgical intervention for wound treatment inevitably causes additional injury. As an alternative, wound therapy via noninvasive approach has been accepted and proven to be effective. In this study, blended nanofibrous membranes were prepared by an electrospinning technique with Gelatin as the filament-forming polymer, and curcumin, an insoluble but a reported potential wound healing agent, as the treatment drug. Analysis of the blended nanofibrous membrane by scanning electron microscopy, X-ray diffraction and attenuated total reflectance Fourier transform infrared spectra revealed that curcumin was well distributed in the nano-fibers in the form of amorphous nanosolid dispersions. Results from *In vitro* release profile proved that the blended nanofibrous membrane produced desired control-release kinetics of the entrapped curcumin as compared to the pure drug. Furthermore, wound healing test and histological evaluation revealed that the topical application of curcumin loaded nanofibrous membrane accelerates wound healing, which might be through the anti-inflammatory activity and *in-situ* cell mobilization (fibroblasts) of curcumin. The study provides evidence of potential strategies to develop composite drug delivery system as well as promising materials for future therapeutic biomedical applications.

#### Predicting Cell Damage using Multi-scale Finite Element Modelling of 3D Cell-embedded Collagen Constructs under Equiaxial Strain

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It is significantly important to gain a physical understanding of the mechanical stress and deformation milieu within the tissues and resident cells that governs the interaction between molecular and structural events. Yet, it is extremely difficult to gain thorough understanding from only experimental observations, especially for three-dimensional (3D) systems. The objective of this study was to understand the effect of equiaxial mechanical strain applied on 3D adipose derived stem cells (ASC)-embedded collagen constructs in tissue (macro-scale) and in multi-cellular (micro-scale) levels using multi-length scale finite element model (MSM). Based on that understanding, we could predict the degree of cell damage induced by different strains. The MSM simulated 3D ASC-collagen construct exposed to different equiaxial strains using a custom-built mechanical loading platform, called EQUicycler. The EQUicycler's working mechanism is based on creating cyclic equiaxial strain by eccentric cam system on silicon post hosting collagen annular disc around it. Various physiological equiaxial strain values experienced by various tissues including myocardium tissue were applied to the constructs using EQUicycler and were simulated numerically using MSM. The MSM approach utilized in this work provided detailed information for the effect of position and cell distributions on the cellular level strains. That information were used with stochastic simulations after defining a damage criterion to predict mechanical loading-induced cell damage. The stochastic simulations predictions agreed well with experimental viability studies conducted using flow cytometry. These findings can provide a methodology to predict cell damage induced by equiaxial strains in the range of both physiological and pathological conditions.

#### Cell Response to Calcium Phosphate Doped Alginate/Fibrin Coatings on Titanium Surfaces

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Different surface modifications have been proposed to promote beneficial interactions at bone-implant interfaces to enhance adhesion, proliferation and differentiation of bone cells. Our aim is to modify titanium (Ti) surfaces with thin alginate/fibrin hydrogel scaffolds serving as carriers for bioactive hydroxyapatite (HA) or  $\beta$ -tricalcium phosphate (TCP). Ti surfaces were chemically treated following by covalent immobilization of neridronate (BP) and alginate (Alg) monolayer as a coupling interface via carbodiimide chemistry. Composite alginate/fibrin hydrogel films containing HA or TCP were deposited via ionically crosslinking. SaOS-2 cell lines, human mesenchymal stem cells (HMSC-bm) and mononuclear cells obtained from buffy coat were used for *in vitro* study.

Composite coatings stimulated lower inflammatory cytokine production (multiplex proteomic analysis RayBio Human Inflammation Array) than Ti controls. Mononuclear cells produced preferentially factors of nonspecific immunity, HMSC-bm produced mostly chemokines activating predominantly chemotaxis, monocytes, granulocytes and neutrophils whereas SaOS-2 cells were insufficiently sensitive. SaOS-2 cells differentiated distinctively better on composite Alg/HA or Alg/TCP surfaces than on Ti; TCP supported early cell differentiation whereas HA promoted expression of osteocalcin. Coatings containing fibrin augmented significantly cell adhesion and proliferation. Developed bioactive composite alginate/fibrin coatings can serve as a tool to support metabolic and differentiation activity of bone cells on Ti implants.

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### Production of Elastic Chitosan/Chondroitin Sulphate Multilayer Films For Tissue Regeneration

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Membrane-like scaffolds are suitable to induce regeneration in many and different anatomic sites, such as periodontal membrane, skin, liver and cardiac tissues. In some circumstances, the films should adapt to geometrical changes of the attached tissues, such as in cardiac or blood vessel tissue engineering applications. In this context, we developed stretchable two-dimensional multilayer constructs through the assembling of two natural-based polyelectrolytes, chitosan (CHT) and chondroitin sulphate (CS), using the layer-by-layer methodology. The morphology, topography and the transparency of the films were evaluated. The influence of genipin, a natural-derived cross-linker agent, was also investigated in the control of the mechanical properties of the CHT/CS films. The water uptake ability can be tailored by changing the cross-linker concentration, which influenced the young modulus and ultimate tensile strength. The maximum extension tends to decrease with the increase of genipin concentration, compromising the elastic properties of CHT/CS films: nevertheless using lower cross-linker contents, the ultimate tensile stress is similar to the films not cross-linked but exhibiting a significant higher modulus. The *in vitro* biological assays showed better L929 cell adhesion and proliferation when using the cross-linked membranes and confirmed the non-cytotoxicity of the CHT/CS films. The developed free-standing biomimetic multilayer could be designed to fulfill specific therapeutic requirements by tuning properties such as swelling, mechanical and biological performances.

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### Comparison of Urine Stem Cell and Adipose Stem Cell

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Adipose tissue derived stem cells (ADSCs) would be an attractive autologous cell source. However, ADSCs require invasive procedures, and has potential complications. Recently, urine derived stem cells (USCs) have been proposed as an alternative stem cell source. In the current study, we compare USCs and ADSCs from same patient on stem cell characteristics and capacity to differentiate into various cell lineages to provide a useful guideline for selecting the appropriate type of cell source for use in clinical application. The urine samples were collected from upper urinary track by catheterization, and adipose tissue was obtained from subcutaneous fat tissue from the same patient (n=10). Cell proliferation, colony formation, cell surface markers expression, immune modulation, chromosome stability and multi-lineage differentiation capability were analyzed for each USCs and ADSCs at cell passage 3, 5, and 7. USCs showed high cell proliferation rate, enhanced colony forming ability, strong positive for stem cell markers expression, high efficiency for inhibition of immune cell activation compared to ADSCs at cell passage 3, 5, and 7. In

chromosome stability analysis, both cells showed normal karyotype through all passages. In analysis of multi-lineage capability, myogenic, neurogenic and endogenic differentiation rate of USCs were higher, and osteogenic, adipogenic and chondrogenic differentiation rate were lower than ADSCs. USCs possessed superior cell proliferative ability, immune suppressive effect, and myogenic, neurogenic and endothelial differentiation potency compared to ADSCs. (2014R1A1A3049460)

### Targeting Biomimetic 3D Soft-Hard Tissue Interfaces: Formation of a Calcified Spherical Shell with a Double Diffusion Bioreactor

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**Introduction:** Entheses are the interfaces between bone and soft tissues, such as articular cartilage, meniscus and labrum fibrocartilage, intervertebral disc, tendon, and ligament. Formation of such interfaces normally involves local biochemical processes combining calcium and phosphate to form hydroxyapatite-reinforced materials. In contrast, engineering of such interfaces involves both the initiation and diffusion-limited growth of hydroxyapatite-like structures. Previous studies established the ability of flat-ended double diffusion bioreactors, providing calcium and phosphate from opposite sources, to form a calcified flat structure within hydrogels and at tissue interfaces. The objective of the present study was to test if such a reactor could be extended to a 3D configuration to generate such interfaces in a more complex geometry, specifically a spherical shell.

**Methods & Results:** Diffusion-reaction modeling in spherical coordinates allowed determination of a general relationship between bath concentrations, location of the formed calcified interface, and the rate of hydroxyapatite formation.

Experiments on a 3D-printed prototype and a CNC-machined spherically symmetric double-diffusion bioreactor incubated for four days, and micro-computed tomography analysis of the resultant material, indicated the ability to rapidly form an internal radiopaque shell, with a thickness of ~100 um and positioned at a distance of ~2-4 mm from the internal and external hydrogel surfaces.

**Discussion:** These results indicate that a double diffusion bioreactor, for forming calcified tissue plates and interfaces, can be extended from creating flat geometries to partial-spherical shell geometries, which are typical of certain entheses. Further control of the sample boundary conditions may enable creation of even more complex plates and interfaces.

### Real-time Visualization of Blood Flow for Monitoring in Regenerative Surgery using a Smartphone Application

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**Background:** Free flap monitoring is of paramount importance to ensure that the vascular supply is not compromised over the post-operative course. Early detection of arterial or venous failure facilitates salvage. The ideal monitoring system has been described as continuous, non-invasive, quantitative, easy to administer, highly sensitive, and highly specific. A variety of currently used technologies provide proxies to assess flap perfusion, however are expensive and fall short of the ideals. Other attempts at using smartphones for microsurgical free flaps do not provide real-time monitoring.

**Methods:** We have developed an Android-based smartphone application that utilizes the Eulerian Video Magnification (EVM) [3] algorithm to directly visualize skin perfusion of non-buried free flaps in real-time and provides immediate analysis of tissue. The EVM algorithm allows us to amplify small changes that are not discernable to the human eye, such as the change in color of skin in systole and diastole. Our application has modified this algorithm to assess arterial

and venous occlusion in models using hands with various applied tourniquet pressures.

**Results:** This smartphone application enables the visualization of perfusion to the skin. Our testing shows that we can both qualitatively and quantitatively identify arterial and venous occlusion in our simulated environments. We have also demonstrated its use intraoperatively and postoperatively for free flap monitoring.

**Significance:** This serves as proof-of-concept for real-time, non-invasive monitoring of microsurgical free flaps with a smartphone. Our application meets many of the requirements of the ideal monitoring system: continuous (real-time), non-invasive, quantitative and easy to administer.

### Osteogenic Induction in Human Muscle Grafts via Bmp-2 Gene Therapy

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The healing potential of a ruptured anterior cruciate ligament (ACL) is considered to be extremely poor. One of the strategies to enhance tendon graft-bone healing in anterior cruciate ligament reconstruction is gene therapy. Recombinant bone morphogenetic proteins are the most potent osteoinductive agents available today and have demonstrated efficacy in promoting bone healing in tibial fractures and spinal fusions in prospective randomized controlled trials. Using "same day" *ex vivo* regional gene transfer it is possible to create human skeletal muscle cells that produce BMP-2 and subsequently return muscle graft at the place of healing with the surgery done within two hours. Using adenoviral vector carrying the luciferase reporter gene (Ad.Luc 2) we determined optimal virus concentration (107 plaque forming unit) and period of contact (30 min) since time is the limiting parameter. The presence of CaCl<sub>2</sub> and LnCl<sub>3</sub> did not help bypass the inefficiency of receptor-dependent uptake of the vector. The human muscle grafts form ten patients were transduced with adenoviral vector encoding bone morphogenetic protein-2 (Ad.BMP-2) according to the developed protocol and osteogenic potential was evaluated by quantitative PCR. The amount of released BMP-2 protein from transduced muscle grafts was determined by ELISA. Taken together, our data introduce adenoviral vectors as a powerful tool in regenerative medicine and the clinical potential of a novel "same day" *ex vivo* regional gene therapy approach to promote bone repair, which may make gene therapy cost-effective when adapted for human use.

### Shockwave Treatment Enhances Proliferation and Improves Wound Healing via Purinergic Signaling Linked Erk1/2 Pathways

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In clinics shockwave treatment (SWT) accelerates poor wound healing of e.g. burn wounds or diabetic ulcers, though the underlying principles of this beneficial effect remain to be fully elucidated. Therefore the aim of this study was to identify signaling pathways involved in the proliferative and wound healing effect of SWT.

Primary human adipose derived stem cells, mouse mesenchymal stem cells, and a human Jurkat T cell line were subjected to *in vitro* SWT and ATP release was measured. Proliferation after SWT was determined via BrdU incorporation. Western blot analysis was performed to evaluate MAPK pathway activation. An *in vivo* rodent ischemic excision wound healing model was used to assess the dependency of SWT induced wound healing on ERK1/2 signaling.

SWT dose-dependently released ATP in all three cell types and significantly increased the amount of proliferating cells. Hydrolysis of released ATP with apyrase diminished the proliferative effect of SWT. Shockwaves significantly activated phospho-ERK1/2, which was prevented by the P2 receptor antagonist suramin as well as by ATP depletion. Our *in vivo* study confirmed that SWT induced wound healing in an ERK1/2 dependent manner.

We conclude that *in vitro* SWT releases cellular ATP, activating downstream ERK1/2 signaling via purinergic receptors, ultimately causing the proliferative effects of SWT. Our *in vivo* data endorse the ERK1/2 signaling pathway being essential in the SWT wound healing effect. Thus, this signaling cascade is one of the underlying principles of the beneficial effects of SWT and will hopefully emphasize its application as a routine wound healing treatment.

### Thermally Responsive Core-shell Nanoparticles with Independent Dual Drug Release Profiles

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We developed a dual drug delivery system that can release two drugs independently by thermal responsiveness. The nanoparticles consisting of chitosan oligosaccharide (COS) and carboxyl group-terminated pluronic F127 (F127) was synthesized by covalent cross-linking. Kartogenin was covalently conjugated to COS before the nanoparticle synthesis. Diclofenac was encapsulated in the inner core of the nanoparticle. The nanoparticles were ~125 nm in size at 37 °C and expanded to ~442 nm when cooled to 4 °C in aqueous solutions. Swelling and shrinking of the nanoparticles by thermal responsiveness was controllable by the composition ratio of F127 or kartogenin to COS. The nanoparticles showed immediate and sustained release of diclofenac and kartogenin respectively, which was controlled independently by temperature change. The toxicity of the nanoparticles was found to be negligible. Inflammation in U937 cells and chondrocytes was effectively suppressed by the nanoparticles treated with cold shock. Chondrogenic differentiation of mesenchymal stem cells was also enhanced by cold shock treatment of the nanoparticles. These results suggested that the thermally responsive nanoparticles could provide dual-function therapeutics to quench the inflammation and regenerate damaged tissue when combined with cryotherapy.

### Rheological Characterization of an Extracellular Matrix Hydrogel as a Cell Carrier for Stroke Damage Repair

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Stroke is the most common cause of adult disability in industrialized countries. Neural stem cells injected into a rat brain infarct improved neurological functional deficits, but with limited restoration of brain tissue loss. Current efforts are aimed at embedding human neural stem cells within a biomaterial carrier to provide shielding during implantation, and structural support within the ischemic microenvironment post-injury.

A novel approach for stroke lesion repair using an injectable extracellular matrix (ECM) hydrogel as a cell carrier was investigated. ECM hydrogels provide advantages such as minimally invasive delivery by a catheter or needle (gelling *in situ*), filling an irregularly



sized defect, and mimicking the inherent bioactivity of the native matrix. Urinary bladder matrix (UBM) hydrogel is an ECM hydrogel that promotes human neural stem cell viability and differentiation in 3D culture. Important unanswered questions are: 1) the change in viscoelastic properties of the ECM hydrogel embedded with cells?, 2) the distribution of cells within the ECM hydrogel?, and 3) the maximum cell:ECM concentration for a gel-cell carrier?

UBM of GMP quality (ACell MatriStem) was powdered, enzymatically digested by pepsin/HCl, and gelled by temperature-controlled neutralization. Rheological characteristics of MatriStem gel (4, 6, 8 mg/mL) alone and with cells (20:80 cell:ECM volume) were determined with a parallel-plate rheometer. Cellular distribution within the hydrogel was quantified with ImageJ. Cells were added to MatriStem gel (6 mg/mL) at increasing concentrations to determine the maximum cell:ECM concentration still permissive to hydrogel formation. The present study results inform novel regenerative medicine strategies for stroke patients.

### Development of Islet Organoids from Human Pluripotent Stem Cells

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Diabetes mellitus has become a global epidemic with striking impacts on society and economy. While islet transplantation is a promising treatment for diabetes, it has not been widely available to majority of patients due to the scarcity of donors. Here, we report a new biofabrication technique for creating islet organoids from human pluripotent stem cells. We developed a unique collagen 3D scaffold and discovered that islet organoids can be formed from pancreatically differentiated human embryonic stem cells (HESCs). Immunohistochemistry assay revealed that these cell clusters formed from HESCs exhibited a typical tissue structure of the islets. The real-time PCR and glucose challenging experiments divulged that these cells can secrete insulin and C-peptide upon glucose challenging. Furthermore, TEM indicated the existence of insulin-secretion granules in beta cells consisting of islet organoids, suggesting a high degree of maturation of these cells. This study clearly demonstrated the formation of islet organoids from HESCs *in vitro*. The augment of this technology to other stem cell differentiations will bring cell replacement therapy one step closer to treating many diseases such as diabetes in more controllable clinical settings.

### Extracellular Matrix Hydrogels as Scaffolds for Spinal Cord Injury Repair

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Restoration of lost neuronal function after spinal cord injury (SCI) still remains a big challenge for current medicine. To bridge the lesion and re-establish damaged connections after SCI, extracellular matrix (ECM) hydrogels were prepared from porcine spinal cord (SC), brain and urinary bladder (UB) tissue.

The hydrogels were characterized *in vitro* in terms of their biochemical composition and adhesion, as well as the proliferation and migration of human mesenchymal stem cells (hMSC). In an *in vivo* study, UB- and SC-ECM hydrogels were acutely injected into the spinal cord hemisection and evaluated after 2, 4 and 8 weeks. Histological analysis showed that both hydrogels integrated into the host tissue and stimulated neovascularization and nervous tissue ingrowth into the lesion. On the other hand, massive infiltration of macrophages into the lesion and rapid hydrogel degradation did not prevent cyst formation, which progressively developed over 8 weeks. Gene expression analysis at 2 weeks post-SCI revealed significant down-

regulation of genes related to immune response and inflammation in both hydrogel types, whereas this effect diminished at later time points.

In conclusion, ECM hydrogels are biocompatible and promote *in vitro* the proliferation and migration of hMSCs. When injected into SCI, UB- and SC-ECM hydrogels modulated the innate immune response and provided a stimulatory substrate for *in vivo* neural tissue regeneration. However, fast hydrogel degradation resulting in cyst formation may be a limiting factor for the use of ECM hydrogels in the treatment of acute SCI.

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### Flexible Fiber-based Rapid Prototyping Technology for Implants with Structural Gradings for Regenerative Medicine

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To meet the complex requirements for scaffolds in terms of biomimetics the Net Shape Nonwoven (NSN) method was developed at ITM to implement new custom-designed implant structures with gradings on the material and structural level and thus to create patient specific, complex and scalable implants for the regeneration of tissue interfaces [1]. The NSN method is based on rapid prototyping and has the potential to process short fibers into three-dimensional constructs of arbitrary geometry. NSN structures offer good conditions for the ingrowth of cells due to the excellent ratio of surface to volume of the fibers and the interconnecting pore spaces. The starting material is applied in layers and is solidified by computer aided manufacturing during the process. To realize complex three-dimensional geometries a CNC system with a specially programmed CAD Interface was developed.

Multiple types of fibers (e.g., the biopolymers chitosan or silk) can be processed by selecting appropriate biocompatible binding agents [2]. Due to different process parameter settings a specific adaptation to the target tissue and a controlled cell growth are achieved. Modeling software, developed and used at ITM, allows a detailed simulation of the nonwoven structures. Thus, trial-and-error methods are avoided and the customized structural properties of the implants can be realized as required without wasting the costly biomaterials.

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### Tendon and Ligament Engineering: Recapitulating Embryonic Development Accelerates Tendon/Ligament Healing

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Tendon and ligament injuries greatly impact the patient's quality of life and can, if not treated, contribute to the early onset of osteoarthritis - a more serious conditions for which no effective treatment is available. One of the ligaments most often injured is the anterior cruciate ligament (ACL). Unfortunately the ACL has poor regenerative potential and in most cases surgical intervention is necessary to restore function.

Based on the knowledge gathered during embryonic development of tendons and ligaments we propose a novel strategy whereas signals emanating from the different cells sources known to orchestrate the development of these structure are harnessed to accelerate healing. We tested if bone-, cartilage-, stromal- and/or

muscle-derived signals were capable of enhancing the teno/ligamentogenic program in human hamstring-derived cells. Our results showed that muscle-derived signals led to the up regulation of two genes involved in tendon/ligament specification: tenomodulin and scleraxis. Whole-genome and gene ontology analysis clearly showed an enrichment on extracellular matrix genes with an up regulation of anabolic genes such as collagen family members and a down regulation of catabolic genes such as proteinases. This positive remodelling can aid the regenerate process by allowing a faster and more active rehabilitation.

These results strongly suggest that muscle-derived signals have the potential to accelerate tendon/ligament remodelling paving the way to novel therapies.

#### **A Strain-dependent Computational Model for the Diffusion of Transcription Factors through the Cell Nucleus**

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Nuclear spreading plays a crucial role in stem cell fate determination. We reported evidences of multipotency maintenance of mesenchymal stromal cells cultured on three-dimensional engineered niche substrates fabricated via two-photon laser polymerization (2PP) [1]. We correlated multipotency maintenance to a more roundish nuclear morphology of cells cultured in the 2PP-fabricated niches, with respect to cells cultured on flat substrates.

To interpret these findings, we developed a multiphysics model coupling nuclear strains due to cell adhesion to diffusive transport across the cell nucleus. We reconstructed the cell geometry and relevant boundary conditions from confocal Z-stack images acquired from cells cultured in the niches. On the reconstructed geometry, we estimated volume, surface and shape factors for each cell nucleus. We assigned the cell diffusivity as a function of the local volumetric deformation. The levels of nuclear spreading turned out to depend on the cell localization within the niche architecture. The computational results also indicate that the higher the nuclear deformation (e.g. in spread nuclei), the greater the rate of the nuclear import of small solutes such as transcription factors.

Our results point towards nuclear deformation as a primary mechanism by which the stem cell translates its shape into a fate decision, i.e. through strain-dependent amplification of the diffusive flow of signaling molecules into the nucleus.

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#### **Mineralization and Ultrastructural Analysis of Maxilla Bone in Rat Ovx Model**

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Ovariectomy (Ovx) could cause estrogen deficiency which leads to bone loss and induce osteoporosis in female rats. Most of the studies have focused on the bone loss in long bone and mandibular alveolar bone after Ovx, however, it remains unclear how Ovx affect the mineralization of maxilla alveolar bone with the strength and load-bearing capacity in ultrastructural level. The aim of this study was investigating the bone quality of maxilla in ovariectomized rats in newly formed and old bone area to reveal the effect of Ovx on mineral structure changes on maxillary bone formation. The differences of composition and ultrastructure of maxilla bone mineral were analyzed using advanced analytical

facilities such as scanning electron microscope (SEM), energy dispersive spectrometry (EDS), laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), transmission electron microscope (TEM) and nano-indentation. The results of EDS showed that the ratios of Ca/P were comparable between Ovx and Sham group, while the measurement by nano-indentation and TEM demonstrated that hardness of Sham group was significantly higher than that of Ovx group and mono mineral crystals may exist in Ovx group. These results may indicate that the composition of mineral in maxilla is similar in Ovx and Sham maxilla, but the changes of maxilla after Ovx may occur in the ultrastructure of nano-scale. This study provided some new knowledge in the mineralization process of new bone formation and abnormality in Ovx induced osteoporosis, which will assist the understanding of fracture healing and potential early clinical loading treatment in osteoporosis patients.

#### **Effect of Agarose-based 3D Cellular Microenvironment on Porcine Embryonic Stem Cell Self-renewal**

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This study was conducted to evaluate the effect of three-dimensional(3D) culture using agarose-based hydrogel, which mimics *in vivo* physiological environment, on maintaining self-renewal of porcine embryonic stem cells (ESCs). ESCs retrieved from porcine embryo were cultured within 3D scaffold made of agarose hydrogel and their cell proliferation, alkaline phosphatase (AP) activity and transcriptional expression of self renewal related genes were analysed. We observed that proliferative activity of porcine ESCs was maintained in agarose hydrogel and AP activity was significantly increased in 3D condition, compared with those cultured in 2D with or without MEF feeder cells. Moreover, porcine ESCs cultured in 3D agarose hydrogel showed higher transcriptional levels of all self-renewal-related genes (significantly transcriptional up-regulation in case of OCT4, SOX2, and TERT) than those cultured in 2D. These results demonstrate that self-renewal of porcine ESCs can be maintained more effectively in 3D culture microenvironment than in 2D culture microenvironment.

#### **A Novel Igf-ii-like Synthetic Peptide for Improving Tendon Healing**

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Tendon injuries are an increasingly common clinical problem occurring in otherwise healthy, active people. Primarily caused by overuse or trauma, these injuries significantly affect patient quality of life and are a substantial financial burden to the healthcare economy. While there are currently no clinically accepted biological treatments for improving tendon healing, much research has attempted to identify growth factors capable of aiding this challenging process.

In this study we evaluate vesiculin, a novel synthetic peptide similar in structure to IGF-II, for its ability to enhance tendon healing *in vitro*. We compare this to PDGF, a pleiotropic factor that increases tenocyte function *in vitro*, and improves tendon healing *in vivo*.

Primary tenocytes harvested from rat tails or human bicep tendons were treated with vesiculin and PDGF. Cell growth was determined using alamarBlue<sup>®</sup> assays and collagen deposition measured by Sirius red dye release. Gene expression analysis using real-time PCR was used to study tenocyte differentiation and trans-differentiation.

Vesiculin (10–8M) marginally increased tenocyte cell number (~20%,  $p < 0.05$ ) and significantly increased the expression of tenocyte genes collagen Ia1, collagen IIIa1, decorin, scleraxis, tenomodulin and tenascin-c (all  $p < 0.05$ ), without increasing the expression of chondrocyte or osteoblast marker genes. PDGF, meanwhile, increased tenocyte numbers significantly (~50%,  $p < 0.05$ ), increased collagen production (~40%,  $p < 0.05$ ), but decreased tenocyte (scleraxis and tenomodulin), chondrocyte (aggrecan) and osteoblast (alkaline phosphatase) gene expression.

Overall, vesiculin shows great promise as a novel factor for improving healing outcomes of tendon repair. Future work is required to determine a suitable delivery method for *in vivo* study.

### Microtubular Scaffolds Guides Odontogenic Differentiation and Dentin Formation of Human Dental Pulp Stem Cells

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Dentin, a mineralized dental tissue produced by odontoblasts, is structurally anisotropic, containing highly aligned dentinal tubules. Damage to dentin often causes irreversible changes in the underlying pulp affecting tooth vitality and resulting in tooth loss. The ability to regenerate dentin may offer the exciting opportunity to maintain pulp vitality and prevent tooth loss. Designing scaffolds to closely mimic native tissues can often facilitate tissue regeneration. In this work, microtubular scaffolds of poly(L-lactic acid) (PLLA) were fabricated using a thermally-induced phase separation technique (TIPS) to reproduce the natural dentin-like tubular structure. A range of microtubule diameters was prepared at various temperatures including -20, -80, and in liquid nitrogen. For comparison, PLLA scaffolds with random non-tubular architecture were included in this study. The scaffolds were seeded with human dental pulp stem cells (hDPSCs) obtained from wisdom teeth, and then cell-scaffold constructs were cultured *in vitro*. Using a confocal microscopy, microtubular scaffolds were shown to enhance alignment of hDPSCs and facilitate homogeneous cell distribution, as the parallel-orientated structure guided the cells through microtubules. Furthermore, cells seeded on the scaffolds with smaller microtubules exhibited aligned elongated shape; closely resembling the morphology of columnar odontoblasts. After 14-day cell culture, up regulation of key dentin gene markers was observed in microtubular scaffolds compared to random scaffolds. These results suggest that designing scaffolds to mimic the organization of the extracellular matrix could promote dentin regeneration.

### Structural Nichoids Fabricated by Two-Photon Laser Polymerization Promote Maintenance of Pluripotency During *In Vitro* Expansion of Adult and Embryonic Stem Cells

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Therapeutic application of stem cells requires control over their fate during culture. We developed an innovative substrate mimicking the geometric constraints perceived by stem cells in their native

niche. This “nichoid” substrate is fabricated by two-photon laser polymerization using a zirconium/silicon hybrid sol-gel [1].

We expanded human bone marrow-derived mesenchymal stromal cells (hMSC) and R1 mouse embryonic stem cells (mES) for three weeks on the nichoid substrates. In hMSC, we evaluated multipotency by expression of CD146 and osteogenic/adipogenic lineage commitment by Alizarin Red/Oil red O and by expression of RUNX2 and Bone Sialoprotein (BSP). In mES, we evaluated pluripotency by expression of OCT4 and lineage commitment by expression of GATA-4 and alpha-SMA.

In hMSC, cell density and colony diameter were greater by 36% and 15%, respectively, than in flat-cultured controls. The expression of RUNX2 and BSP after osteogenic conditioning was greater than in flat controls. The number of adipocytes and their lipid content obtained after adipogenic conditioning were comparable to non-expanded cells.

In mES, the expression of OCT4 was greater than in flat controls. The expression of all the lineage commitment markers tested was lower than in both controls and cells cultured on extracellular matrix obtained from de-cellularized renal tissue.

These results demonstrate the ability of the nichoid substrate to promote maintenance of pluri/multi potency of stem cells in culture.

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### Noninvasive Trafficking of Scaffold Degradation and Tissue Regeneration

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Noninvasive imaging of transplanted cells, host tissue infiltrates, and scaffold degradation is of significant importance in the field of tissue engineering. Ideally, the transplanted stem cells with tissue-engineered scaffolds for tissue regeneration need to be monitored longitudinally without sacrificing animals. Currently, however, a large number of samples and animals are required to track biodegradation of implanted scaffolds, and such nonconsecutive single-time-point information from various batches result in inaccurate conclusions. To overcome this limitation, we developed functional biodegradable scaffolds by employing invisible near-infrared (NIR) fluorescence and followed their degradation behaviors longitudinally *in vitro* and *in vivo*. Using optical fluorescence imaging, the scaffold degradation could be quantified in real-time, while tissue ingrowth was tracked by measuring vascularization using magnetic resonance imaging in the same animal over a month. Since light scattering and absorption in human tissue are the major obstacles in optical fluorescence imaging, NIR wavelength can efficiently minimize tissue autofluorescence, resulting in less background interference and enhanced signal-to-background ratio. Moreover, we optimized the *in vitro* process of enzyme-based biodegradation to predict implanted scaffold behaviors *in vivo*, which was closely related to the site of inoculation. This combined multimodal imaging will benefit tissue engineers by saving time, reducing animal numbers, and offering more accurate conclusions.

### Enzyme Resistance and Mechanical Stability of Genipin-crosslinked films

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Intracranial cerebral aneurysms are arterial saccular dilatations in which a weakening of a major blood vessel in the brain is susceptible to rupture. Current treatment options for both ruptured and unruptured aneurysms include surgical clipping and catheter-based intervention. The endovascular approach is less-invasive and includes the placement of foreign materials such as platinum coils into

the aneurysm. However, the primary mechanisms for recurrence in the endovascular coiling procedures are recanalization of the clot (via enzymatic digestion) and mechanical compactions of coils within the sac (due to blood flow). Enzymatic remodeling reduces the mechanical integrity of the protein network. We propose to deliver a bioactive agent, genipin, locally within the aneurysm sac that will induce covalent crosslinking to stabilize the clot. Genipin is a natural biocompatible crosslinking agent extracted from the *Gardenia jasminoides*. It is 10,000 times less cytotoxic than its more commonly used counter agent, glutaraldehyde. In our study, we hypothesize genipin-crosslinked fibrin hydrogel networks could improve the performance of haemostatic materials for minimally invasive occlusion of brain aneurysms. The mechanical stability and enzymatic resistance is evaluated through the incorporation of streptokinase, a bacterial enzyme derived from group C(beta)-hemolytic streptococci. Using rheology, our preliminary data depict mechanical stability in the crosslinked system to be significantly higher than in pristine fibrin gels. These observations suggest that genipin could serve as a biologic to improve minimally invasive embolic treatments.

#### Microengineered Heterospheroids of Pancreatic Islets and Adipose-derived Stem Cells for Effective Cell-based Treatment of Diabetes

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Currently, pancreatic islet transplantation is the only curative therapy of type 1 diabetes; however, donor shortages and cellular damage during the isolation critically limit the use of this approach. In this study, we describe a novel method for creating viable and functionally potent islets by co-culturing single islet cells with adipose-derived stem cells (ADSCs) in three-dimensional (3D) environment, and quantitatively demonstrate the positive effect of ADSCs on islet survival *in vitro* and *in vivo*.

Here, mixtures of single islet cells and ADSCs isolated from Sprague-Dawley rats were seeded to PDMS-based concave micro-well arrays to have heterospheroids with controlled-size and shape. For *in vivo* tests, we encapsulated spheroids in microfiber and implant them into diabetic mice.

When ADSCs were co-cultured with islets in 3D structure, they segregated from the islets, eventually yielding purified islet spheroids. Thereafter, the ADSC-exposed islet spheroids showed significantly different ultrastructural morphologies, higher viability, and enhanced insulin secretion compared to mono-cultured islet spheroids. Also, they showed increased efficacy of *in vivo* experiments, which revealed that co-culture-transplanted mice maintained their blood glucose levels longer than mono-culture-transplanted mice, and required less islet mass to reverse diabetes.

In conclusion, the initial presence of ADSCs effectively improved islet survival while inhibiting apoptosis and supporting better cell-cell interactions between  $\beta$  cells. Our proposed method could potentially help overcome the problem of cell shortages, and could possibly suggest a future platform for the cell-based treatment of diabetes.

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#### Tendon Regeneration Through a Scaffold-free Approach: Development of Tenogenic Magnetic hASCs Sheets

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Tendon's regeneration is limited, demanding for cell-based strategies to fully restore their functionality upon injury. The concept of magnetic force-based TE(1), generally using magnetic nanoparticles may enable, for example, stem cell stimulation and/or remote control over TE constructs. Thus, we originally propose the development of magnetic cell sheets (magCSs) with tenogenic capability, aimed at promoting tendon's regeneration.

A Tenomodulin (TNMD+) subpopulation was sorted from human adipose stem cells (hASCs), using TNMD-coated immunomagnetic beads(2) and used as cell source for the development of magCSs. Briefly, cells were labeled with iron oxide composite particles (Micromod) and cultured for 7 days in  $\alpha$ -MEM medium with or without magnetic stimulation provided by a magnetic device (nanoTherics). CSs were retrieved from the plates using magnet attraction as contiguous sheets of cells within its own deposited ECM.

CSs were evaluated by confocal microscopy, flow cytometry and immunocytochemistry for tendon related markers, and prussian blue staining. Cell metabolic activity and proliferation was also assessed by MTS and DNA assays. Moreover, the genetic expression of collagen I, III, and tenascinC was quantified by real time RT-PCR.

Preliminary results suggest that magnetic actuation improves cell proliferation and influences the synthesis of tendon ECM proteins in comparison to magCSs under static conditions. Ongoing studies on mechanical properties will provide information on the functionality of magCSs versus non-magnetic as tissue-like substitutes for tendon TE.

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#### Extracellular Matrix-based Device for Reconstruction of the Temporomandibular Joint Meniscus

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Currently, no consistently effective option exists to replace a temporomandibular joint (TMJ) disc. The present study examined the use of an extracellular matrix (ECM) device as an inductive template for reconstruction of the TMJ disc in a porcine model, considered the "gold-standard" for TMJ disc research. The device was implanted following bilateral discectomy, leaving the contralateral side empty (control). Animals (n=60) were sacrificed at 2, 4, 12, and 24 weeks and remodeling was assessed by gross morphologic and histologic examination, MRI imaging, and biomechanical analysis (tension and compression). All results were compared to native disc as a control.

The ECM device was remodeled and replaced by a structure highly resembling native tissue by 4 weeks post-implantation in greater than 50% of animals. Immunolabeling showed that mononuclear macrophages and perivascular progenitor cells mediate the early stages (2-4 weeks) of tissue remodeling. The histologic appearance of the remodeled implant at later times (>4 weeks) was characterized by dense, aligned fibrocartilage containing spindle-shaped cells within the area of articulation. Formation of peripheral muscular and tendinous attachments resembling those in native tissue was also observed. MRI confirmed these results. The biomechanical properties of the remodeling tissue approached that of native tissue over the course of remodeling. Protection of condylar surfaces in ECM implanted animals was observed regardless of ECM-mediated formation of new TMJ disc tissue.

This device fills a clinical need for which there are currently no effective treatments and may represent a simple and effective "off-the-shelf" solution for reconstruction of the TMJ disc.

#### Silk Based Antibacterial Nanofibers

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As biomaterials, silk fibroin is a unique material which has remarkable properties including biocompatibility, permeability, biodegradability, minimal inflammatory reaction and proper mechanical properties. Fibroin morphology; random coil (Silk I) or  $\beta$  sheets (Silk II) is a crucial factor that is affecting their mechanical and thermal properties, hydrolytic stabilities and also release of drug/biocides. In this study, silver nanoparticle containing fibroin composite nanofibers (Ag/Fibroin) and antibacterial polyethylenimine/fibroin (PEI/Fibroin) blend nanofibers were produced in both random coil (Silk I) and  $\beta$  sheet (Silk II) morphologies, via glutaraldehyde vapor and methanol post-treatments. Nanofibers with  $\beta$  sheet structure have higher hydrolytic and thermal stability compared to random coil structure, due to crystalline polymer morphology. Antibacterial activity and cytotoxicity results reveal that PEI containing nanofibers exhibit good antibacterial properties and ability to prevent bacterial adhesion over a long period of time. On the other hand, cumulative amount of silver ion released from random coil fibroin morphology was significantly higher than the amount released from  $\beta$  sheet morphology. Besides, Ag/Fibroin nanofibers showed promising antibacterial activity against *P. aeruginosa* even at low Ag content (0.1% Ag, w/v) and slightly active against *S. epidermidis* and *S. aureus* at 1% Ag (w/v) content with minimal cytotoxicity to L929 cells. In summary, silk fibroin based antibacterial bionanotextiles would meet a demand for wound dressings in medical field due to their long-term antimicrobial activity with no developed resistance, minimal cytotoxicity to mammalian cells and biocidal ability.

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### Ceramic-Hydrogel Composites Enhance Bone Regeneration in a Rodent Critical Size Defect Model

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Bone defects can result from trauma, congenital abnormalities, tumor excision and infection. Current clinical methods are often insufficient for reconstruction of large and nonunion shape defects. Tissue engineering has significant potential for bone regeneration. Recently, we have developed porous hydrogels that stimulate vascularization *in vivo*. In this study we examined whether incorporation of ceramic materials within the hydrogel would enhance bone formation.

Degradable porous hydrogels with fibrin were generated using a particulate leaching method. Hydrogel structure was composed of polyethylene glycol diacrylate (PEG-DA) and PEG-poly(L-lactic acid)-DA. Varying ratios of hydroxyapatite (HA) to tri-calcium phosphate (TCP) (0:0, 70:30, 50:50, 30:70%) was incorporated into hydrogel structure to evaluate the optimum ratio for bone regeneration.

In this study, ceramic hydrogel composites exhibited longer degradation times than controls both *in vitro* and *in vivo* (>8 weeks).

A rodent cranial defect model was used to examine the effect of HA and TCP on bone regeneration. Regenerated bone volume was quantified based on micro-computed tomography images for 4 weeks and 8 weeks. Tissue structure was evaluated with histological stains. Ceramic hydrogel composites showed greater bone regeneration than the control at all time points. The highest bone replacement (~75%) was found in the composite hydrogel (50:50%, HA:TCP) at 8 weeks. Our results indicate that supplementing hydrogels with HA and TCP augmented bone regeneration.

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### Silver Nanoparticle-Embedded Polymersome Nanocarriers for the Treatment of Antibiotic-Resistant Infections

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Antibiotics have been extensively used since their commercialization to treat patients suffering from a wide variety of infectious diseases. When utilized correctly, these drugs are extremely effective at reducing mortality rates and healing time, which makes them essential in the clinic today. Unfortunately, however, antibiotics have been used so prevalently over the last 80 years that the bacteria they were designed to kill have begun to evolve and adapt, rendering these drugs ineffective. According to the Center for Disease Control's 2013 report on antibiotic resistance in the United States, at least 2 million people acquire serious infections from antibiotic resistant bacteria each year, and over 23,000 die as a direct result. This study explored the development and optimization of a polymersome nanocarrier formed from a biodegradable diblock copolymer to overcome bacterial antibiotic resistance. Here, polymersomes were synthesized containing silver nanoparticles embedded in the hydrophobic compartment, and ampicillin in the hydrophilic compartment. Results showed for the first time that these silver nanoparticle-embedded polymersomes (AgPs) inhibited the growth of *Escherichia coli* transformed with a gene for ampicillin resistance (*bla*) in a dose-dependent fashion. Free ampicillin, AgPs without ampicillin, and ampicillin polymersomes without silver nanoparticles had no effect on bacterial growth. The relationship between the silver nanoparticles and ampicillin was determined to be synergistic and produced complete growth inhibition at a silver-to-ampicillin ratio of 1:0.64. In this manner, this study introduces a novel nanomaterial that can effectively treat problematic, antibiotic-resistant infections in an improved capacity for a wide range of medical applications.

### Osteoinductive Silk Fibroin/titanium Dioxide Nanoparticle/hydroxyapatite Hybrid Scaffold for Bone Tissue Engineering Application

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The present study demonstrated the fabrication that incorporation of titanium isopropoxide (TiO<sub>2</sub>) and hydroxyapatite (HA) nanoparticles into the silk fibroin (SF) scaffolds. In this process, we prepared TiO<sub>2</sub> nanoparticles using sol-gel synthesis and the porous structure was developed by salt-leaching process. Homogeneous distribution of TiO<sub>2</sub> and HA nanoparticles were confirmed by images of VP-FE-SEM and those equipped with energy dispersive X-ray spectrometer. Structural characteristics of the porous SF/TiO<sub>2</sub>/HA hybrid scaffold were also determined using FTIR analysis and X-ray diffractometer. In this study, the porous SF/TiO<sub>2</sub>/HA hybrid scaffold showed similar porosity, enhanced mechanical property, but decreased water binding abilities, compared with the porous SF scaffold. For evaluation of the osteogenic differentiation of rat bone marrow mesenchymal stem cells, alkaline phosphatase activity and osteogenic gene expression were employed. Our results revealed that the porous SF/TiO<sub>2</sub>/HA hybrid scaffold had improved osteoinductivity compared with the porous SF scaffold. These results suggest that the osteogenic property as well as mechanical property of the porous SF/TiO<sub>2</sub>/HA hybrid scaffold could be better than the porous SF scaffold. Therefore, the porous SF/TiO<sub>2</sub>/HA hybrid scaffold may be a good promising biomaterial for bone tissue engineering application.

### Synthesis and Characterization of Ph-responsive Hydrogels as a Biocompatible Drug Carrier Based on Chemically Modified Tragacanth Gum Polysaccharide

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The preparation of pH-responsive hydrogels based on Tragacanth gum (TG) polysaccharide and glycidyl methacrylate (GMA) was investigated. Ring opening reactions between epoxide and hydroxyl groups of GMA and TG, respectively, led to preparation of TG-GMA which was then reacted with K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> through radical polymerization to achieve hydrogels. The obtained hydrogels were characterized using techniques such as FT-IR, scanning electron microscope (SEM), zeta sizer and thermogravimetric analysis (TGA). The gel content of hydrogels showed dependence on the weight ratio of TG/GMA. According to the results, the swelling behavior of the prepared TG-GMA hydrogels showed significant dependence on the gel content, pH, and immersion time. The loading and *in vitro* release of Quercetin as a model drug was investigated at a pH of 7.0 and 2.2. At higher pH values, the water transport profile became more dependent on polymer relaxation. This effect was attributed to the increase in the ionized groups of tragacanth acid segments, which contributed to electrostatic repulsion among the groups and led the gel polymer network to expand. Furthermore, the investigation shows that pH-responsive TG-GMA hydrogels can be a good candidate for further tests as drug carriers.

### Efficient and Simple Method to Prepare High Quality Cell Spheres -a Novel Culture Surface with Multiple Open Holes

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Cell sphere formation is an important procedure in regenerative medicine and numerous high quality cell spheres of uniform size are required toward therapeutic practice. Floating culture is easy but cannot control sphere size. In culture surfaces with multiple holes of various size and depth, a paradox exists between stability and easy harvest of formed spheres, depending on the depth of the holes. To resolve this problem, we invented a novel culture surface that allows secure sphere formation and easy harvest. The culture area is filled with funnel-like holes of 1 mm square opening and the bottom of it is perforated as a 0.5 mm square opening. A prototype with 680 open holes was made as an insert for 6-well plate and its performance was examined.

**Methods:** Medium suspending mouse ES cells in different cell density was poured into the device. Cells sank to and accumulated on the downward medium surface formed at the lower opening of each hole and formed a cell sphere. To harvest cell spheres after 1–3 day culture, the downward medium surface was broken by touching it to a thin medium layer prepared in another culture dish and medium flowed out with spheres by hydrostatic pressure.

**Results:** Cell spheres of fairly uniform size were harvested. The average size was controllable by changing the density of cell suspension. Endodermal differentiation was induced from these cell spheres as well as from those made by time-consuming hanging drop method.

**Conclusion:** Our novel culture surface is expected to contribute future cell sphere technologies.

### 3D Bioprinting of Soft-Tissue Substitutes from Cell-Seeded Stimuli Responsive Hybrid Hydrogels

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Three dimensional bioprinting is being applied to tissue engineering and regenerative medicine as a manufacturing tool to produce 3D tissues and organs suitable for transplantation. In the present study, we use micro-extrusion based 3D bioprinting of hydrogels embedded with genetically corrected keratinocytes from recessive dystrophic epidermolysis bullosa (RDEB) 1 to create a skin grafts. We first transfected the cells with highly efficient non-viral transfection vectors to generate collagen type VII expressing RDEB keratinocytes that were previously incapable of producing the protein 2. The cells are then seeded into a printable thermoresponsive hybrid polymeric hydrogel from methacrylate monomers and collagen that support drug delivery, cell survival, attachment and proliferation. Moreover, we have printed micro-vessels within the hydrogel structure to represent the blood vessels in the skin. The 3D printing presented an added advantage over conventional hydrogel preparations by replicating the

wound bed structure and incorporating composite hydrogels for improved blood circulation and anti-inflammatory drug delivery.

This combination of 3D bio printing, biodegradable, printable hydrogels and gene corrected skin cells will yield skin grafts capable restoring missing proteins in chronic wounds of RDEB patients and accelerate the wound healing process in our clinical disease model. The current project will also be tested on other chronic wounds such as diabetic ulcers and burn wounds.

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### Highly Robust Chitosan Hydrogels via a Fast, Simple and Biocompatible Dual Crosslinking-based Process

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Load-bearing soft tissues such as cartilage, blood vessels and muscles are able to withstand a remarkable compressive stress of several MPa without fracturing. Interestingly, most of these structural tissues are mainly composed of water and in this regard, hydrogels, as highly hydrated 3D-crosslinked polymeric networks, constitute a promising class of materials to repair lesions on these tissues. Although several approaches can be employed to shape the mechanical properties of artificial hydrogels to mimic the ones found on biotissues, critical issues regarding, for instance, their biocompatibility and recoverability after loading are often neglected. Therefore, an innovative hydrogel device made only of chitosan (CHI) was developed for the repair of robust biological tissues. These systems were fabricated through a dual-crosslinking process, comprising a photo- and an ionic-crosslinking step. The obtained CHI-based hydrogels exhibited an outstanding compressive strength of ca. 20 MPa at 95% of strain, which is several orders of magnitude higher than those of the individual components and close to the ones found in native soft tissues. Additionally, both crosslinking processes occur rapidly and under physiological conditions, enabling cells' encapsulation as confirmed by high cell survival rates (ca. 80%). Furthermore, in contrast with conventional hydrogels, these networks quickly recover upon unloading and are able to keep their mechanical properties under physiological conditions as result of their non-swell nature.

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### 3D Printing and Plasma Coating of Nerve Guide Conduits (NGC)

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**Introduction:** Polymer NGC are increasingly used in peripheral nerve repair. As existing FDA approved materials are bio-inert, microtopographical features and surface functionalisation may improve neuroregeneration<sup>1-2</sup>.

**Methods:** NGC were produced from photocurable polycaprolactone (PCL) by microstereolithography (μSL) using a 405 nm laser and digital micromirror device (DMD). Laser light was reflected from an image loaded onto the DMD and focused onto the liquid prepolymer, causing crosslinking. 3D shapes were produced by lowering a stage within the prepolymer.

Plasma chambers were used to coat substrates with acrylic acid (AAc), allylamine (AAM) or maleic anhydride (MA).

NG108-15 neuronal and RN22 Schwann cells were cultured on surfaces. MTT assay was used to confirm cell viability. After 72 hours, cells were fixed and immunolabelled. Cells were imaged by confocal microscopy.

Grooved and smooth NGC were implanted in *thy-1-YFP-H* mouse common fibular nerve and compared with nerve autograft.

**Results & Discussion:** Guidance features < 100  $\mu\text{m}$  were created on NGC inner surfaces. Increased cell viability and neurite growth occurred on AAc coated surfaces. Cells adhered to other surfaces but neurite growth was less extensive. Micro geometries showed significantly improved regeneration than smooth NGC and were comparable to autograft.

**Conclusions:** We have produced NGC with user-defined topography and surface chemistry. Plasma treatment may be used to enhance neuronal and Schwann cell response to bio-inert materials. Topographical features enhance peripheral nerve regeneration in an animal model.

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## Engineering EMT using 3D Micro-scaffold to Promote Hepatic Specification for Drug Hepatotoxicity Screening

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Accompanied with decreased hepatic functions, epithelial-mesenchymal transition (EMT) was observed in primary hepatocytes *in vitro* culture on two dimensional (2D) substrates with elongated morphology, loss of strong cell-cell interaction and hepatic polarity. Upgrade to three dimensional (3D) culture has greatly improved and prolonged synthetic and metabolic functions of the hepatic cells, but the mechanisms of how the 3D culture enhances hepatic function are not well-understood. Here we achieved biomaterial-engineered EMT by utilizing non-adherent and adherent micro-scaffolds which can result in spheroid 3D (SP-3D) and stretched 3D (ST-3D) culture of HepaRG respectively. Improved expressions of representative hepatic functional markers (e.g. albumin), epithelial markers (e.g. E-cadherin), and depression of mesenchymal markers (e.g. Vimentin) were achieved in SP-3D culture compared with ST-3D and 2D culture which was proved through blocking EMT of HepaRG. Transcriptome analysis of over 60,000s genes revealed that there were about 3000 differentially expressed genes (DEG) when compared cells in the SP-3D and ST-3D. In particular, histone deacetylases (HDACs), (an enzyme for epigenetic chromatin remodeling and modifications of DNA), was identified via classic edgeR analysis of DEG as a key mediating factor in regulating EMT and hepatic differentiation. To prove the potency of this micro-scaffold-based 3D culture for application in drug safety evaluation, hepatotoxicity and metabolism assays of two hepatotoxins (i.e. N-acetyl-p-aminophenol and Doxorubicine) were performed which showed more biomimic toxicity response than 2D culture. These findings indicate that optimal regulation of EMT should be vital criteria for consideration in designing 3D hepatocyte culture configuration for basic research and application.

## Direct-write Assembly of 3d Hydroxyapatite for Segmental Mandibular Bone Reconstruction

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Custom-made, patient-specific scaffold can play crucial role in mandibular reconstruction for critical-size defect. Porogen-leached porous scaffolds or titanium plates fail to meet patient-specific requirement. In this study quality and quantity of bone formation was evaluated using custom-made 3D hydroxyapatite constructs for segmental mandibular bone reconstruction, in comparison to filling with hydroxyapatite granules.

Powdered hydroxyapatite was used for filling bone defects in critical size mandibular defect in 2 patients. 3D hydroxyapatite microperiodic architectures was fabricated by Direct-write technique. Anatomically shaped tray-like structure was formed by fusing the constructs using bone cement. Bone marrow chips from iliac crest were placed into the construct. Following surgical excision of a recurrent locally malignant tumor of mandible (size 7 cm x 3 cm), this construct was used for reconstructing the mandible. Integration of construct/powders was assessed over 4 months with radiological bone formation characteristics and clinical follow up using OHQOL score.

Mandibular reconstruction with powdered hydroxyapatite fillings fail to induce osteo-integration. But continuity of mandible bone is maintained in the patient receiving 3D microperiodic constructs 4 months post-surgery. Function of the mandible was satisfactory (chewing, talking, swallowing). OHQOL score was recorded. Aesthetics was satisfactory, according to the patient. The structure has shown no signs of rejection.

Although one patient was treated, clinical form, function and aesthetics were satisfactory. The patient is on regular follow up for evaluating quantity, quality of bone formation and osteointegration. Radiological evidence showed successful integration of construct to surrounding tissue. Direct-write technique offers a versatile tool to efficiently create patient-specific grafts.

## Spatially Controlled Proliferation, Migration and Differentiation of Neural Stem Cells on Novel 3D Conductive Scaffolds

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Several studies on the regeneration of the central nervous system have demonstrated the importance of combining neural stem cells (NSCs) and polymer scaffolds. In fact, expandable sources of neurons and properly engineered scaffolds, which can provide neural differentiation to specific lineage, can be instrumental for the effective repair of the neural tissue. [1] Specific functionalization of substrates with proteins from the extracellular matrix, such as laminin, are generally required for adhesion and proliferation of NSCs. [2] In this study, we developed electrically conductive polymer scaffolds, constituted by randomly distributed or aligned nanofibers, which are able to promote the proliferation and differentiation of NSCs without laminin functionalization. The scaffolds were fabricated by electrospinning technique which provides nanofibers that mimic the extracellular matrix. [3,4] The NSCs, seeded on the 3D scaffolds, not only attached and proliferated, but also were able to migrate and infiltrate inside the porosity of the fibrous mats. In this way, 3D cellular networks were created. Furthermore, the alignment of the fibers induced the growth of NSCs along one specific direction. In fact, the cells were able to highly elongate following the direction of fibers, creating aligned parallel patterns. The obtained results demonstrate that the developed scaffolds are characterized by exceptional biocompatibility and that the topographical cues can be advantageously used in neural tissue engineering.

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### Fabrication of an Osteochondral Graft with PLGA and Substance blended Alginate using a Solid Freeform Fabrication

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Current approaches for the engineering of osteochondral grafts are associated with poor tissue formation and compromised integration at the interface between the cartilage and bone layers. Many researchers have attempted to provide osteochondral grafts of combined cartilage and bone for osteochondral repair to help overcome the limitations of standard procedures. SFF system is recognized as a promising tool for creating tissue engineering scaffolds due to advantages such as superior interconnectivity and a highly porous structure. This study aimed to develop a 3D plotting system to enable the manufacturing of a biphasic graft consisting cartilage and subchondral bone for application to osteochondral defects. The material advantages of PLGA and alginate polymers were combined for a supporting frame and cell printing. Specifically, in order to promote the maturity of the osteochondral graft in our study, cartilage-derived ECM (cECM) or hydroxyapatite (HA) substances blended with alginate was plotted together with human fetal-derived stem cells (HFPCs) in the cartilage or subchondral bone layer under a multi-nozzle deposition system. Notably, a plotted biphasic graft shows good integration between cartilage and subchondral bone layers without structural separation. Furthermore, structural collapse of the scaffolds was not observed during the tissue culturing process. The non-toxicity of the cECM and HA substances were proved from a live/dead assay of plotted cell-laden alginate. A fabricated osteochondral graft with cECM and HA substances showed dominant cartilage and bone tissue formation in a differentiation assay. Future studies should be done to modify the alginate physical properties for long-lasting structural stability.

### Cell Based Pharmaceuticals for Veterinary Use - Pharmaceutical Classification, Criminal and Veterinary Law Pitfalls from a European Perspective

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In Europe, cell based therapies have been applied in veterinary practice for years. The cell product used is by legal definition within the European Union a veterinary medicinal product. Therefore, the application of such cell products requires market authorisation as well as production authorisation. Without these authorisations, the production, placing on the market and application of cell based veterinary medicinal products are legal only under defined exemptions. Acting against these regulations may have legal consequences for the treating veterinarian. Taking Germany as an example, at the time being, the application of cell based therapies under the umbrella of these exemptions cannot be legally performed, and the importation of such product is prevented by the lack of legal regulations for it. The only possibility for the production and application of cell based therapies in accordance with EU veterinary medicines legislation is to perform the necessary steps of production and application under the direct supervision of the responsible veterinarian in his practice.

### Effect of HUVEC/hMSC Pre-culture on Vascularization of Fibrin-Loaded Poly(propylene Fumarate) Scaffolds *In Vitro* and *In Vivo*

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Vascular networks are vital to the development, healing, and function of bone. Extensive intercellular interactions between human mesenchymal stem cells (hMSCs) and endothelial cells (ECs) promote angiogenesis and osteogenesis for tissue engineering applications. Pre-vascularized tissue-engineered bone grafts could result in improved outcomes. This study aimed to investigate the effect of *in vitro* pre-culture on *in vivo* vascularization of composite fibrin/poly(propylene fumarate) (PPF) scaffolds. A single spheroid containing 5000 cells of 50% HUVEC/50% hMSCs was encapsulated in each fibrin gel loaded into 3D-printed, cylindrical, porous PPF sleeves and cultured *in vitro* for 1–3 weeks. Immunofluorescent staining for CD31 and  $\alpha$ SMA showed extensive cellular sprouting and formation of vascular networks increasing with culture time. During *in vivo* studies, the scaffolds were implanted for one week in SCID mice into four subcutaneous pockets surgically created proximally on all four limbs. The following groups were studied: a) No pre-culture, b) 1 week pre-culture, c) 2 weeks pre-culture, d) 3 weeks pre-culture, and, e) Control, i.e., PPF/fibrin composite scaffold without spheroid. Preliminary findings suggest increased neo-vascularization occurs inside the scaffolds with longer pre-culture times as indicated by positive human-specific CD31 staining. The results from H&E and Masson's trichrome staining indicate neo-tissue formation and integration with host tissue.

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### 3d Microfluidic *In Vitro* Culture System for Cornea Disease Research Model

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TGFBI (transforming growth factor- $\beta$ -induced gene)-linked corneal dystrophy is an autosomal dominant disorder caused by a point mutation in TGFBI gene on chromosome 5q31. Age-dependent progressive accumulation of mutant-TGFBI protein in the corneal stroma is a hallmark of TGFBI-linked corneal dystrophy and interferes with corneal transparency. Ideally the effect of pathologically determined this disease should be examined in the whole cornea of a living organism. However, no animal model of TGFBI-linked corneal dystrophy replicates the accumulation of mutant-TGFBI protein in cornea as the complex pathophysiological changes seen in patients. This is a severe limitation in the pathological and therapeutic implication studies of TGFBI-linked corneal dystrophy. Additionally, development and analysis of animal models can also be time-consuming and costly. Miniaturization technology, in particular microfluidics, has shown promise in overcoming these limitations and disadvantages. We report simple microfluidics 3-dimensional (3D) compartmentalized culture systems which have employed mixed culture of Keratocyte cells and epithelial cells in 3D gels (e.g., collagen I). This microfluidics system enables information-rich *in vitro* assays. We presented a simple compartmentalized 3D culture model that supports the Keratocyte and Epithelial cell *in vitro*. In conclusion, our PDMS-based 3D microfluidics cell culture system may help overcome barriers to a limitation in a scientific method to investigate pathogenesis and treatments of TGFBI-linked corneal dystrophy.



### Functional Bioabsorbable Braided Filament with Anti-inflammatory Drug Delivery System Using PLGA Microspheres

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The goal of the present study was to develop an absorbable braided filament incorporating poly(lactic-co-glycolic acid) (PLGA) particles loaded with dexamethasone (DEX) as an anti-inflammatory drug for surgical suture applications. DEX-loaded PLGA (DEX/PLGA) particles, prepared using a water-in-oil emulsion method, were electrostatically immobilized onto the surface of absorbable sutures. The surfaces of these DEX/PLGA particles were coated with positively charged polyethyleneimine (PEI) molecules, which imparted a net positive surface charge. These modified PEI-coated DEX/PLGA (PEI/DEX/PLGA) particles were then immobilized on negatively charged absorbable suture surfaces by electrostatic attraction. The results obtained showed that DEX was efficiently loaded into PLGA particles and that PEI was successfully coated on the surfaces of DEX/PLGA particles. PEI/DEX/PLGA particles were well dispersed and immobilized onto suture surfaces. Furthermore, PEI/DEX/PLGA particles remained adherent to suture surfaces *in vitro* and demonstrated sustained DEX release in phosphate-buffered saline (pH 7.4) at 37°C for up to 28 days under static conditions. The tensile strength and elongation at break of PEI/DEX/PLGA particle-treated sutures were almost the same as that of non-treated control sutures. This study shows that various therapeutic drugs could be efficiently incorporated into absorbable sutures using biodegradable polymeric particles, and suggests that the devised absorbable, drug-eluting, sutures offer a promising basis for a novel absorbable surgical suture system.

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### Preparation and Characterization of Glucose-Responsive Hyaluronate-Based Nanoparticles

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Functionalized polymer nanoparticles have shown potential in cancer detection and therapy, as they can be designed and fabricated to recognize cellular and molecular processes in the body and they can reduce side effects that may occur with conventional diagnostic and/or therapeutic agents. Hyaluronate-based nanoparticles have been widely utilized due to excellent biocompatibility and low immunogenicity. Here, we report the preparation and characterization of hyaluronate-based nanoparticles that respond to the change of glucose concentrations in microenvironments. Phenylboronic acid was chosen as a hydrophobic moiety and chemically introduced to the backbone of hyaluronate, and nanoparticles were prepared by self-assembly of the polymer amphiphiles in water. Various physicochemical characteristics of nanoparticles were investigated *in vitro*. Changes in the size as well as internal structure of nanoparticles depending on the glucose concentration were investigated. This approach may provide a useful means to design functional polymer nanoparticles for efficient diagnosis and therapy of many diseases, including cancer.

### Physiologically Relevant Drug Testing *In Vitro* - An Integrated Multiple Organoid-on-a-Chip Approach

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To create a system for achieving physiologically accurate responses of human organs to drugs, chemical agents, and biologicals, we have developed a modular platform, consisting of 3-D human-derived organoids, housed in a microfluidic system, comprising a “Body-on-a-Chip” platform. To date, this platform consists of bioprinted primary human liver organoids and human IPS-derived cardiomyocyte organoids. To verify the physiological accuracy of organoid drug response, a variety of independent liver-only and cardiac-only experiments were performed, followed by an integrated organoid system test. Liver organoids responded in a dose-dependent manner to administration of increasing acetaminophen (APAP) concentrations, showing decreased viability, and albumin and urea production. N-acetyl-L-cysteine, an APAP-overdose treatment for human patients, effectively counteracted the APAP-induced toxicity, as evidenced by albumin, urea, lactate dehydrogenase, and alpha-glutathione-S-transferase assessments. Cardiac organoid drug response was assessed by epinephrine and propranolol treatments. Rapid response to epinephrine was observed (< 5 min) despite several cm of microfluidic tubing between the media/drug reservoir and organoids, and slow fluid flow rates. Organoids responded with dose dependent increases in beating rate, ranging from 1- to 2-fold with increasing epinephrine concentrations. Initial incubation with propranolol concentrations ranging from 0 to 20 uM resulted in dose dependent decreases in beating rate increases after administration of 5 uM epinephrine. Finally, this system demonstrated an integrated organoid response, in which the cardiac response to epinephrine hinged on the presence of the liver organoid, which if present in the system, was able to metabolize the propranolol, negating its blocking effect.

### Three-dimensional Cellular Microarray Platform for High-throughput Screening of Human Neural Stem Cells

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Drug discovery and toxicology use animal models for many purposes such as identifying therapeutic targets, assessing the efficacy of drug candidates and testing the safety of potential therapeutics and commercially available chemicals. While animal studies are valuable for these purposes, they are associated with high costs, low-throughput and results that do not translate well to humans. There is, therefore, significant interest in developing *in vitro* systems that use human cells to reduce costs, increase throughput, and improve reliability and predictability in drug and toxicity screening processes. Importantly, differences in toxicity between different types of human cells, such as stem cells compared to terminally differentiated cells, is largely unstudied. To this end, we have developed a microarray chip (microchip) platform for three-dimensional cell culture and high-throughput screening. Human neural stem cells (hNSC) were grown and differentiated on the microchip for periods up to two weeks and used to screen for chemical toxicants or modulators of differentiation. The cytotoxic dose responses and IC<sub>50</sub> values for 12 chemicals was measured for hNSC and their differentiated progeny on the microchip platform. Lower IC<sub>50</sub> values were observed for all-*trans* retinoic acid, 5-fluorouracil and cytosine arabinoside against hNSCs in comparison to their differentiated counterparts, which were predominantly astrocytic. This work demonstrates progress in developing *in vitro* screening systems for predicting human adult and developmental neurotoxicity. In addition, this work begins to address how different cell types have differential sensitivities to chemicals, which should be considered when performing screens.

### Treatment of Canine Atopic Dermatitis with Allogeneic Adipose Tissue-derived Mesenchymal Stem Cells

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Canine atopic dermatitis (CAD) is a chronic skin disease of dogs, wherein, immune dysregulation of T cells appears to be a significant defect. Based on the immunomodulatory properties of mesenchymal stem cells (MSC), we have initiated a clinical trial to assess whether the infusion of allogeneic MSC represents a potential option therapeutic for CAD. In this study allogeneic cells from fat and expanded *ex vivo* (AD-MSC), were obtained from healthy donors dogs. Five doses of  $0.5 \times 10^6$  cells/kg once per week were injected intramuscularly in the gluteal of dogs diagnosed with CAD by Canine Atopic Dermatitis Lesion Index. After infusion, the safety and efficacy were assessed over a period of 6 months. The results showed that intramuscular infusion of allogeneic AD-MSC in this cohort of dogs with atopic dermatitis, was safe and devoid of procedural complications. In turn, the effectiveness evaluation reveals that in all patients, infusion of allogeneic AD-MSC eliminates and/or reduces most of the symptoms of CAD, as well as most of the clinical signs associated. The outcome of this clinical trial put forward the notion that the use of allogeneic *ex vivo* expanded MSC appears to be a safe and effective therapeutic approach for the treatment of CAD in dogs.

### Novel Method for the 3D Printing of Biomedical Devices

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With the advancement of 3D printing new and creative filament materials are being quickly introduced into the marketplace. These filaments are commonly manufactured with a large batch manufacturing method used to ensure that large product volumes can be rapidly manufactured with cost efficiencies. Small batch manufacturing is a more promising approach offering the potential for developing new and novel patient treatment modalities. These can be designed, prepared, and applied locally rather than having specialty implants fabricated offsite, with a significant savings in time and cost and the ability to provide customized treatments. In this study, commercially available 3D equipment (extruders and printers) and the role they may play in onsite small batch manufacturing was examined. Characterization of 3D printed constructs was performed to study the effects of using bioactive compounds in these fabrication processes and to ensure that bioactive reactivity of the dopant compounds was maintained throughout the manufacturing process. Preliminary results have shown that commercial 3D equipment can be used successfully for both antibiotic and chemotherapeutic doping of biodegradable polymers already in use with commercial 3D printers, and that these manufactured implants show greater elution properties than commercial-grade PMMA/MMA bone cements. This novel method permits the fabrication of heterogeneous physical objects or structures of high complexity, without loss of resolution, and permits control over many design features including stack orientation, dopant and polymer composition. Doped constructs were also shown to be effective in inhibiting bacterial growth and reducing osteosarcoma cell proliferation.

### Neural Spheroid Culture System on Advanced Surface-Modified Substrates of 3D Microwell Chips

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For cell physiology and behavior, neural cells in three-dimensional (3D) spheroidal culture system may better mimic the conditions and properties of our body than two-dimensional (2D) cultures. Neural spheroids can be generated due to the tendency of adherent cells to aggregate and establish 3D communication networks with neighboring cells through chemical and physical cues of *in vivo* environment.

We developed 3D neural spheroid cell culture system on the hemispherical concave microwell chips by utilizing the surfaces modified for chemical and electrical stimulation. For this, we immobilized 3-aminopropyltriethoxysilane (APTES) on our manufactured polydimethylsiloxane (PDMS) microwell chips by covalent attachment and then investigated the neuronal differentiation and behavior of a neural progenitor cell.

Here we present that neural progenitor cells successfully formed the neuronal spheroids with the size of about 200 micron in five days and neural network formation and neurite extension of the neuronal spheroids were significantly enhanced on this substrate, compared to the simple PDMS chips. It is likely that adhesion of neuron cells sensing nanotopography and chemicals on the surfaces might stimulate ECM molecules of cell surfaces and then leads various cell signaling pathways for neurite outgrowth.

These results highlight an innovative surface-modified 3D platform for neuronal culture and differentiation and may allow the development of enabling technologies for tissue engineering and screening applications.

### Challenges and Strategies in Bioreactor Development - An Engineers Point of View

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In the field of tissue engineering, bioreactors are utilized to mimic *in-vivo* conditions *in vitro*. To achieve the desired effects in cultivation, many aspects have to be taken into account. Based on publications and on our own experience, we gathered and summarized the experiences, challenges and needs in bioreactor technology of the last years.

So far, the simulation of all environmental conditions is not possible. Consequently, specific solutions have to be found for each application. Thus, modularity should be one of the basic requirements. The impact on flexibility, response time and long-term costs is significant. Sterility has always been a prerequisite. In the past the focus was set on providing a sterile environment and designing sterilizable or disposable components. Providing the means to sterilize the complete bioreactor would prevent known issues during assembly. Providing and controlling a physiologic environment is clearly another key feature. Measurement of O<sub>2</sub>, pH and CO<sub>2</sub> is essential. In case of flow stimulation, pressures, flow characteristics and flowrate have to be monitored as well. With an increasing amount of process parameters, it becomes of utmost importance to optimize the usability of bioreactor systems. Providing a graphical user interface with the means of monitoring, controlling and documenting the complete operating process is pivotal. To allow an easy modularity in regard of sensors and actuators, hardware-software-interactions should be handled by real-time-capable programmable logic controls.

Bioreactor development increasingly requires the knowledge of mechanical and software engineering combined with medical knowledge. Thus it represents a prime example of medical engineering.

### Elastic Silk-collagen-i Tyrosine Crosslinked Hydrogels for *In Vitro* Engineering of Dynamic Human Tissues

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Collagen-I is a widely used ECM protein for tissue engineering research, but collagen scaffolds undergo contraction, limiting their use as biomaterial for long term *in vitro* tissue culture. Formaldehyde, glutaraldehyde, genipin and isocyanates have been used to chemically crosslink collagen gels, but can leave toxic residues within the hydrogels post processing. Herein, we take advantage of tyrosine amino acids in silk (5% tyrosine) and collagen-I (1% tyrosines) to form dityrosine crosslinked silk-collagen-I composite protein biomaterials in the presence of peroxidase and H<sub>2</sub>O<sub>2</sub>. Tyrosine crosslinked silk-collagen-I scaffolds are mechanically stronger, elastomeric, can be maintained longer in culture, and possess improved optical properties compared to pure collagen scaffolds. Rheological characterization of these materials for mechanical properties showed that silk concentration dictated the storage modulus of the hydrogels and collagen concentration altered the rate of hydrogel crosslinking. Storage moduli of silk-collagen-I hydrogels were significantly higher compared to collagen only gels of the same concentration. Hydrogels underwent up to 40% strain before onset of plastic deformation and light transmission were significantly higher in silk-collagen-I cell laden hydrogels compared to pure collagen scaffolds. Finally, human pulmonary fibroblasts were encapsulated in silk-collagen-I hydrogels and subjected to uniaxial strain. Alamar blue and live dead staining showed good cell viability and immunohistochemical staining confirmed that fibroblasts interacted with the silk-collagen-I matrices via  $\alpha 1\beta 1$  integrins and form focal adhesion complexes via vinculin proteins. In conclusion, these new silk-collagen-I enzyme-mediated crosslinked hydrogels overcome limitations of pure collagen-I scaffolds and provide a unique biomaterial system for engineering mechanically dynamic human tissues.

#### Co-Implantation of Auricular Chondrocytes and Mesenchymal Stem Cells for Auricular Cartilage Generation

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The encapsulation of bovine auricular chondrocytes (AuCs) in collagen has successfully formed auricular cartilage *in vivo*, however this technique requires a large number (>200x10<sup>6</sup>) of cells. Human AuCs can be isolated from donor tissue but lose chondrogenic potential when cultured beyond initial passages. Mesenchymal stem cells (MSCs) can differentiate into chondrocytes and have been shown to promote cartilage formation when cultured with articular chondrocytes. Here, human AuCs and MSCs were mixed with 10 mg/mL type I collagen hydrogels in ratios of 100:0, 50:50, and 0:100 AuC:MSC at a density of 25x10<sup>6</sup> cells/mL. Disc constructs 2 mm thick by 8 mm diameter were implanted subcutaneously in nude mice for 1 and 3 months. Gross inspection showed that 100% AuC and 50:50 constructs developed cartilage-like color and appearance at 1 and 3 months, while 100% MSCs contracted significantly after 3 months. Safranin-O and Verhoeff's staining to analyze GAG content and elastin fibers, respectively, revealed the deposition of both molecules in 50:50 constructs at 1 month and increased presence at 3 months in 100% AuC and 50:50 constructs. Picrosirius red staining of 50:50 mixed cell constructs displayed a collagen-rich perichondrial layer characteristic of auricular cartilage by 3 months. The maintenance of size, the deposition of elastic cartilage molecular components, and the organization of auricular cartilage microstructure demonstrates the success of co-implantation of MSCs with AuCs for the formation of auricular cartilage using 50% fewer chondrocytes. The use of MSCs to supplement chondrocytes is a critical step toward the clinical viability of tissue engineered auricular reconstruction techniques.

#### Thermosensitive Hydrogels for the Intranasal Administration of Drugs to the Central Nervous System

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The pharmacological treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases is hampered by the presence of

the blood brain barrier (BBB). However, recent literature has highlighted the effectiveness of the intranasal route as a non-invasive method for drug delivery to the central nervous system (CNS). The intranasal administration route transports drugs directly to the CNS through the olfactory and trigeminal nerve pathways, by slow intra-axonal transport or by a faster transfer through the perineural space surrounding the nerve cells, hence bypassing the BBB. Thermosensitive hydrogels have been proposed to favour drug administration by the intranasal route, as they avoid rapid clearance and favour drug delivery to the nasal mucosa [1,2]. In this work, amphiphilic polyurethanes were synthesised from a thermosensitive macrodiol (such as Pluronic F127 or polyester-polyethylene glycole-polyester block copolymer), hexamethylene diisocyanate and N-Boc serinol and modified with dihydroxyphenylalanine (DOPA) to favour mucoadhesion. In addition, Pluronic F127/Chitosan-glycerophosphate blends were also prepared for the purpose.

Hydrogels were characterised for their sol-to-gel transition, rheological properties and *in vitro* cyto-compatibility. They were then tested for the release of anti-oxidant and anti-inflammatory drugs *in vitro*, such as curcumin. Finally, *in vivo* tests in mouse models were performed to evaluate the effectiveness of the hydrogels in releasing curcumin to the CNS.

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#### The Predictable Animal Model for Guided Tissue Regeneration Procedures in Oral and Maxillofacial Surgery

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For the development of new surgical solutions/techniques suitable animal models are required and the available ones are not always good enough.

The aim of this study was to develop a reliable, predictable and safe animal model intended for research related to dental implantology.

The experiment was performed on mandibles obtained from 5 miniature pigs. The mandibles were cleaned of soft tissue. Next, 15 potential locations of the implant-systems were evaluated in order to mimic the implantation in humans as closely as possible. The pattern of the proposed implants locations was applied on the bone surface. Titanium dental implants were inserted into drilled cradles in the designated points - 15 implants on each side of the mandible were introduced. Technical capability to carry out the surgery - mainly the surgical access, the ability to drill an implant cradle, the possibility of implants insertion were analyzed. The bone thickness: cortical and spongiosa layer at the selected locations, implants position in relation to dental roots and germs as well as to inferior alveolar nerve and vessels were analyzed by the computed tomography (CT). Primary stabilization of the implants was measured.

As a result, the optimal position of implant insertion into the jaw was chosen and set as guidelines for *in vivo* experiments. Six-weeks long experiment performed in the proposed model brought a good basis for a comparison of two different modifications of the implantation procedure. It confirmed utility of the developed model.

#### Central Nervous System Regeneration *In Situ*: Reprogramming Neural Cells

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The field of tissue engineering has made steady progress in translating various tissue applications. Although classical tissue engineering strategy - which involves the use of culture-expanded cells and scaffolds to produce a tissue construct for implantation - has been validated, this approach involves extensive cell expansion steps, requiring a lot of time and laborious effort before implantation. To bypass this *ex vivo* process, a new approach has been introduced: *in situ* tissue regeneration uses the body's own regenerating capacity by mobilizing host endogenous stem cells or tissue-specific progenitor cells to the site of the injury. This approach relies on development of a target-specific biomaterial scaffolding system that can effectively control the host microenvironment and mobilize host stem/progenitor cells to target tissues. An appropriate microenvironment provided by implanted scaffolds would facilitate recruitment of host cells that can be guided to regenerating structural and functional tissues.

In this work we have used aligned Polylactic acid nanofibers to recapitulate development and recover the neurovascular stem cell niche after a traumatic brain injury in mice. We demonstrated that PLA70/30 and L-lactate are required to maintain the metabolism and self-renewal of neurogenic progenitors. 3D architecture of the cell environment modulates its morphology and differentiation state. The appropriate orientation of vascular sprouts and radial glia in the regenerating tissue was only achieved when the topology of the PLA nanofibers reproduced that of embryonic radial glia organization. Random PLA nanofibers did not allow vascular invasion inside the scaffold, demonstrating the relevance of scaffold topology in CNS regeneration.

#### Ecofriendly and Biocompatible Chitosan/Diatomaceous Earth Composite Membranes for Dual Drug Release

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Marine origin materials have gained an increasing interest in a wide range of applications, both due to their abundance and the vast range of interesting properties they possess [1]. In this work,  $\beta$ -chitin was isolated from the endoskeleton of giant squid and further converted to chitosan (CHT) by deacetylation [2], a biopolymer exhibiting interesting properties for biomedical applications. In addition, chitosan was combined with diatomaceous earth (DE), a natural porous material, mainly consisting of SiO<sub>2</sub> and originated from fossilized diatoms, a hard-shelled unicellular alga [1,3]. These are both easily accessible, cheap and highly biocompatible materials, very interesting for the preparation of drug delivery systems (DDSs). Accordingly, composite CHT-DE membranes were prepared by the solvent casting technique, including different proportions of these materials, up to 10% (w/w) of DE. These membranes were characterized by Fourier Transform Infrared Spectroscopy, Thermogravimetric Analysis and Scanning Electron Microscopy and Energy-dispersive X-ray Spectroscopy, for the determination of their chemical composition, mineral content and morphological features, respectively. The results confirmed the incorporation of DE in the membranes, being deposited into the lower surface of the membranes,

forming a hybrid structure. Finally, two drugs with different water solubility index, dexamethasone and gentamicin, were loaded into the membranes, by immersion methods. The release profiles demonstrated the membranes ability to act as dual DDSs of these two interesting drugs.

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#### Tenogenic induction of Equine Mesenchymal Stem Cells by means of Growth Factors and Low Level Laser Technology

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Tendons are characterized by an elevated extracellular matrix density, low cellularity and vascularity, all properties that give elevated stiffness and elasticity but confer scarce regenerative potentials. In Veterinary Medicine several therapies are used in the horse tendon regeneration and, within cellular therapies, tenocytes play a pivotal role although their limited proliferative potential and difficulties in their isolation. The aim of the present research was to find a straightforward way to induce the differentiation of equine mesenchymal stem cells (MSCs) into tenocytes. The MSCs were isolated from equine peripheral blood (n=4) and induced toward the tenogenic fate using the low-level laser irradiation (LLLI) combined with a cocktail of different growth factors (GF) such as TGF $\beta$ 3, FGF2, EGF2 and IGF1. The most effective combination, detected by means of a Real Time analysis was the following: FGF2 + TGF $\beta$ 3, FGF2 + TGF $\beta$ 3 + LLLI. The latter mixture was able to influence, in particular, the mRNA expression of Early Growth Response Protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN) genes, all involved in the tenogenic pathway. Future investigations will be necessary to check the efficacy of MSC-induced cells in regenerating *in vivo* studies.

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#### Automatic Closed Cell Culture Equipment for Cell Sheet-based Tissue Engineering

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Epithelial cell sheets have been utilized for treatment of ocular surface diseases and prevention of esophageal stricture after endoscopic submucosal dissection. At present, cells are fabricated by manual operations in general. For mass production and quality stabilization in the future, automation technology is necessary. In this study, an automatic closed cell culture equipment for esophagus regeneration has been developed and oral mucosal epithelial cell sheets were cultured and evaluated. The automatic closed cell culture equipment includes ten closed culture vessels. The vessels have double-layered structure. All components including ten vessels are connected as a single closed circuit to avoid biological contamination. During culture period, medium/gas changes were performed and cells were observed with a phase-contrast microscopy. After cultivation, the vessels were taken off by sealing the connected tubes to the vessels, and kept their sterility during the delivery from a cell

manufacturing facility to operating rooms. The cell sheets cultured by the equipment were evaluated. The cell number, viability, histology and expression of specific protein markers of cell sheets cultured automatically were equal to those of manually cultured sheets. These results suggest that the automatic closed cell culture equipment is effectual for human oral mucosal epithelial cell sheet fabrication. This work was supported in part by the Formation of Innovation Center for Fusion of Advanced Technologies 'Cell Sheet Tissue Engineering Center (CSTEC)', from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

### Biofunctionalized Carbon Nanotubes as Substrates for Drug Delivery and Neural Regeneration

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The biomimetic materials for nerve regeneration require (1) desired substrates with proper topographical surfaces, (2) improved attachment to the neuronal cells for proliferation or differentiation, (3) sustained release of the biofunctional factors for neuroregeneration and (4) also indispensable factors including mechanical integrity, controllable biodegradability, and good biocompatibility. For innovative approaches to new biomaterials in nerve regeneration, carbon nanotubes (CNTs) take great attractions due to their nanoscale features, modifiable chemical functionality, and electrical property.

To demonstrate an improved substrate for drug capture system and neuronal regeneration, we immobilized polyethylene glycol (PEG) functionalized CNTs directly on the commercial culture dishes by covalent attachment and then investigated the neuronal differentiation of a neural progenitor cell with or without drugs. We show the attractive effects of CNTs on neural regeneration in its conductive nanotopographic dimensions that can modulate networking characteristics within and between the neuronal cells. In particular, neural extension and cell proliferation of the PC-12 neuronal cells were significantly enhanced by release of surface-entrapped bpV(phen) on CNT substrates, compared to the simple functionalized CNT substrates. It is likely that adhesion of neuron cells sensing nanotopography and chemicals on the surfaces of CNTs might stimulate ECM molecules of cell surfaces and then leads various cell signaling pathways by drug-loaded biofunctionalized CNTs.

These results highlight the promise of the electroactive and drug-releasing CNT biomaterials for neuronal culture and differentiation, and suggest their potential for future neural regeneration applications.

### Fabrication of Silk Fibroin Scaffolds for Osteogenesis with Demineralized Bone Particle

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Silk fibroin has been widely used as a potential biomaterial in the field of tissue engineering due to its remarkable biocompatibility and biodegradability. Herein, we fabricated silk fibroin scaffold with demineralized bone particle (DBP) in order to improve its structural connectivity and mechanical strength. Demineralized bone particle is well known as an inducer of osteogenesis and the effects of DBP were previously studied by our group. Structural characterization were performed using SEM, which shows smaller pores in DBP/silk scaffold compared to pure silk fibroin scaffold. Cell proliferation activity and osteogenesis were evaluated by MTT and alkaline phosphatase assay. Various mRNA ( $\beta$ -actin, collagen I, osteocalcin and runx-2) from scaffolds were also collected and studied. Furthermore, histological staining demonstrated that a remarkable en-

hancement in the nucleation sites of bone marrow mesenchymal stem cells. *In vivo*, bone formation with cranium defect was studied using micro CT images which shows well-formed bone volume, trabecular thickness and density etc. Collectively, the enhancement in mechanical properties result in formation of bone matrix and cell growth through establishment of structural connectivity of the fabricated silk fibroin-DBP scaffolds. This research was supported by Bio-industry Technology Development Program (112007-05-1-SB010), Technology Commercialization Support Program (814005-03-1-HD020), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea and the Brain Korea 21 PLUS Project, NRF.

### Immobilization of Bone Morphogenetic Protein on Titanium Surface using Mussel Adhesive Protein Fused with RGD Peptide to Improve Osseointegration and Bone Regeneration

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Recombinant human bone morphogenetic protein-2 (rhBMP-2) is known to have the ability to induce bone formation and enhance osseointegration by stimulating osteoblast differentiation. Mussel adhesive proteins (MAPs) secreted from mussels have been suggested as friendly adhesives for use in tissue engineering and medicine. RGD peptide as an important constituent of extracellular matrix (ECM) molecules previously was fused with MAP to create a new recombinant adhesive, MAP-RGD, by us. In this study, MAP-RGD was used to immobilize rhBMP-2 on titanium surfaces, and then we investigated the osteoinductivity of the mixture of MAP-RGD-rhBMP-2 on titanium surfaces *in vitro* and *in vivo*. It was found that cellular behaviors of mouse pre-osteoblast MC3T3-E1 cells such as adhesion and proliferation were significantly increased on titanium surfaces coated with MAP-RGD-rhBMP-2 and MAP-RGD compared to non-coated surface. Furthermore, MAP-RGD-rhBMP-2 titanium surface showed the highest ability of osteogenic differentiation among the three types of titanium surfaces. Also, we found that the mRNA expressions of osteogenic differentiation marker genes were upregulated in MC3T3-E1 cells on MAP-RGD-rhBMP-2 titanium surface. In addition, though micro-computed tomographic analysis and histological evaluation, both MAP-RGD-rhBMP-2 and MAP-RGD titanium surfaces displayed improved new bone formation in rat calvarial defect model compared to non-coated surface. From the above we can see that the mixture of RGD-fused MAP and rhBMP-2 is a promising biomaterial for implantology and bone tissue engineering with enhanced osteoinductivity.

### Development of Organic Solvent-Free Polymeric Scaffolds for Musculoskeletal Regeneration

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Biomaterial scaffolds have received keen interest in the tissue engineering research field. Whether the scaffolds are fabricated from synthetic polymers, naturally derived sources, or a combination of the two, the aim of the scaffold is to assist the human body to regenerate tissues and organs, repair the injuries and recover lost functions of the body. Favourable characteristics of synthetic materials include being able to tailor their design properties to suit specific applications, high mechanical strength, reproducibility and mass production. Currently, there are various methods by which synthetic biomaterial scaffolds can be fabricated. However,

most synthetic fabrication techniques involve the use of organic solvents. Hence, there is an interest in fabricating scaffolds that are completely organic solvent-free. The novel micro-/nano-fibrillar composite (MFC/NFC) technique produces scaffolds that are completely free from organic solvents.

The cytocompatibility of these materials have been tested *in vitro* using osteoblast-like cells and primary rat tenocytes. Live/dead staining and alamarBlue assays demonstrated cell numbers increase over the culture period, inferring cytocompatibility. Gene expression analysis of primary rat tenocytes cultured on MFC/NFC scaffolds show collagen III, tenomodulin and scleraxis coincide with tenocytic behaviour. Histological studies show collagen has been deposited on the scaffolds and increases over time.

This study presents the potential of producing completely organic solventfree scaffolds using the MFC/NFC technique to fabricate scaffolds capable of hosting musculoskeletal cells, with the end goal of providing a graft material that can be used in applications such as rotator cuff repairs, to improve healing outcomes.

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#### Image-based Profiling of Mesenchymal Stem Cells for Quality Assessment

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Morphology of cells had long been used as an important indicator of cell quality. However, such experience-based morphological importance has not been quantitatively examined to answer the correlation between their quality decay and morphological changes. For the cell therapy, non-invasive technique to assess the quality of stem cells is strongly required for its industrialization by the growth of successful stem cell applications. Human bone marrow-derived stem cells (hBMSCs) and adipose-derived mesenchymal stem cells (hADSCs) had been widely studied and applied to clinical cell therapies with their multipotency and feasibility as the cell source. However, it is also known that such stem cells require highly skilled cell culture to carefully maintain their quality for their undifferentiated status. In our research, we introduced bioinformatic machine learning strategy to build a prediction model, which links "the cell morphology profile" and "the experimentally determined cellular quality". By modeling information from the time-lapse phase contrast images of more than 20,000 time-lapse images, we found that the experimentally defined staining results of differentiations was able to be profiled only from their morphological information. We also show the biological mechanism of such morphological profiling by analyzing total expression profile by microarray.

#### PHB-HV 3D Scaffold Supports Osteoblast Growth

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**Introduction:** Bone tissue engineering requires a biocompatible scaffold that supports cell growth and enhances the native repair process. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-HV) is a biodegradable 3D scaffold with  $88.1 \pm 0.3\%$  porosity and pore size of  $163.5 \pm 0.1 \mu\text{m}$ . Previous studies demonstrated the potential of PHB-HV as a scaffold in spinal cord repair. The aim of this study was

to evaluate PHB-HV as a scaffold for bone regeneration by assessing the cytocompatibility of this scaffold.

**Methods:** PHB-HV scaffolds were manufactured through an emulsion freezing/freeze-drying technique. For cytocompatibility assays, primary rat osteoblasts were seeded onto the surface of PHB-HV scaffolds at a density of  $2.5 \times 10^4$  cells/ $1.9 \text{ cm}^2$  scaffold in 24 well tissue culture plates and cultured for 21 days. Assessment of cell viability was performed using the alamarBlue<sup>®</sup> assay (Invitrogen), accompanied by Live/Dead<sup>®</sup> Cell Viability assay (Life Technologies). Migration of cells through the scaffold was assessed by DAPI staining and fluorescent imaging across a transverse plane.

**Results:** Over the 21-day culture period, there was a significant increase in the number of viable osteoblasts cultured on the PHB-HV scaffolds ( $p < 0.0001$ ). Live/Dead<sup>®</sup> and DAPI staining demonstrated viable osteoblasts colonised the surface of the 3D scaffold and began migrating through the porous scaffold.

**Conclusions:** PHB-HV is cytocompatible to primary osteoblasts and our initial data suggests that PHB-HV is a suitable scaffold for bone tissue engineering. An *in vivo* study utilising the rat critical-sized calvarial defect model is underway to further evaluate the therapeutic potential of PHB-HV in bone regeneration.

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#### Improved Biodistribution and Efficiency of Endogenous Radiotherapy by Means of Functionalized Hydrophilic Nanogels

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Most advancement in radiotherapy has been made in external beam treatments but it is still limited by the effects it induces in the surrounding healthy tissues and its limitations in patients with disseminated disease. Though endogenous radiotherapy allows a direct delivery of radiation to the tumor by means of radiolabelled antibodies, peptides, or isotopes of halogens that are organified (like radioiodine therapy of thyroid cancer), only few of these approaches are implemented in standard patient treatment, since the biodistribution of these small molecules is not ideal and leads to relevant toxic doses in healthy tissue (critical organs are liver, kidneys and intestine). In order to overcome these limitations by increasing selective delivery of radiation to cancer manifestations, our approach focuses on the development of hydrophilic nanogel-carriers coupled with chelators for theranostic radioisotopes. Such water swollen nano-carriers offer advantages, as their open structure barely interact with proteins and cells featuring i) long blood pool circulation, ii) variability of the carrier and targeting function and iii) responsive properties to environmental factors like enzymes, pH, ionic strength. Thus tumor targeting by the nanogels is supported by their stealth properties in combination with the enhanced permeability and retention effect (EPR effect) and is complemented by the incorporation of ligands of receptors overexpressed by tumor cells. Hence, first step in targeting results in the accumulation of the nanogels in tumor tissue by utilizing the EPR effect followed by the second step, which is targeting of tumor cells by addressing their specific surface property.

#### Geometrically Confined Pluripotent Stem Cell Differentiation and Migration for Human Developmental Toxicity Detection

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Unintended exposure to developmentally toxic compounds leads to various birth defects; however current animal-based testing is limited by time, cost and high inter-species variability. Here, we developed a human-relevant *in vitro* model, which recapitulated two cellular events characteristic of embryogenesis, to identify developmentally-toxic compounds. We spatially directed mesoendoderm differentiation, epithelial-mesenchymal transition and the ensuing cell migration in micropatterned human pluripotent stem cell (hPSC) colonies to collectively form an annular multicellular pattern populated with Brachyury+ cells. Developmentally toxic compounds could disrupt the two cellular processes to alter the morphology of the mesoendoderm pattern. Image processing and statistical algorithms were developed to quantify and classify the developmental toxic potential. We could not only measure dose-dependent effects but also correctly classify species-specific drug (Thalidomide) and false negative drug (D-Penicillamine) in mouse embryonic stem cell test. This model offers a scalable screening platform to mitigate the risk of developmentally toxic exposures in human.

#### Human Buccal Fat Pad as a New Cell Source for Dedifferentiated Fat Cells

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The aim of this study is to clarify whether human buccal fat pad could be an alternative cell source for dedifferentiated fat (DFAT) cells. Buccal fat pad was harvested from 10 patients, minced into small pieces, and then dissociated into individual cells with collagenase solution. DFAT cells were separated from mature adipocytes using a ceiling culture technique based on buoyancy. Isolated DFAT cells were characterized at passage 3: i.e., cell surface marker expression, specific gene expression, colony-forming unit fibroblast (CFU-F), proliferation activity, and multilineage differentiation potential. In CFU-F assay, colony formation was observed in DFAT cells culture 10 days after plating. Flow cytometric analysis showed a high level of expression of MSC markers such as CD13, CD73, CD90, and CD105. mRNA expression of c-Myc, Klf-4, Oct3/4, Runx2, Ppar $\gamma$ 2, and Sox9 was also demonstrated in DFAT cells by RT-PCR. DFAT cells under osteogenic induction exhibited calcium deposition positive in alizarin red S staining. Also shown was intracellular accumulation of oil red O-positive lipid droplets in DFAT cells after adipogenic induction. DFAT cells prepared from human buccal fat pad possessed cellular characteristics and differentiation potentials equivalent to those of DFAT cells from ordinary subcutaneous fat pad. These results indicate that buccal fat pad could be an another potential cell source for DFAT cell preparation.

#### Correlation of Mechanical and Histological Results for Osteogenesis in a Porous Titanium Scaffold

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Bone loss may be a consequence of tumours, traumas or various diseases. Although bone is able to regenerate when injured, an appropriate scaffold is required when the defect exceeds a critical size for spontaneous repair. Moreover, scaffolds must have an adequate stiffness to ensure load sharing with the surrounding bone. Finding such a structure, with adequate mechanical properties and great compatibility, is still a great challenge to reconstructive surgery.

Here, we investigated the *in vivo* osteoconductive properties of a new porous titanium scaffold implanted in a ewe's mandible and compared it with a non porous scaffold (same outer shape and Young modulus). This new porous implant was obtained by Selective Laser Melting, a rapid prototyping method. Micro-computed tomography imaging, histological (undecalcified samples) and mechanical (cantilever bending test) analyses were performed on explanted hemimandibles after 12 weeks of implantation.

Experimental deflection curves showed different solidity behaviours for the two implants, reflecting a stiffer and better bone/implant interface with the porous implant.

Both scaffolds were surrounded by unstructured bone in active remodelling process, most likely formed through direct ossification rather than osteochondral ossification. The porous scaffold was filled with this same remodelling bone, in close contact with the titanium surface. The non porous implant remained empty and a layer of fibrous tissue could be seen between the external bone and the titanium.

This preliminary study confirms that the use of porous implant improve osseointegration and bone ingrowths and demonstrates the usefulness of porous titanium scaffolds for reconstruction of load-bearing bones.

#### Osteogenic Differentiation of Bone Marrow Stem Cell in Mg-Ca Particles and Synthetic Body Fluid Loaded Silk Fibroin Scaffold

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An ideal tissue engineering scaffold following characteristics such as biocompatibility, suitable porosity, pore size, sufficient mechanical strength and good cell affinity. Silk as a tissue engineering material has excellent biocompatibility, bioactivity, low immunity and ease of fabrication. On the other hand, We were also attraction attention from scientist as Mg-Ca alloys a biodegradable biomaterials for orthopedic applications. In this study, silk-fibroin hard and soft condition scaffolds were prepared coated with Mg-Ca particles Synthetic Body Fluid solution for bone tissue engineering. Structured mechanical characteristics all coated and non-coated samples by Gross image, FT-IR, SEM, Compressive strength analysis. Osteogenic differentiation of Bone Marrow Stem Cells was further confirmed through MTT, ALP, RT-PCR, SEM. Results suggest that Mg-Ca/SBF solution helps osteogenic differentiation of BMSC, and can be considered for potential applications as scaffolds for future tissue regeneration. This research was supported by Bio-industry Technology Development Program (112007-05-1-SB010), Technology Commercialization Support Program (814005-03-1-HD020), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, BK 21 PLUS.

#### Silk Films for Bio-engineering of Neo-corneal: Degumming Time & Cellular Interactions

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Silk is a biodegradable natural polymer with low immunological rejection after transplantation. As well, silk can be manufactured in various forms and adjusted to the thickness of several micrometers depending on the manufacturing method. Silk shows high transparency in the form of film, high water and oxygen permeability.

However, studies have cited some drawbacks of silk, such as inflammatory response due to presence of silk protein(sericin) and its another disadvantage of silk requires a long period of time to decomposition after transplantation. In this study, silk films with various degumming time (0, 20, 40 and 60 minutes) were fabricated in order to evaluate the effect of sericin in learns of permeability enhancement, inflammatory responses, etc. Corneal endothelial cells were seeded on the silk films and examined to evaluate the degree of adhesion in films, cell's proliferation and specific mRNA expression. This study showed the degumming time of silk film fabrication which

is a parameter for regeneration of corneal endothelial cells. This research was supported by the Brain Korea 21 PLUS Project, NRF, NRF-2012M3 A9C6050204 and Bio-industry Technology Development Program (KMIFAFF,112007-05-3-SB010).

#### Age-related Change in The Osteoinductive Activity of rhBMP-2

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**Introduction:** Bone morphogenetic proteins may hold broad potential for use in the reconstruction of bone defects resulting from tumor resection or trauma and in assisting bone healing thanks to methods enabling the synthesis of recombinant human bone morphogenetic protein-2 (rhBMP-2). The aim of the present study was to investigate the effect of aging on osteoinduction by rhBMP-2.

**Methods:** rhBMP-2 was implanted with type I collagen as a carrier into the calf muscles of 3-, 8-, and 48-week-old Wistar/ST male rats (n = 12). After 21 days, the formation of ectopic neoplastic bone was examined in soft X-ray imaging, and the mineral content (BMC), bone area (BA) were measured by dual-energy X-ray absorptiometry (DXA). Their bone mineral density (BMD) was calculated as BMC per BA. In addition, hematoxylin-eosin, proliferation cell nuclear antigen (PCNA) immunostaining and von Kossa staining were performed. Data are presented as mean  $\pm$  SD.

**Results:** BMD values determined by DXA were  $29.40 \pm 5.47$ ,  $24.15 \pm 2.33$ , and  $19.01 \pm 2.02$  mg/cm<sup>2</sup> in the 3-, 8-, and 48-week-old rats, respectively, demonstrating that BMD significantly decreased with aging (P < 0.05). The number of PCNA-positive cells also decreased significantly with aging (P < 0.01). The von Kossa stain-positive area decreased significantly with aging (P < 0.01).

**Discussion and Conclusions:** The ectopic bone induction ability of rhBMP-2 decreased with aging, demonstrating the effects of aging on bone induction by rhBMP-2. These findings will be of considerable benefit in the bone regeneration of oral and maxillofacial tissue clinically in elderly patients.

#### Mineral-Polymer-Fiber Composite Scaffolds Guide Differentiation of Ligament Fibroblasts for Periodontal Regeneration

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Periodontitis is a highly prevalent chronic inflammatory disease characterized by the destruction of the tooth-supporting structures, ultimately leading to tooth loss. The periodontium is comprised of soft (periodontal ligament (PDL), gingiva) and hard (root cementum, alveolar bone) tissues. The PDL anchors the root cementum to alveolar bone, providing tooth anchorage. This attachment is characterized by a soft-to-hard tissue (fibro-osseous) interface. Regenerative options for treatment of periodontitis do not routinely achieve the integration of multiple tissues. This work aims to develop a calcium phosphate (CaP)-polymer-fiber scaffold to guide PDL cells towards periodontal regeneration. CaP of varying composition (hydroxyapatite (PLGA-HA) and amorphous CaP (PLGA-SBF)) are incorporated into electrospun poly(lactic-co-glycolic acid) (PLGA) fibers and scaffolds are also coated (SBF) in the amorphous CaP6. It is hypothesized that through modulation of substrate composition, the growth and differentiation of cells can be guided towards multi-tissue regeneration required for periodontal healing. Scaffolds were seeded with human PDL cells and cultured for 28 days. Proliferation was significantly enhanced on PLGA-HA. Interestingly, after 28 days, collagen production was significantly enhanced on SBF scaffolds. Furthermore, the expression of osteoblastic markers osteocalcin and osteopontin were upregulated on the SBF scaffolds compared to PLGA-HA. These differences in response may be attributed to the biomimetic nature of amorphous CaP to the mineral found in cementum and bone. These results demonstrate

that CaP-containing scaffolds are promising materials for periodontal regeneration and culture on fibers with amorphous CaP stimulates an osteoblastic phenotype necessary to promote the regeneration of the periodontal soft-to-hard tissue interface.

#### Tailor Design Self-assembling and Stimulus-Responsive Polypeptide-based Nanoparticles for Active Targeted Drug Delivery

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Polymeric nanoparticles have shown promising potential as carriers for drug delivery. The structural stability of delivery vehicle and effective release of encapsulated therapeutic drugs are crucial for drug delivery system. In this study, the biodegradable pH-sensitive nanoparticles composed of natural polypeptides and calcium phosphate (CaP), have been developed. We utilized two different amphiphilic sequences, poly(ethylene glycol)3400-*aconityl* linkage- poly(L-glutamic acid)- poly(L-histidine)-poly(L-leucine) and LyP1-poly(ethylene glycol) 1100-poly(L-glutamic acid)- poly(L-histidine)-poly(L-leucine), to self-assemble into nanoparticles in aqueous phase. The biostable nanoparticles provide three distinct functional domains: the hydrated PEG outer corona for prolonging circulation time, the anionic PGlu shell for CaP mineralization, and the protonation of PHis shell for facilitating anticancer drug release at target site. The active targeting ligand, LyP-1, is served to bind to lymphatic endothelial cells in tumor for the reduction rate of metastasis. The resulting mineralized Dox-loaded particles (M-DOX NPs) with negative charge ( $-21.9 \pm 1.6$  mV) have a smaller size  $179.4 \pm 33.9$  nm at pH 7.4, but particles at pH 5.0 have a doubled size ( $291.2 \pm 25.1$  nm) and positive charged ( $21.7 \pm 2.1$  mV), implying the protonation of poly-histidine. From the release profile, M-DOX NPs effectively reduce the leakage at physiological pH value comparing to DOX NPs, and both nanoparticles facilitate the encapsulated drug release at acidic condition. The biocompatible pH-sensitive drug carriers can effectively release anti-cancer drug in acidic condition to obtain sustained controlled release as promising carriers for anti-tumor drug delivery.

#### 3-D Bioprinting of Tissue Organoids for Body-on-a-Chip Drug Screening Platform

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Development of a three-dimensional (3-D) human organoid system that mimic the human body on a chip is an attractive option for drug screening and toxicology studies. In this study, we developed tissue organoid constructs using the 3-D bioprinting technology. This technology is able to fabricate organoid structures by concurrent patterning of cell-laden hydrogel bio-ink and synthetic thermoplastic polymer. The hydrogel patterning provides a 3-D biological environment for cell proliferation and differentiation. The synthetic polymer serves as a physical and structural support. In this study, poly( $\epsilon$ -caprolactone) (PCL) and gelatin-based cell carrier materials were used for the construction of the organoid structures, including hepatic and cardiac tissues. Cell carrier material was prepared by mixing gelatin and natural polymers, such as fibrin and alginate, for the production of extracellular matrix (ECM) environment. This composite hydrogel was mixed with hepatocytes or cardiomyocytes at 37°C and used for patterning the organoid constructs. The 3-D bioprinted tissue organoids maintained viability and cellular function up to 4 weeks under a microfluidic condition. This 3-D bioprinted organoid system provides new tissue models and platforms for use in drug discovery and basic research, while supplying the means for the advancement in *ex vivo* organ systems.

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### Relative Motion Affects Tissue Growth and Osteoclastic Gene Expression in *In-Vitro* Bone Tunnel - Ligament Model

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Poor bone tunnel healing and enlargement have been reported after anterior cruciate ligament reconstruction surgery. Motion between the grafts and the bone tunnel is associated with these phenomena. However, its effect on cellular and molecular events driving the healing process leading to graft integration is unknown. We hypothesised that motion affects graft to bone tunnel healing by hindering cell and tissue ingrowth and inducing bone tissue resorption by activating osteoclast development by means of the upregulation of RANKL expression.

To test this, epiphysis cells seeded onto polyester ligament scaffolds were subjected to cyclic motion for 1 hour/day using in-house developed bioreactor. Cell proliferation (days 1–7), tissue growth (at 5 weeks) and the expression of genes relevant to osteoclastogenesis including RANKL and OPG were determined.

Cyclic longitudinal motion, in contrast to unloading, induced significant reduction of cell and tissue growth around the ligament scaffolds. Furthermore, longitudinal motion induced a significant increase of RANKL expression but had no effect on OPG expression compared to control group.

The deficiency of cell and tissue on-growth around the scaffold under mechanical loading may explain the direct involvement of motion in poor healing of the graft-bone tunnel. The change in the RANKL/OPG ratio in favour of RANKL suggests that motion could support the activation of bone resorption via differentiation and activation of osteoclasts.

Improved Grafts surgical fixation, combined with post-surgery immobilisation or conservative rehabilitation regimes in early post-operative period might negate the negative effects described above and favour improved graft-to-bone healing with reduced bone tunnel widening.

### Alterations to ECM Stiffness and Composition Impact the Progression of Fibrosis by Cardiac Fibroblasts

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The native reparative response following acute myocardial infarction (MI) involves a complex inflammatory process that is initially beneficial for stabilizing the necrotic tissue, but ultimately leads to scar formation and subsequent heart failure in patients with large MIs. This fibrotic response is generally characterized by an accumulation of fibrillar collagens which are crosslinked by lysyl oxidase (LOX) to form mature collagen fibers. While the scar formation following MI leads to an increased bulk tissue modulus, we recently combined nonlinear microscopy techniques and mechanical testing of decellularized tissue to find that individual collagen fibers in the scar are actually weaker, and have lower levels of crosslinking, than their healthy counterparts. We hypothesize that continued scar formation is the result of abnormal stimuli at the cellular level and strategies that alter the mechanical and compositional stimuli may lead to better functional outcomes. We have thus replicated the mechanical and compositional stimuli *in vitro* to determine the effects of these stimuli on the expression of fibrotic genes and progression of fibrosis by cardiac fibroblasts (CF). CF plated on collagen I exhibited a 2-fold increase in LOX gene expression, 3-fold increase of  $\text{col1}\alpha 1$ , and 2-fold increase of  $\alpha\text{SMA}$ . Ultimately, this research can be translated to treatment of MI through providing an altered environment for resident CF to control the ECM remodeling response and prevent progression of scar formation. Additionally, understanding the effect of various substrate properties on the remodeling response of CF is important for appropriate scaffold designs for cardiac tissue engineering applications.

### Effects of Nozzle Diameter during Drop-on-Demand Cell Bioprinting

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Three-dimensional (3D) printing is an attractive biomanufacturing technology because it is an automated and industrial scalable approach that allows spatially accurate delivery and simultaneous deposition of several cell types and biomaterials into a 3D environment. With the obvious advantages of being inexpensive as well as its ability to print with high speed, inkjet printing of living cells has been used to print biomolecules onto substrates resulting in the creation of DNA chips, protein arrays and cell patterns. However, the printing factors must be carefully examined for it to be a viable biomanufacturing technology. The risk of exposing cells and materials to thermal and mechanical stress, low droplet control characterized by non-uniform droplet size (also known as the coffee-ring effect) as well as frequent clogging of the nozzle are considered disadvantages for the use of these 3D bioprinters.

In this study, we aimed to study the effect of nozzle diameter in a piezoelectric-based inkjet dispensing system to quantitatively print of cells at high resolutions. Human ovary adenocarcinoma expressing multiple fluorescent markers were studied to appreciate the effect of cell concentration on the droplet formation process. Our results showed that (1) as the cell concentration of bioink increases, the droplet size decreases, and (2) increasing nozzle diameter the bioink tends to improve droplet formation, shorten breakup time and improve cell viability.

### Modeling the Synergy between Hematopoietic Stem Cells and the Bone Marrow Niche for *In Vitro* Culture

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The development of all blood and immune cells in the body is maintained via hematopoiesis. Hematopoietic stem cells (HSCs) regulate this process in concert with several components of the bone marrow niche. The *in vivo* regulation of HSCs is therefore a dynamic process involving feedback loops and complex signaling cascades. Efforts to imitate these interactions *in vitro* to guide stem cell fate have not been successful party due to the large number of niche parameters influencing cell biology. A computational approach has the potential to simplify and systematize these interactions and to develop a dynamic model that adequately reflects the system's complexity and parameter sensitivity.

We utilize the STELLA modeling and simulation software (see systems) in order to simulate the interactions between HSCs and mesenchymal stem cells (MSCs), an important niche cell type. The more primitive HSC populations hold clinical significance for their repopulating ability and the model investigates the dynamic balance between HSC self-renewal and differentiation on a 2D *in vitro* platform. The effect of MSCs on this balance is also explored. Experimental data is used to validate and complement the model, improving its accuracy. Although such an approach has been demonstrated before, it has not been implemented on a three-dimensional (3D) platform. Therefore, our model further attempts to capture the complexity imparted by a 3D matrix on the determination of HSC fate. Such a system can help us better understand the kinetic relations between niche components while isolating key parameters for a controlled HSC culture platform.

### Extracellular Matrix Differentiation in a Novel Model of Human Oral Mucosa Generated with Alternative Stem Cell Sources

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**Objective:** The aim of this work was to generate and evaluate an artificial human oral mucosa substitute using an alternative stem cell source obtained from the human umbilical cord.

**Methods:** Two models of human oral mucosa were generated by tissue engineering. Both models contained stromal fibroblasts immersed within fibrin-agarose biomaterials, but the epithelial layer was generated with native oral mucosa keratinocytes (OM samples) or with alternative Wharton's jelly stem cells -HWJSC- (hOM samples). Tissues were analyzed by light and electron microscopy to determine cell and tissue differentiation markers at the *ex vivo* and *in vivo* levels.

**Results:** The results showed that oral mucosa stromas presented an organized fibrillar pattern after *in vivo* grafting. Increased synthesis of collagen was observed after 3 weeks of *ex vivo* culture OM and hOM. No elastic or reticular fibers were found. When samples were grafted *in vivo*, we observed an increased expression of glycoproteins at the epithelial-stromal layer. Expression of decorin, versican and aggrecan was related to the presence of a well-structured epithelium on top and strongly dependent on the environment *in vivo*. Finally, the use of HWJSC was associated to an increased synthesis of versican.

**Conclusions:** These results suggest that the use of fibrin agarose biomaterials may allow the generation of an efficient human oral mucosa substitute. As previously suggested, the *in vivo* environment and the epithelial-mesenchymal interaction are necessary for an adequate differentiation of the bioengineered stroma.

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### Morphological Assessments for Regenerated Sciatic Nerve using Different Nerve Conduits

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Nerve injury leads to profound functional loss in human body. However, the nerve regeneration is difficult due to poor self-repair and prevalence of neuropathy. The neural progenitor cells (NPCs) can become different mature cells in neural lineage. Recently, we discovered the modification of cell culture surface by chitosan has potential to induce the transdifferentiation of adipose-derived stem cells (ASCs) into NPCs. This study aim to investigate the therapeutic effect of a new strategy by combining chitosan-coated conduit and NPCs (C+N). Promotion of nerve regeneration will be assessed by morphological assessments using histological staining and transmission electron microscopy (TEM). We created a 10-mm gap on rat sciatic nerve and bridged with chitosan-coated or non-coated conduit. In addition, the transplantation of NPCs was also designed to evaluate the combination of NPCs and chitosan-coated conduit. 6 weeks after surgery, the nerves were harvested to observe the myelin structure and the anti-inflammatory mechanism. Neuroma and scar were found in the regrowing area using non-coated conduit which can be inhibited by C+N treatment. Furthermore, the C+N therapy promoted the axon regeneration and myelin sheath formation. The degeneration of myelin sheath in non-coated conduit was confirmed by TEM. Re-myelination was observed in C+N treatments using toluidine blue staining and TEM. Immunohistochemistry staining revealed the possible therapeutic mechanism of chitosan conduit by inhibiting the injury-induced interleukin-1 beta and 5-lipoxygenase inflammatory signaling. In summary, this study demonstrated the combined treatment have an optimal result in nerve repair which was capable of inhibiting inflammation and boost nerve regeneration.

### 3D Bioprinting Facilitates Scalable Endochondral Bone Formation *In-Vivo*

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Cartilaginous tissues engineered using mesenchymal stem cells (MSCs) form bone *in-vivo* by executing an endochondral programme offering a promising route for bone regeneration. Potential limitations associated with such endochondral strategies for repairing large bone defects within load bearing regions include the relatively poor initial mechanical properties of engineered cartilaginous tissues and achieving vascularisation when scaling up such constructs. The objective of this study was to bioprint a MSC laden degradable gamma-irradiated alginate hydrogel alongside a polycaprolactone backbone to mechanically reinforce the construct, and to subsequently chondrogenically prime this construct *in-vivo* to provide a template for endochondral bone formation *in-vivo*. The MSC laden alginate hydrogel was deposited within every second fibre spacing, thereby creating an interconnected network of channels to facilitate vascularisation. Small (5 mm×3 mm) and large (10 mm×6 mm) constructs were bioprinted to assess whether the process was scalable and a solid MSC-laden alginate hydrogel without channels or a polycaprolactone structure was implanted as a control.

After 4 weeks *in-vivo* the 3D bioprinted cartilaginous templates had proceeded along the endochondral pathway, as demonstrated by vascularisation and mineralization of the constructs ( $\mu$ CT, H&E). After 12 weeks *in-vivo* the bioprinted constructs were well vascularised, with both small and large constructs supporting bone formation throughout the depth of the constructs ( $\mu$ CT, H&E). Solid control constructs remained relatively non-vascularised, with significantly lower vessel infiltration and bone formation after 12 weeks (Histomorphometric analysis). This study demonstrates that 3D bioprinting of chondrogenically primed templates can facilitate the development of scalable, mechanically reinforced constructs suitable for endochondral bone regeneration strategies.

### Medical Imaging-guided Additive Manufacturing of Human Osteochondral Tissues

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**Objectives:** In this study, we aim to develop a technique for PSL-fabricated osteochondral scaffolds based on medical imaging for focal and total articular cartilage surface repair. The principal fabrication method involves a visible light-based photocrosslinking-based PSL protocol developed in our lab (1). Poly(D,L-lactide)-poly(ethylene glycol)-poly(D,L-lactide) (PDLLA-PEG)/hyaluronic acid (HA) hydrogels loaded with cells and TGF $\beta$ 3 were used to form the cartilage layer, while poly( $\epsilon$ -caprolactone) (PCL)/gelatin hydrogels infused with hydroxyapatite were used to form the bone layer.

**Methodology:** Human adipose stem cells (hASCs) were isolated from lipoaspirate with Institutional Review Board approval (University of Pittsburgh and University of Washington). Explanted joint tissue with iatrogenic osteochondral defects created after tissue harvest was incubated with Hexabrix<sup>TM</sup> and scanned by micro-CT. Image contrast were adjusted to enhance and distinguish bone versus cartilage. Micro-CT imaging was converted into 3D models and used as the template for PSL fabrication of osteochondral tissues including hASCs.

**Results:** We successfully fabricated hASCs-laden osteochondral tissues, using the micro-CT imaging as the template, which had the same anatomical architecture as the original scanned native tissues. After fabrication, hASCs showed high viability (84%) and their ability to undergo osteogenesis or chondrogenesis in the bone or cartilage scaffolds, respectively, was also confirmed. We are currently using a goat model to test the applicability of this technology for the repair of osteochondral defects.

**Significance:** Our findings suggest a promising procedure for effective treatment of post-traumatic osteoarthritis and other major articular joint injuries.

**Reference:** (1) Lin *et al.* Biomaterials, 2013, 34(2):331–9.

### Strategies for Temporal Control of Biophysical Signals in Biomimetic Hydrogels for Tissue Engineering

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For the past decade researchers have been motivated to develop biomimetic hydrogels in pursuit of a blueprint to bridge the gap between biomaterials and living systems. Recent advances highlight methacrylated gelatin (Gel-MA) as a versatile hydrogel platform for regenerative medicine with its inherent biological complexity [1]. However, tailorability of Gel-MA degradation dynamics is limited [1], thereby reducing the ability for temporal delivery of biochemical and biophysical signals to cells, which ultimately restricts its clinical application. Previous studies in our lab have shown that addition of thiol moieties into Gel-MA hydrogels affected their degradation rates, but little is known about using this as a strategy to introduce temporal control of biophysical signals in biomimetic hydrogels. Therefore, the aim of this study was to investigate the relationship between thiolated molecules and degradation kinetics of Gel-MA hydrogels. Hydrogel constructs were fabricated by irradiating 10 wt% Gel-MA in PBS, containing dithiothreitol (150 Da) or thiolated-heparin (19 kDa) at varying molar ratios (0.41–4.6 SH/MA), for 15 minutes with ultraviolet light (365 nm, 3.5 mW/cm<sup>2</sup>) with the addition of 0.05 wt% photoinitiator (Irg2959). Hydrogels with tuneable degradation rates ranging from 6 h to 10 weeks were successfully fabricated. Mass loss and swelling data showed that adding higher thiol molar ratios or lower molecular weight molecules significantly accelerated construct degradation. Our current studies are investigating the degradation mechanism, along with the capacity of these degradable hydrogels to guide cell development. We envision that these innovative compositions and physico-chemical properties of Gel-MA can serve as a platform for procuring spatio-temporal control of cell function and drug or growth factor delivery.

1. Schuurman, W. *et al.* *Macromolecular Bioscience*. 13(5):551–561, 2013.

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### Biodegradable 3D Printed Vascular Grafts with Enhanced Elasticity

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The most common birth defect in the world is congenital heart disease and severe cases require extensive surgery using vascular grafts. However, the gold-standard grafts come from premade sizes that do not allow for the long-term growth of patients. To minimize complications associated with permanent synthetic grafts, researchers are turning to biodegradable tissue engineering scaffolds. These strategies are especially attractive when coupled with 3D printing technologies to fabricate custom-tailored vascular grafts. We investigated 3D printable biodegradable poly(propylene fumarate) (PPF) grafts. These grafts exhibited mechanical properties similar to native blood vessels and supported cell viability. We implanted the grafts in the inferior vena cava of mice. Grafts remained patent after one year. The grafts were next implanted in the carotid arteries and veins of sheep. While they functioned in the carotid vein, grafts failed in the arterial environment due to fracturing. To mitigate these issues, we have improved our 3D printing resin by utilizing a PPF and poly(caprolactone) (PCL) copolymer. 1,2 This material exhibits more elasticity than PPF, which may alleviate mechanical failure issues. Cytotoxicity assays indicated no statistically significant difference between PPF and P(PF-co-CL) materials in regards to cell viability. Mechanical properties were tuned by adjusting the ratio of included PCL, leading to elastic moduli ranging from 6.3–175.0 MPa and ultimate tensile strengths of 2.0–16.6 MPa. Printing resins based on this copolymer may prove to be a valuable platform for more complex 3D printing vascular graft technologies.

(1) Wang S *et al.* *Biomaterials*. 2008.

(2) Wang, S *et al.* *Biomaterials*. 2006

### Wnt Signalling and Stem Cell Mobilisation in Tissue Injury Repair

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Repair of bone and skin are efficient processes when the lesions are small, but inefficient when lesions are large. Repair of other tissues such as cartilage and ligament is less efficient, even for small lesions and in general repair efficiency decreases with age. A thorough and detailed understanding of the individual processes required for tissue repair, their initiation, integration, co-ordination and cessation is clearly required in order to develop ways of promoting healing in situations where it is inefficient.

We use a novel experimental model - the mouse molar tooth - to investigate the cellular and molecular details of tissue repair. The cellular ontogeny, anatomical location, and precise identity of dental pulp stem cells (DPSCs) is largely unknown. In addition, the signalling events that co-ordinate the recruitment, proliferation and differentiation of DPSCs into odontoblasts, the cells that produce reparative dentin, is poorly understood. The Wnt signalling pathway plays multiple essential roles in all the stages of wound healing. We have found that Wnt signalling is activated in the tooth following damage and that manipulating Wnt levels alters the rate of repair. In addition, we have evidence that pericytes are one source of stem/progenitor cells that are mobilised to generate new odontoblasts, which produce reparative dentin in response to damage.

### Active Mechanics of Cytoskeletal Networks of Single and Collective Cells

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Cells are multifunctional building blocks of complex organisms and cytoskeletal proteins are basic building blocks of cells. These basic components exhibit fundamental mechanical and kinetic properties, including binding kinetics and motor force generation, that enable function, and large networks of them confer complex behaviors that enable various physiological phenomena, from intracellular transport to cell migration to collective cell dynamics. We use an integrated experimental and computational approach to decipher the dynamics and mechanics of networks of biological components, starting at the subcellular scale. Using particle tracking microrheology, we measured the internal fluctuations of cells in 2D, 3D, and within multicellular aggregates and we characterized passive and active (motor-driven) features. We found that the nature of the culture environment can modulate the internal mechanical properties of cells. For example, cells in 3D exhibit more subdiffusive intracellular motions compared to cells in 2D. Furthermore, compaction of cells appears to alter internal motions. Through Brownian dynamics simulations, we investigated the roles of the most fundamental components of the cytoskeleton - actin, actin crosslinkers, and myosin motors (all active and dynamic) - in generating stress and modulating morphological states and phase transitions. We found that diverse functional capabilities can be conferred by networks of these interacting components, from tuning stress magnitudes and fluctuations to large contractile events. Our integrated approach enables us to probe into the fundamental mechanics of complex biological systems.

### Mesoporous Bioactive Glass/CaP Bone Cement Composites for Protein Delivery

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Loading of proteins, e.g. growth factors into biomaterials and tissue engineering scaffolds and their subsequent release has recently come into focus of tissue engineering. Protein delivery into a specific tissue defect could foster defect healing by recruiting tissue-specific cells, stimulating their differentiation and activity and inducing vascularization. Such a local approach meets drawbacks of systemic application, e.g. low targeting efficiency. In bone defects, calcium

phosphate cements (CPCs) [1] are excellent carriers due to their excellent biocompatibility and bio-resorbability. We established a composite material based on a hydroxyapatite-forming CPC and mesoporous bioactive glass (MBG [2]) granules. *In vitro* cytocompatibility of the composite was demonstrated. The intrinsic nanoporosity of sol-gel derived MBG with pores in the range of 5–10 nm allowed the loading and diffusion-limited release of proteins from the composite while preserving the biological activity of the protein. Besides bovine serum albumin and lysozyme as differently charged model substances we investigated loading and release kinetics of brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF). A gradual release was obtained that could be tailored by the protein/MBG ratio and total MBG loading of the composite. The biologic activity of BDNF and VEGF after release was confirmed. Therefore, CPC/MBG composites offer the possibility to deliver controlled amounts of biologically active, sensitive protein molecules into a specific defect location and thus could help in bone defect regeneration.

### References

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### CEST MRI for High-resolution *In Vivo* Tracking of Implanted Stem Cells

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The traditional tissue engineering paradigm implants cells in a scaffold with growth factors in the hope that the construct will result in regenerated tissue. The notion that transplanted stem cells directly differentiate to regenerate tissue has been a subject of some controversy, with alternate hypotheses suggesting the cells contribute to repair via other modes, such as secretion of trophic factors. To elucidate the precise role transplanted stem cells play in tissue engineering, a high-resolution cell tracking technology is required. Ideally, the tracking modality is also longitudinal. Here, we demonstrate the use of chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) using transduction of the herpes simplex virus thymidine kinase 1 (HSV1-tk) for longitudinal tracking of implanted stem cells *in vivo*. The CEST approach allows for specific tracking of implanted cells and takes advantage of the high resolution in MRI, in contrast to the lower resolution with imaging modalities such as luciferase-based optical imaging. The CEST probe used here also has the advantage of expression only by transduced live cells, precluding adventitious imaging of macrophages that have taken up the probe. The objectives of this study are to (1) confirm visibility of transduced human adipose-derived stem cells (ASCs) *in vitro* under CEST MRI, (2) determine the effect of probe transduction on cell viability and potency, and (3) use CEST MRI to track ASCs longitudinally in a murine calvarial defect. Successful application of this technique would provide a powerful tool for investigating mechanisms of cell-mediated regeneration.

### Fabrication of Scaffolds Mimicking the Architecture of Articular Cartilage using Direct-write Electrospinning

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Near-field electrospinning (ESP) and melt ESP have been developed to overcome the limitations of traditional ESP techniques in terms of design of scaffold's architecture and densely packed fibrillar meshes. Despite these methods proved to obtain an exquisite control over single-fiber deposition at the micron scale, they are still lacking flexibility in controlling multi-fiber deposition. Here, we developed a direct writing (DW) ESP technique for the fabrication of microfibrillar structures in a precise and controllable

way. The developed technology allows engineering fiber deposition from single fibers to bundles of fibers by controlling the processing parameters. A scaffold mimicking the zonal organization of articular cartilage was produced by this method and its performance on cartilage tissue formation was evaluated through the chondrogenic differentiation of seeded human mesenchymal stromal cells *in vitro*. The scaffold revealed a better seeding efficiency than other reported 3D scaffolds and was able to direct tissue organization and fibrillar matrix orientation, as opposed to a typical electrospun mesh. RT-PCR results revealed that expression of the chondrogenic markers Sox9 and ACAN in DW scaffolds was significantly enhanced compared to conventional ESP scaffolds at day 14 (3.5-fold and 17-fold, respectively) and day 21 (4.6-fold and 67-fold, respectively). The formation of cartilage like matrix on DW scaffolds was further confirmed by Alcian Blue staining at day 21. This work not only shed light on the potential of DW ESP for cartilage regeneration, but can also be used in other regenerative medicine applications where anisotropy patterns are of importance.

### 3D Bioprinting of Human Chondrocyte-laden Nanocellulose Hydrogels for Patient-specific Auricular Cartilage Regeneration

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Auricular cartilage tissue engineering (TE) aims to provide an effective treatment for acquired or congenital auricular defects. Bioprinting has gained attention in several TE strategies for its ability to spatially control the placement of cells, biomaterials and biological molecules. Although there are considerable advances in bioprinting of complex 3D tissue analogues, the development of bioinks with good printability and bioactive properties must improve for the clinical application of 3D bioprinting. In this study we evaluated the biological functionality of a bioink, composed of nanofibrillated cellulose and alginate (NFC-A), for application in auricular cartilage TE. 3D bioprinted NFC-A auricular constructs, laden with human nasal chondrocytes (hNC), were cultured for up to 28 days. Redifferentiation capacity of hNCs and deposition of cartilage specific components was evaluated on gene expression as well as protein level. The presented 3D bioprinting process using NFC-A bioink facilitated the biofabrication of cell-laden, patient-specific auricular constructs with an open inner structure, high cell density and homogenous cell distribution. The high porosity improved the nutrition supply of the embedded hNCs, as indicated by the increased cell viability and proliferation during 3D culture. After bioprinting and 3D culture, constructs offered an excellent shape and size stability and supported the redifferentiation and chondrogenesis of hNCs; as demonstrated by gene expression, immunohistochemical and biochemical analyses. We demonstrated that non-cytotoxic NFC-A bioink provides a biologically relevant environment promoting redifferentiation of hNCs while offering excellent printability and bioactive properties, making it a promising tool for auricular cartilage TE and many other biomedical applications.

### Application of High Pressure Engineering to Maintain and Inactivation of Congenital Melanocytic Nevi Extirpation

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Extirpation of the skin is fundamentally required for treatment of the skin tumor disease such as giant congenital melanocytic nevi. However, therapeutic approach contains a difficulty in preparation of the alternative skin graft. To overcome this problem, we have developed a new approach to transplant a cell-inactivated autologous skin prepared by high hydrostatic pressure 1, 2. The skin graft

would be able to be re-transplanted after inactivating the tumor cells. In this study, we investigated the cellular morphology, mitochondria activity, and membrane permeability of mammalian cells treated with various pressure condition under *in vitro* models. The fibroblast, smooth muscle, and endothelial cells were used. When the cells were treated with more than 200 MPa pressure for 10 min., the cells were not attached and spreaded out. Mitochondria activity was also suppressed. Membrane permeability of cells treated with above 500 MPa was disturbed. From these results, the pressure of 200 MPa was enough to induce cell killing through mitochondria inactivation.

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## Plasmonic Lipogels Responsive to Near Infrared Light for Remote Control of Drug Release

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The clinical use of most conventional chemotherapeutics is often limited due to inadequate delivery of therapeutic drug concentrations to the targeted tumor or to severe and harmful toxic effects in healthy tissues. Lipid-based nanocarriers offer the advantage of a high drug payload, enhanced drug accumulation at tumor sites and decreased drug toxicity. In order to optimize time, location and amount of drug release, so much effort has been devoted to the development of liposomal systems capable of releasing drug in response to a specific stimulus. Examples of this are lysolipid-based thermosensitive liposomes (LTSL), which lipid membranes rapidly change structure and create pores at the gel-liquid crystal phase transition temperature (T<sub>c</sub>). LTSL formulation tuned for a T<sub>c</sub> in the mild hyperthermia (HT) range of 40–42°C are very attractive carriers for chemotherapeutics as doxorubicin (DOX), which antitumor effect is enhanced by mild HT. In this work, we incorporated DOX-loaded LTSL (DOX-LTSL) to *in situ* polymerizable fibrin hydrogels (lipogels) filled with plasmonic gold nanostructures that enable them to transduce photonic energy delivered by near-infrared (NIR) light of the “therapeutic window” into heat. Treatment of developed plasmonic lipogels with an 808 nm laser restricted drug release from NIR-targeted areas in the construct, enabling spatiotemporal control of DOX release that effectively reduced cell viability in an *in vitro* model of human epithelial carcinoma.

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## Systematic Evaluation of the Mesh Size of Poly(ethylene glycol)-based Hydrogels for Biomedical Applications

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Poly(ethylene glycol) (PEG) hydrogels are one of the most widely utilized biomaterial systems, thanks to their intrinsic resistance to protein adsorption and the ability to be covalently linked to multiple functional moieties. This opens a wide range of design options for the synthesis of substrates with tunable chemistry and bioactivity. Moreover, the hydrogel mesh size can be finely controlled to regulate the molecular trafficking within the polymer matrix. The primary objective of this work was to explore the mesh size of poly(ethylene glycol) diacrylate (PEGDA)-based hydrogels obtained by photocrosslinking. Aqueous solutions differing for polymer concentration and type of photoinitiator were crosslinked by ultraviolet (UV) exposure for different time lengths. Free swelling and uniaxial compression measurements were performed to evaluate the hydrogel mesh size, based on classical theoretical models of swelling and rubber elasticity. The calculated values of mesh size were then experimentally validated by protein diffusion studies. Further aim of this work was to assess the gradual increase of the mesh size resulting from hydrolytic degradation, upon incubation at 37°C for 10 weeks. The experimental results showed that, for the hydrogel formulations tested, the mesh size could be reliably estimated by both swelling and mechanical measurements. Remarkably, the evaluation of the mesh size following hydrolysis allowed detection of significant differences in the degradation rate of the samples, while providing pivotal information on the dynamic change of their diffusive properties. Such a systematic evaluation of the mesh size allows the careful design of PEG-based hydrogels for medium to long-term biomedical applications.

## Fabrication of Silk Fibroin using Centrifugal Casting Technique for Corneal Tissue Engineering

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Films prepared from silk fibroin have shown potential as biomaterials in tissue engineering applications for the eye. Here, we present a novel process for fabrication of silk fibroin films for corneal application. In this work, fabrication of silk fibroin films was simply achieved by centrifugal force. In contrast to the conventional dry casting method, we carried out the new process in a centrifuge with a rotating speed of 1000 rpm, where centrifugal force was imposed on an aluminum tube containing silk fibroin solution. In the present study, we also compared the surface roughness, mechanical properties, transparency, and cell proliferation between centrifugal and dry casting method. In terms of surface morphology, films fabricated by the centrifugal casting method have less surface roughness than those by the dry casting method. For elasticity and transparency, silk fibroin films obtained from the centrifugal casting method had favorable results compared with those prepared by dry casting method. Furthermore, primary human corneal keratocytes grew better in films prepared by centrifugal casting method. Therefore, our results suggest that this new fabrication process for silk fibroin films offers important potential benefits for corneal tissue regeneration.

## A Non-contact Wide Field Method to Evaluate Epithelialization: UV Fluorescence Excitation Imaging

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**Background and Objectives:** Methods for evaluation of epithelialization are often subjective, invasive, difficult to implement and limited to microscopic regions. We take advantage of the fluorescence of molecules native to skin to image cellular proliferation and collagen cross-links upon UV fluorescence excitation. The present

study demonstrates the utility of using UV fluorescence excitation imaging to evaluate epithelialization of human skin and a skin substitute *in vitro*.

**Study Design:** Circular 3 mm diameter full-thickness wounds were created in *ex vivo* skin. A commercial acellular dermal matrix (AlloDerm) was implanted into the wounds and cultured in saline or DMEM (EGF+). Circular 3 mm diameter superficial wounds subjected to the same culture conditions were used as controls. Wound size and keratinocyte proliferation was quantified longitudinally using fluorescence imaging. Histology (H&E) and immunohistology (pan-keratin, Ki67) were used to confirm the source of fluorescence.

**Results:** Temporal variations in fluorescence distribution across the wound surface show that AlloDerm in DMEM was fully epithelialized in 16 days, as compared to 12 days for controls in DMEM. In saline, a fluorescence distribution in the shape of a ring by the wound edge did not change in size and shape, indicating a non-healing wound. Evaluations of newly formed epidermis by histology and pan-keratin and Ki67 staining are in agreement with fluorescence distributions.

**Conclusions:** We have demonstrated the utility of using UV fluorescence excitation imaging to evaluate epithelialization *in vitro*; our method provides a non-destructive, non-contact, wide field, objective and direct method for quantitative measurements in wound healing.

### Controlled pH Through Automated Medium Exchange Supports Chondrogenic Capacity of Human Nasal Chondrocytes

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Automation and online monitoring in bioreactors offer great advantages over conventional manual production of cell-based therapies, providing non-invasive data relevant to both process and product. Through feedback control, online sensing can also allow optimal control of culture parameters, facilitating product standardization. Here we developed an automated medium exchange system with feedback control to maintain pH of the culture medium within a user-defined range. A pH sensor is integrated in the medium reservoir of a perfusion-based bioreactor. Once a pH user-defined threshold is reached, the medium is automatically replaced through a dedicated hydraulic circuit. The system can fit any bioreactor set-up provided with luer-lock connectors. Operating parameters are set through a user-friendly control panel. Data are displayed online and logged, accessible via remote wireless connection. To validate the system, human nasal chondrocytes were expanded on 3D collagen scaffolds in the perfusion-based bioreactor. In one group, the pH was kept between 7.2–7.4 through the automatic medium exchange system. In another group, medium was changed manually at regular intervals. Cells were then enzymatically retrieved from the scaffolds, analyzed by RT-PCR and re-differentiated in micro-masses. Cells expanded at pH-controlled conditions showed higher expression of cartilaginous matrix genes, and higher glycosaminoglycans deposition by Safranin-O staining and GAG/DNA quantification. In conclusion, our automated medium Exchange system provided a controlled physico-chemical environment favourable for the re-differentiation of human nasal chondrocytes. The implementation of such a system in the manufacturing of cell-based products will greatly improve safety and standardization, facilitating regulatory compliance and cost-effectiveness in the long-term.

### The Influence of BMP-2 on Signaling Mechanisms and Differentiation Fate of Human Adipose Stem Cells

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Bone morphogenetic protein (BMP)-2 is a growth factor used to stimulate bone regeneration in clinical applications. However, there are contradicting reports on the functionality of BMP-2 in human adipose stem cells (hASCs). In this study we analyzed the effects of BMP-2 on Smad1/5 signaling, proliferation and differentiation in hASCs, *in vitro*. BMP-2 induced Smad1/5 activation was analyzed in different serum conditions (fetal bovine serum; FBS and human serum; HS) with hASC lines derived from several donors by Western Blotting of phosphorylated Smad1/5 protein (p-Smad1/5) and by immunofluorescence microscopy of the p-Smad1/5 nuclear translocation. The BMP-2 induced differentiation of hASCs was analyzed in basal growth medium (BM) and osteogenic medium (OM) by quantitative alkaline phosphatase assay (qALP), Alizarin Red mineralization assays, Oil Red O lipid formation assays and expression of osteogenic and adipogenic marker genes. Our results indicate that the functionality of BMP-2 is strongly dependent on serum and culture conditions. BMP-2 induced Smad1/5 activation and nuclear translocation in all cell lines studied, but Smad activation was prominent only in HS conditions compared to FBS conditions. Interestingly, BMP-2 exhibits a dual role in differentiation process of hASCs, and the effect on differentiation fate is donor cell line dependent. BMP-2 stimulated osteogenic effect in some cell lines by inducing ALP activity and mineralization, whereas in other donor lines, BMP-2 induction clearly promoted adipogenic differentiation. These results partially explain the existing contradictory in results of the previous BMP-2 studies and indicate variability in functional mechanisms of BMP-2 signaling in hASCs.

### Angiogenesis from Biofabricated Hierarchical Vasculature within Engineered Tissue Flaps

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Large wounds caused by trauma, burn, or disease are very common and require voluminous tissue for reconstruction. While de novo pre-vascularized tissues would dramatically improve outcomes for these patients, significant challenges remain in creating clinically relevant tissue volumes with embedded vasculature. In this study, we utilize extrusion-based biofabrication strategies to create large volume hydrogels with prescribed vascular channel geometries. We compared the effects of cell source and fabricated vessel size on endothelialization presence and stability and angiogenesis propensity in culture. Vascular channels were created within bulk hydrogel (collagen, gelatin/hyaluronic acid) using Pluronic F127 as sacrificial templates. Large (1–1.5 mm) and small (25–100 μm) diameter vessels were generated by 3D bioprinting or manually positioned microfilaments. HUVECs were perfusion seeded into channel lumens alone or subsequent to pre-seeding with HASMC, fibroblasts, and/or pericytes. Constructs were cultured up to 28 days in static or fluid flow conditions. Incorporation of small vessels showed sequestration of HASMC to larger vessels and HUVEC within smaller vessels. Large vessels yielded few angiogenic sprouts, but smaller vessels revealed robust angiogenic response. HASMC-supported vasculature destabilized, while HASMC/pericyte/fibroblast seeded channels maintained endothelial coverage. Flow studies indicated similar vascular resistance between biofabricated constructs and native tissue flaps. These results demonstrate that clinically sized hierarchical vascularized tissue flaps can be biofabricated with angiogenic potential, and mural cells are critical to maintain endothelial integrity and vessel stability. This implantable vascularized tissue platform enables further study on the engineering of pro-angiogenic and homeostatic vascular templates to accelerate and improve healing in challenging wounds.

### Methods for Monitoring New Elastin Formation During Elastogenic Therapy

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Elastic fibers are essential to the function of many biological tissues including skin, lung, ligaments, and arteries. Consequently, when damage occurs to these fibers, disease can result. Interestingly, for the case of elastic fibers, it is rarely the acute damage that leads to disease but a progressive loss of elastin through a vicious cycle of destruction. Current methods for analyzing elastin content and function in an example tissue, arteries, span the disciplines of protein chemistry, microscopy, and biomechanics. Full hydrolysis of arteries in a basic environment leaves insoluble elastin, which can be quantified by assays including acid hydrolysis/ninhydrin, liquid chromatography to identify cross-linking moieties, or immunoidentification. Arteries can be analyzed by multi-photon microscopy directly for elastin identification, or sectioned and stained for elastic fiber components. Mechanical testing of explanted arteries can be performed to determine the extent of the elastic toe region on the stress-strain curve. However, all of these current methods necessitate destruction of the blood vessel, which makes experiments with animal models wasteful and potential clinical therapies impossible. In my laboratory, I have been investigating techniques for monitoring elastin production and destruction at multiple scales. This includes the earliest timepoint in elastin production, transcription, as well as a novel dye-based reporter of elastin assembly into the vascular matrix. We can also monitor levels of destructive enzymes using peptide substrates and zymographic techniques. The long term goal of this work is to perform elastin monitoring noninvasively as a complement to developing pro-elastogenic therapies.

#### Automated Bioreactor for Biomimetic Adaptive Culture and *In Situ* Mechanical Characterization of Cardiac Tissue Models

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The development of cardiac tissue models that recapitulate properties of the normal and diseased heart is of great interest in myocardial tissue engineering and drug discovery. In order to provide a suitable environment for the *in vitro* maturation and investigation of these constructs, a novel automated bioreactor with real-time integrated monitoring for biomimetic adaptive culture has been developed.

The system is composed of: 1) a culture chamber, for housing annular or patch constructs; 2) an integrated monitoring unit, with sensors for online pH and oxygen measurements and a load cell for both real-time assessment of construct physical responses and *in situ* non-destructive mechanical characterization; 3) a biomimetic stimulation unit, for providing tunable physiological/pathological-like cyclic stretch (1–200% deformation, 1–10 Hz frequency); 4) a control unit, with a purpose-built software, for controlling the stimulation. Furthermore, a feedback loop between the monitoring and control units has been designed to automatically adapt the stimulation to the construct maturation phase (measured in terms of mechanical properties).

In-house tests confirm the biocompatibility, sterility maintenance, and automation performances of the system. The development of cardiac tissue models is ongoing for investigating in the near future the effects of mechanical stimulation on cardiac cell/tissue organization and maturation.

In conclusion, by enabling real-time monitoring, non-destructive mechanical characterization, control of individual parameters (separated from *in vivo* systemic effects), and automated adaptive dynamic culture, the proposed bioreactor will provide a reliable biomimetic environment for production and investigation of cardiac tissue models, and for disease modelling and drug screening studies.

#### T2 and ADC of MRI Reflect Maturation of Tissue-engineered Cartilage Subcutaneously Transplanted in Rats

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In cartilage regenerative medicine, autologous chondrocyte implantation (ACI) has been applied clinically for partial defects of joint cartilage or nasal augmentation. To make treatment with ACI more effective and prevalent, modalities to evaluate the quality of transplanted constructs noninvasively are necessary. In the present study, we compared the efficacy of several noninvasive modalities for evaluating the maturation of tissue-engineered cartilage containing biodegradable polymer scaffold. We first transplanted tissue-engineered cartilage consisting of human auricular chondrocytes, atelocollagen gel and a poly-L-lactic acid (PLLA) porous scaffold subcutaneously into the back of asthymic nude rats. Eight weeks after transplantation, the rats were examined by MRI, X-ray and ultrasound as noninvasive modalities. Then, the excised constructs were examined by histological and biochemical analysis including toluidine blue staining, content of glycosaminoglycans and ELISA of type II collagen. Among the modalities examined, transverse relaxation time (T2) and apparent diffusion coefficient (ADC) of MRI showed quite a high correlation with histological and biochemical results, suggesting that these can effectively detect the maturation of tissue-engineered cartilage. Since these noninvasive modalities would realize time-course analysis on the maturation of tissue-engineered cartilage, our present study would provide substantial insights to improve the quality of tissue-engineered cartilage, leading to improvement of the quality and technique in cartilage regenerative medicine.

#### A Scalable Bioreactor System for Stem Cell Cultivation on Nanofiber Scaffolds

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Nanofiber scaffolds for neural regeneration are able to provide physical guidance for neuronal tissue. Therefore the use of such nanofiber mesh as scaffolds for neural stem cells (NSC) is a promising strategy to mimic the natural cell niche, contributing to cell attachment and orientation, as porosity allows for effective nutrition and oxygen diffusion. Functionalized materials can provide additional specific cell response or function and tissue organization. Cell based therapies usually require substantial number of cells, but as NSC are relatively rare, *ex-vivo* cultivation is usually required prior to transplantation.

A novel stirred “plate and frame” bioreactor, able to accommodate a nanofiber scaffold for the production of neural tissue constructs, was designed as a scalable system for NSC expansion and differentiation. Computational fluid dynamics analysis suggested that a volume of 30 mL and agitation rates above 45 rpm promote efficient mixing. A human NSC line (ReNcell VM) was cultivated in the bioreactor, containing aligned polycaprolactone nanofibers, functionalized with adhesion motifs. A 7-fold increase in cell number was achieved after 10 day culture, with a uniform expansion of the cells along the nanofibers and a maximum non-toxic lactate concentration of 5 mM. Expanded cells were successfully differentiated into neurons and glial cells as evaluated by immunocytochemistry. These results are promising for scaled up production of aligned cells for stem cell biology research, regenerative medicine or other biomedical applications.

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#### From Synchrotron Radiation to the most recent breakthroughs in Clinical Regenerative Dentistry

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In recent years there has been an increasing interest in a novel approach to evaluate human bone biopsy specimens by means of synchrotron micro-tomography (SCT). Using SCT, bone regeneration subsequent to grafting hosting sites with different types of bio-materials (with or without stem cells seeding) is recently explored. Evaluation of the amount of bone formed is usually based on 2-D histomorphological data obtained from one or several histological sections.

If the regenerative potential of neighboring tissues with different morphology (alveolar process, unmineralized extracellular matrix involvement, regenerated vessels, etc.) on a defect or space to regenerate is not clearly verified or unknown, 3-D analyzing methods like high resolution SCT are indicated to explore the dynamic and spatial distribution of regenerative phenomena in such complex atomic structures.

Moreover the use of advanced techniques like phase contrast tomography (PCT) and holotomography (HT) allow to visualize components with low attenuation coefficient, like blood vessels.

In the present lecture the most recent breakthroughs in Clinical Regenerative Dentistry will be shown, demonstrating the unique capabilities of the SCT in offering not only an advanced characterization of different biomaterials (to understand the mechanism of their biological behavior as bone substitute) but also to investigate the growth kinetics of regenerated bone in different dental implants retrieved from humans.

Implant survival, bone regeneration, graft resorption, neo-vascularization and morphometric parameters (including anisotropy and connectivity index of the structures) were evaluated by microCT and HT at different times from implantation or grafting in human bone defects.

#### Development of Biomimetic Microengineered Hydrogel Fibers for Tendon Regeneration

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Musculoskeletal diseases are one of the leading causes of disability worldwide. Tendon injuries are responsible for substantial morbidity, pain and disability. Tissue engineering strategies aim at translating tendon structure into biomimetic materials. The main goal of the present study is to develop microengineered hydrogel fibers through the combination of microfabrication and chemical interactions between oppositely charged polyelectrolytes. For this, methacrylated hyaluronic acid (MeHA) and chondroitin sulfate (MeCS) were combined with chitosan (CHT). Hydrogel fibers were obtained by injecting polymer solutions (either MeHA or MeHA/MeCS and CHT) in separate microchannels that join at a y-junction, with the materials interacting upon contact at the interface. To evaluate cell behavior, human tendon derived cells (hTDCs) were isolated from tendon surplus samples during orthopedic surgeries and seeded on top of the fibers. hTDCs adhered to the surface of the fibers, remaining viable, and were found to be expressing CD44, the receptor for hyaluronic acid. The synthesis of hydrogel fibers crosslinkable through both physical and chemical mechanisms combined with microfabrication technology allows the development of biomimetic structures with parallel fibers being formed towards the replication of tendon tissue architecture.

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#### Development of Novel Biodegradable and pH-sensitive Nanocarrier based on Self-assembling Polypeptides

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Polymeric nanoparticles have shown promising potential as carriers for drug delivery. The structural stability of delivery vehicle and effective release of encapsulated therapeutic drugs are crucial for drug delivery system. In this study, the biodegradable pH-sensitive nanoparticles composed of natural polypeptides and calcium phosphate (CaP), have been developed. We utilized two different amphiphilic sequences, poly(ethylene glycol)400-acetyl linkage- poly(L-glutamic acid)- poly(L-histidine)-poly(L-leucine) and LyP1-poly(ethylene glycol) 1100-poly(L-glutamic acid)- poly(L-histidine)-poly(L-leucine), to self-assemble into nanoparticles in aqueous phase. The biostable nanoparticles provide three distinct functional domains: the hydrated PEG outer corona for prolonging circulation time, the anionic PGlu shell for CaP mineralization, and the protonation of PHis shell for facilitating anticancer drug release at target site. The active targeting ligand, LyP-1, is served to bind to lymphatic endothelial cells in tumor for the reduction rate of metastasis. The resulting mineralized Dox-loaded particles (M-DOX NPs) with negative charge ( $-21.9 \pm 1.6$  mV) have a smaller size  $179.4 \pm 33.9$  nm at pH 7.4, but particles at pH 5.0 have a doubled size ( $291.2 \pm 25.1$  nm) and positive charged ( $21.7 \pm 2.1$  mV), implying the protonation of poly-histidine. From the release profile, M-DOX NPs effectively reduce the leakage at physiological pH value comparing to DOX NPs, and both nanoparticles facilitate the encapsulated drug release at acidic condition. The biocompatible pH-sensitive drug carriers can effectively release anti-cancer drug in acidic condition to obtain sustained controlled release as promising carriers for anti-tumor drug delivery.

#### Implementation of Nanorough Surface Treatments to Improve Bone-Anchored Hearing Aid Integration

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Bone-anchored hearing aids (BAHA) are used in patients with hearing loss that cannot be resolved using typical air-conduction hearing aids. These devices work using sound processors mounted outside of the skull that transmit amplified sound into an implant (often titanium screws) embedded into the mastoid, which conducts sound waves directly to the cochlea, bypassing the external and middle-ear pathology. In percutaneous implants, up to 17% of patients have adverse skin reactions around the implant despite strict hygiene practices, causing revisional surgery and possible explantation of the device. This is particularly problematic with pediatric patients, who also experience implant failure (up to 15%) due to inadequate osseointegration. For children, whose hearing cognition and speech/language development are in a critical stage, failure rates up to 37% with up to 25% requiring implant explantation. To address this problem, we investigated the use of nano-featured surfacing for these implants to both reduce infection and inflammatory responses while improving osseointegration. Nanomaterials have been found to have a profound effect upon cell-material interactions, including the prevention of bacterial proliferation and biofilm formation as well as heightened mammalian cell growth for tissue regeneration. To implement this, we employed ion-beam assisted deposition



(IBAD), a vapor deposition process which produces extremely well-adhered and dense films, to impart nanorough features onto titanium which we previously found to reduce bacterial adhesion and improve osteoblast proliferation. With varied IBAD parameters we have demonstrated topographical control of these surfaces and observed changes in bacterial and osteoblast adhesion and integration.

#### National Institute of Dental and Craniofacial Research Support for Research Training in Tissue Engineering and Regenerative Medicine

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The National Institute of Dental and Craniofacial Research (NIDCR) has primary responsibility for meeting the biomedical and behavioral workforce needs in dental, oral, and craniofacial health research. NIDCR research training programs span the career stages of scientists. Support is provided for basic and translational research training and career development activities employing bioengineering approaches for the reconstruction, remodeling, repair and regeneration of the oral and craniofacial tissues damaged as a result of disease or injury. NIDCR supports individual fellowships (F30, F31, F32), and research career development (K08, K23, K25), research career transition (K99/R00), and research career enhancement (K18) awards. Individuals eligible for support include: (1) predoctoral students pursuing a PhD or dual DDS/DMD-PhD degrees; (2) dentists earning a PhD; (3) individuals with clinical degrees (e.g., DDS, DMD, MD) seeking protected time for research career development; (4) postdoctoral students (e.g., individuals with DDS, DMD, MD, PhD) pursuing research training; (5) individuals with quantitative (e.g., mathematics, statistics, economics, computer science), imaging, informatics, physics, chemistry and engineering backgrounds seeking to integrate their expertise with NIDCR-relevant research; and (6) mid-career or senior scientists seeking to enhance their research program in new areas of research or research methodology, with a focus on behavioral and social sciences, and genetic and genomic research. NIDCR also supports institutional research training programs and supplements to grants to enhance diversity in the research workforce. Individuals from underrepresented groups in biomedical and behavioral science are encouraged to apply for NIDCR research training and career development awards.

#### Bone Tissue Engineering for Cleft Lip and Palate Patients

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Cleft lip and palate (CLP), affects the alveolar bone in the great majority of the cases, and the reconstruction of this defect still represents a challenge in the rehabilitation of these patients. The gold standard in alveolar bone reconstruction is autogenous bone grafts. However, these surgical procedures may be subjected to complications such as donor area morbidity, post-surgical reabsorption and infections. Researchers have been focusing on the development of bone tissue engineering strategies with minimal or no donor site morbidity for bone grafts. In order to use non-invasive source of stem cells, we have used dental pulp stem cell (DPSC) obtained from deciduous teeth of 5 CLP patients to make bone tissue engineering (clinical trial). These cells, through flow cytometry analysis, were mainly positively marked for five mesenchymal stem cell antigens (CD29, CD90, CD105, CD73, CD166), while negative for hematopoietic (CD45) and endothelial cell marker (CD31). After induction under appropriate cell culture conditions, these DPSC were capable to undergo chondrogenic, adipogenic

and osteogenic cell differentiation, as evidenced by immunohistochemistry. We also demonstrated that DPSC together with a biomaterial composed of collagen and hydroxyapatite lead to bone tissue reconstruction in alveolar cleft defect of these patients. In conclusion, we showed that DPSC have phenotypic and behavior characteristics similar to other adult stem cells in concern to bone tissue engineering and they can be used associated with biomaterial to perform alveolar bone tissue engineering for these patients open new avenues to perform their treatment removing the pain of bone donor area.

#### Ultrathin Collagen/Silk Fibroin/Gelatin Film for Corneal Endothelial Cells Regeneration

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Biomaterial is a crucial factor for construction of artificial corneal equilibrium in tissue engineering because it can serve as a supporter and a microenvironment. In addition, incorporating physical, chemical, and biological conditions to guide cells into functional tissues via cell adhesion, migration and differentiation is important. We focus on silk and collagen for corneal endothelium due to possible efforts in tissue engineering field. Herein, we designed optically transparent collagen/silk fibroin/gelatin film, as an alternative scaffold source for bioengineering cornea because of facing formidable challenges to make novel scaffolds. Silk from *B. mori*, collagen from duck feet and gelatin were used to fabricate the ultrathin and transparent films. The morphological and structural properties were analyzed by FESEM, FTIR, contact angle, etc. *In vitro* biological compatibility was studied such as morphology, initial attachment, proliferation, mRNA expression, and proteins related functions on culture primary corneal endothelial cells. Collagen and gelatin are not affected in transparency but these materials affected thickness of fabricated films. Any significant differences on initial attachment, proliferation, and well-expressed their functional proteins which are regulated functions of corneal endothelium showed on each different films. Overall, the results suggest fabricated C/SF/G equilibrium offer good environment and become a proper delivery that can be able to replace worldwide limited number of donor cornea as high quality alternative for corneal tissue expansion and transplantation. This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government (MEST) (NRF-2012M3A9C6050204) and BK21 PLUS.

#### Influence of Chm-I Knockout on Ectopic Cartilage Regeneration and Homeostasis Maintenance

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Ectopic ossification of mesenchymal stem cell (MSC) regenerated cartilage have greatly restricted its application in repairing subcutaneous cartilage defects. Different from MSCs, chondrocytes can maintain stable ectopic chondrogenic phenotype, which was speculated to be related with anti-angiogenic factors such as Chondromodulin-I (Chm-I). Therefore, elucidating the influence of Chm-I on characteristics and functions of chondrocyte as well as ectopic cartilage regeneration and its stability may help to solve the problem of MSC ectopic ossification. The current study demonstrated Chm-I knockout did not obviously influence articular cartilage development *in situ*. However, native articular cartilage from Chm-I knockout (KO) but not wild type (WT) mice showed obvious ossification after subcutaneously implanted into nude mice for 16d. Interestingly, cell morphology, cartilage ECM expression, and pellet culture demonstrated that Chm-I knockout had no obvious influence on phenotype, function, and chondrogenic ability of chondrocytes *in vitro*, except cells in WT group proliferated a little faster than those in KO group. Nevertheless, Chm-I knockout directly interfered with *in vivo* ectopic cartilage regeneration when chondrocytes were subcutaneously injected into nude mice with matrigel. Moreover, Chm-I knockout obviously compromised ectopic stability of *in vitro* regenerated cartilage after subcutaneous implantation. These findings indicated that Chm-I was an indispensable factor for ectopic cartilage regeneration and the maintenance of cartilage homeostasis,

which may provide a clue for solving the stability problem of MSC regenerated cartilage in ectopic niche. In addition, this study also provides a novel model based on tissue engineering strategy to properly evaluate the function of other targeted genes.

### Notch Inhibition Regulates Neuronal Differentiation of Human Pluripotent Stem Cells

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Highly pure sources of neurons are desired in many regenerative medicine applications, including formation of neural tissue constructs. Identification and perturbation of relevant signaling pathways present during neural development can lead to more efficient and accelerated *in vitro* differentiation protocols. Notch signaling is critical throughout development and promotes glial populations over neuronal cell types. Thus, our hypothesis was that inhibition of Notch during stages of *in vitro* neural differentiation could increase the neuronal population. Human induced pluripotent stem cells (iPSCs) were seeded in mono-layer and cultured up to 17 days in neural induction medium initially with SMAD inhibition to bypass neural rosette formation. Retinoic acid and purmorphamine, a sonic hedgehog agonist, were added starting at day 7 for neuralization. A downstream Notch inhibitor, DAPT, was added at different concentrations and time points throughout differentiation. Neuronal populations increased in the presence of DAPT observed by increased  $\beta$ -Tubulin class III ( $\beta$ -TubIII) expression and decreased Pax6 expression compared to groups without Notch inhibition.  $\beta$ -TubIII expression was observed as early as day 7 and increased throughout the duration of the differentiation with presence of DAPT expediting the emergence of the  $\beta$ -TubIII phenotype. The number of  $\beta$ -TubIII positive cells present was dependent on the duration of Notch inhibition with continuous DAPT treatment resulting in the most pure neuronal populations. In addition to timing, DAPT-induced promotion of neuronal differentiation was dependent upon cell density. These results indicate Notch signaling is an important regulator of iPSC neural differentiation that can be used to promote neuronal phenotypes.

### Encapsulated Tumoral Cells for Bioprocessing

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Encapsulation of single cells within semi-permeable biodegradable shell is an attractive procedure for many biomedical and biotechnological applications, including bioprinting and regenerative medicine. The use of cationic polymers, such as poly-L-lysine (PLL), for encapsulation has been of great interest as gene/drug delivery systems, therapeutic applications and recently for the electrostatic stabilisation of bioinks and tissue engineering. The encapsulation of single viable osteosarcoma cells (U2OS) within PLL shells with preservation of long-term cell functioning was established by evaluating different concentrations of the poly-cation. The cell viability was assessed using MTT and Live/dead assays, the cell morphology and polymer uptake was evaluated through confocal and TEM microscopy. Four poly-cation concentrations were evaluated: 400  $\mu$ g/ml, 200  $\mu$ g/ml, 100  $\mu$ g/ml and 50  $\mu$ g/ml. The best cell survival was obtained for the two lower concentrations. Encapsulated cells with these two polymer concentrations were able to recover and self-renew within three days post-encapsulation, achieving after one week <70% of cell viability in contrast to <10% survivability level achieved for the highest PLL concentration. By confocal and TEM, the formation of the initial PLL capsule was confirmed as well as the polymer vacuolisation. Polymer endocytosis was seen after 4 hours to three days of encapsulation. These results indicate that the increase in cell viability and preservation of cell function after PLL-encapsulation are closely related to the polymer concentrations and further cell-internalisation. Thus, engineering a protective cell surface coating without causing damage to the cell membrane or death is possible by controlling the poly-cation concentration.

### Bi-content Micro-collagen Chip Innovates Contraction-based Biomechanical Readout for Phenotypic Drug Screening with Expanded and Profiled Targets

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Phenotypic screening regains new momentum in pharmaceutical industry owing to its exceeded success over target-based screenings. Most phenotypic screenings rely on nonspecific biochemical readout regarding cellular viability which hampers the discovery of novel druggable mechanism of actions (MOAs). Here we present Contractility-based bi-Content micro-Collagen Chip (3CChip), which innovates cellular contractility as a biomechanics-related readout for drug screening for the first time. As a proof-of-concept study, the fibrosarcoma cell line HT-1080 comprising tumor model is investigated in parallel to primary cardiac fibroblasts comprising fibrosis model. Bi-content analysis on cell contractility and viability of the same sample is achieved by gel area calculation (imaged by iPhone and analyzed by homemade software) and Alamar Blue measurement respectively. Molecular investigation on cellular response upon drug administration revealed that the label-free contractility-based analysis might facilitate discovery of extended drug hits, in particular targets involved in cytoskeleton, cell adhesion or cell migration, which are difficult to be detected by traditional cell viability assays. Six typical readout patterns of drug response are summarized according to relative positions of contraction/viability curves and drug targets are profiled into three categories (biomechanical, biochemical and house-keeping) by 3CChip, which would benefit subsequent target identification. The simple-to-use and effective 3CChip offers a robust platform for micro-tissue-based functional drug screening and we envision that the biomechanical phenotype based 3CChip will excite great interest in the context of preclinical pharmaceutical research and synergize with the rapid growing field of pharmacogenomics.

### Acute Mitochondrial Dysfunction in Cartilage Following Mechanical Injury

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Mitochondrial (MT) dysfunction mediates the pathogenesis of many complex diseases. Cartilage trauma can initiate chondrocyte death and matrix degeneration leading to osteoarthritis (OA), however the mechanisms are not fully understood and the role of MT in early OA pathogenesis is unclear. Our goal was to evaluate MT dysfunction in the peracute response of cartilage to mechanical injury.

Cartilage explants were impacted using validated injury model, then divided for use in 3 assays; 1) MT function was assessed by microscale respirometry using a Seahorse XF24 analyzer for a total of 245 minutes. Baseline oxygen consumption rate (OCR) was measured, then respiratory control was assessed by sequential addition of MT inhibitors. 2) MT membrane potential was determined by the intensity ratio of a polarity-insensitive to a polarity-sensitive fluorescent MT probe (MitoTracker Green:TMRM) and 3) chondrocyte viability was evaluated by live/dead staining on confocal microscopy.

Baseline OCRs were higher in control versus impacted samples. Impacted explants displayed impaired respiratory control, with a 61% (43–71) decrease in maximum respiratory capacity. Cell viability decreased by an average of 20% (5–38) in injured samples and impact resulted in a loss of MT potential, with a 34% (3–54) decrease in fluorescent intensity ratio.

MT dysfunction is a peracute response of chondrocytes to injury. Impact resulted in decreased basal respiration and reduced maximal respiratory capacity, indicating likely inhibition of electron transport in the respiratory chain. This model allows kinetic monitoring of MT function in whole cartilage and the opportunity to test drugs to prevent/reverse MT dysfunction.

### Effect of Local Delivery of VEGF-loaded Hydrogel on Bone Healing in Rat Medication Induced Osteonecrosis Model - A Pilot Study

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**Objectives:** Zoledronic acid (ZA), a potent bisphosphonate is used in bone depleting conditions like metastatic bone cancer, Paget's disease and osteoporosis. However, ZA is known to cause a debilitating side-effect in jaw bones termed medication-related osteonecrosis of jaws (MRONJ), especially after surgical manipulations. A significant pathogenic mechanism involved is aberrant angiogenesis, with reduction of VEGF levels both locally and in systemic circulation. This study aims to evaluate the effect of locally delivered VEGF-loaded Hydrogel in a rat MRONJ model.

**Methodology:** Sprague Dawley rats were exposed to weekly, intraperitoneal ZA administration over three weeks, and the maxillary molars were extracted to induce MRONJ. Immediately following extraction, hydrogel containing VEGF was placed in the tooth socket. ZA was continued until the rats were sacrificed. Gross healing was assessed over 4 weeks followed by sacrifice and harvesting of the maxillae. MicroCT was carried out to evaluate socket bone healing and histological evaluation was performed on decalcified paraffin sections.

**Results:** On gross evaluation, control maxillae showed significantly altered bone wound healing with signs of bone necrosis. In contrast, complete wound closure was evident in maxillae which received VEGF hydrogel. MicroCT confirmed significant bone destruction in the control group and normal bone healing in the VEGF hydrogel group. Also, initial histological evaluation showed signs of osteonecrosis including sequestration and diminished vascularity in the no gel controls compared to the gel group.

**Significance:** Locally delivered VEGF-containing hydrogel could be a valuable approach in preventing medication-induced osteonecrosis in patients who require dental manipulation while on anti-resorptive medications.

### Comparison of Tenogenic Differentiation of Equine Bone Marrow-, Adipose-, and Tendon-derived Stem Cell Seed Decellularized Tendon Constructs in a Dynamic Bioreactor

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Tendon is an important target for regenerative medicine in horses due to its frequent involvement in injury in performance horses. Tendon healing under normal conditions is a slow process resulting in mechanically inferior extracellular matrix, often leading to re-injury of the tendon. Evidence suggests that treating tissues with a poor healing ability, such as tendon, with mesenchymal stem cells (MSCs) improves healing via the ability to self renew, supply growth factors, and release immunomodulatory cytokines. MSCs exhibit phenotypic heterogeneity based on the type of donor tissue used, and the efficacy of cell-based treatments may be improved by optimizing cell source. In order to evaluate the relative regenerative potential of equine bone marrow (BM), adipose (AD) and tendon (TN)-derived MSCs, this study applied a previously used bioreactor protocol. Cells were expanded in monolayer prior to seeding on decellularized tendon scaffolds (DTS), and then placed in a bioreactor designed to mimic the native biophysical environment of tendon. It was hypothesized that of these cell sources, TN MSCs would exhibit the most tenocytic phenotype in response to an 11-day conditioning period involving cyclic mechanical stimulation. BM, AD and TN MSCs all integrate into DTS, express tenocytic marker genes, and increase construct failure stress in response to cyclic mechanical stimulation in a bioreactor. TN cells proliferate most rapidly, express more SCX, COL-I and COMP, most efficiently deposit GAG, and increase construct failure stress. The results suggest that TN MSCs possess a predisposition to form tendon and are thus ideal for cell-based regenerative medicine therapies.

### An *In Vitro* Method to Measure Antigenicity of Acellular Matrices

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**Introduction:** Acellular animal derived tissue matrices are commonly used for tissue regeneration and repair. However, implantation of animal derived products in humans always invokes an immune response. A mild response may be inconsequential, and even beneficial in some cases, but an excessive response can prevent suitable regeneration or repair and cause additional damage. Given that, it's important to accurately characterize the immunogenicity of acellular matrices. Here, we describe an *in vitro* method to measure antigenicity of various tissue matrices using animal serum.

**Materials and Methods:** Sub-optimally processed acellular dermal matrices were implanted into rat and primate models. Serums were collected from the animals at different timepoints for antibody titer measurements. High titer serum was then used in an ELISA assay to measure the antigen contents of different acellular matrices, which were also implanted in animal models.

**Results:** Both immunized rat and primate serum can be used to measure different antigenicity levels between completely and incompletely processed matrices as well as in various tissue matrices. The antigenicity levels measured by the *in vitro* assay also correlate with *in vivo* performances of the different matrices; those containing high or low antigen contents also induced high or low antibody titers respectively when implanted *in vivo*.

**Discussions and Conclusions:** We have developed an *in vitro* method that accurately measures antigenicity levels in tissue matrices which can be used to aid in the selection of available tissue products and/or development of new products.

**Disclosures:** All authors are employees of Acelity.

### Two Novel Approaches to Assess Repair after Laryngeal Nerve Injury in a Canine Model

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Injury to the recurrent laryngeal nerve (RLN) results in vocal fold immobility, dysphonia, and potentially life-threatening airway obstruction with major consequences for speech, mobility and quality of life. Outcome measures to determine the degree of reinnervation after nerve repair in a widely accepted canine model of RLN injury are sparse. We describe two novel methods for longitudinal evaluation of innervation to the laryngeal abductor and adductor muscles. Eight dogs were anesthetized and repeated bursts of transient hypercapnia (60 seconds with end tidal CO<sub>2</sub> of 90 mmHg) used to stimulate abductor function and glottic opening. A laryngeal mask airway was used in combination with high speed video endoscopy to evaluate laryngeal function. Transient hypercapnia produced a highly repeatable response with 43–55% increase in glottic area ( $p < 0.001$ ) within 10s of end of the hypercapnic burst. In a second study, transection-coaptation of the RLN was performed in 6 dogs. Six months after injury, the amplitude of the evoked compound motor action potential (CMAP) was determined at the vocalis muscle using a minimal invasive technique. CMAP amplitude correlated closely with the muscle weight and muscle fiber diameter of the intrinsic laryngeal musculature (both  $R^2 > 0.7$ ). These approaches allow longitudinal assessment of the response to regenerative therapies to promote repair of the RLN.

### Bevacizumab Can Promote Chondrogenesis in Osteoarthritis Rabbit Model

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Osteoarthritis (OA) is one of the leading degenerative diseases associated with aging, obesity, and sports injuries. The inhibition of angiogenesis may promote the cartilage generation as a developmental mechanism in endochondral ossification. Bevacizumab (Avastin<sup>®</sup>), a humanized monoclonal anti-VEGF antibody, is known as an anticancer agent that inhibits angiogenesis. In this study, we used Avastin<sup>®</sup>, under the concept of drug repositioning strategy, to explore its bioapplication through the action of inhibiting angiogenesis and evaluate its effect in cartilage regeneration. We conducted *in vitro* molecular analysis as well as *in vivo* studies to confirm the effect of bevacizumab on cartilage regeneration. Chondrocyte and bone marrow derived mesenchymal stem cells (BM-MSCs) from rabbit, and the human umbilical vein endothelial cells were treated with Avastin<sup>®</sup> in a dose-dependent manner and we evaluated their proliferation rates and chondrogenic gene expression levels through the proliferation assay and real-time PCR, respectively. Pellet culture was also performed to investigate their chondrogenic differentiation potentials. Histological analysis of pellets revealed that bevacizumab could promote chondrogenesis of BM-MSCs. *In vivo* studies, intra-articular injection of Avastin<sup>®</sup> in OA rabbit model induced the cartilage regeneration in comparison to the control group. Therefore, our data suggest that bevacizumab as a promising drug that can promote chondrogenesis of BM-MSCs.

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#### Theoretical Framework to Enhance External Solute Transport to Cells in Radial Flow Packed-bed Bioreactors for Bone Tissue Engineering Based on a Stationary 2D Transport Model

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**Aim:** Perfusion of osteogenic cells in 3D annular porous constructs in radial flow packed-bed bioreactors (rPBBs) holds promise to engineer clinical-scale bone tissue substitutes. rPBB design seldom accounts for the effect of resistance to solute transport in pores from bulk medium to cell surface on oxygen and nutrients delivery. In this paper, a 2D axisymmetric transport model for rPBBs with coaxial inlet and outlet ports is proposed to investigate the importance of external transport resistance in rPBBs for bone tissue engineering (TE).

**Methods:** Steady-state outward flow and oxygen transport was described by means of Navier-Stokes equations in the inner hollow cavity and peripheral annulus, the Darcy-Brinkman equation in the porous construct, and by the diffusion/dispersion-convection equation. Michaelian kinetics were used for oxygen consumption. Oxygen mass transfer coefficient at medium/cell interface was estimated for a bed of Raschig rings transport-equivalent to typical scaffolds for bone TE. Equations were numerically solved for conditions used for bone TE.

**Results and Conclusions:** The model predicts that external transport resistance limits oxygen delivery to cells with high metabolic activity, but limits glucose delivery only under hypoxic conditions. High radial perfusion velocities effectively enhance transport and reduce external resistance at high cell concentrations and/or metabolic activity, as occurring when cells proliferate and differentiate. We conclude that the model may help design rPBBs enabling physiological osteogenic cell culture in large bone constructs.

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#### Anti-inflammatory Drug-loaded Pcl Mats for the Treatment of Immune-mediated Liver Diseases

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Recently, electrospinning technology has been widely used as a processing method to make fibrous scaffolds in tissue engineering and drug delivery systems because of its unique features, such as easy fabrication process and high surface area. The unique features of electrospun fibers lead to ease of fabricating the delivery vehicle in the required form and efficient drug release. For these reasons, the present study was designed to develop anti-inflammatory drug-loaded fibrous mats that can release anti-inflammatory drug effectively for treatment of immune-mediated liver disease using electrospinning technology. In the present study, anti-inflammatory drug-loaded methoxy poly(ethyleneglycol)-b-poly( $\epsilon$ -caprolactone-co-L-lactide) (MPEG-PCLA) fibrous mats were prepared by electrospinning. Then, three-dimensional fibrous structures in anti-inflammatory drug-loaded MPEG-PCLA mats were confirmed by scanning electron microscope (SEM). Anti-inflammatory drug loaded in MPEG-PCL was determined for a long period of time *in vitro* by a high performance liquid chromatography (HPLC). In experimental liver damages in mouse models, anti-inflammatory drug loaded MPEG-PCLA mats significantly reduced liver fibrosis. In the present study, it is achieved to fabricate anti-inflammatory drug-loaded fibrous mats that can release anti-inflammatory drug effectively *in vitro* and *in vivo*. This approach, anti-inflammatory drug-loaded fibrous mats, will be useful for development of new treatment models toward immune-mediated liver disease.

#### *In Vitro* (static Vs. Dynamic) and *In Vivo* Bone Formation Potential of Surface Calcium Phosphate-coated Polycaprolactone Scaffold and Polycaprolactone/Bioactive Glass Composite Scaffold

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Polycaprolactone (PCL) -based composite scaffold containing 50 wt% of 45S5 Bioglass<sup>®</sup> (45S5) or strontium-substituted bioactive glass (SrBG) particles were fabricated into scaffolds by means of additive manufacturing for bone tissue engineering purposes. Additionally, PCL scaffolds were surface coated with calcium phosphate (CaP) to enable further comparison of the bone formation potential of PCL (control) vs. PCL/CaP-coated vs. PCL/50-45S5 vs. PCL/50-SrBG scaffold. The loading of 50 wt% BG particles into the PCL bulk did not affect the scaffold overall morphology but decreased the scaffold's compressive Young's modulus. CaP coating on PCL scaffold had negligible effect on the scaffold's porosity and compressive Young's modulus. Under accelerated degradation condition *in vitro*, the degradation rate follow the order of PCL/50-SrBG > PCL/50-45S5 > PCL/CaP = PCL. When immersed in culture media, BG dissolution ions (Si<sup>4+</sup> and Sr<sup>2+</sup>) were detected up to 10 weeks in the immersion media and surface precipitates were formed on both PCL/50-45S5 and PCL/50-SrBG scaffold surfaces, indicating good *in vitro* bioactivity. *In vitro* cell studies were conducted using sheep bone marrow stromal cells (BMSCs) under non-osteogenic or osteogenic conditioned media, and under static or dynamic culture environment. All scaffolds were able to support cell adhesion, growth and proliferation. Under a dynamic culture environment, the rate of cell growth, proliferation and osteoblast-related gene expression was enhanced across all scaffold groups. Subcutaneous implantation of PCL/CaP, PCL/50-45S5 and PCL/50-SrBG scaffolds, with or without cells show no bone formation after 8 or 16 weeks, indicating that all scaffolds did not have osteoinductive properties.

#### Development of an Equine Model of Talocrural Post-Traumatic Osteoarthritis

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Approximately 1 in 10,000 people/day sprain their ankle. At least half will sustain cartilage damage that can lead to posttraumatic osteoarthritis (PTOA) years or decades later. Prevention requires immediate intervention; therefore understanding early cellular events after cartilage injury is critical to developing effective therapies. No *in vivo* ankle PTOA models exist. Our objective was to validate an equine talocrural cartilage injury model.

For *ex vivo* studies, bilateral tali were harvested from 8 horses. Cartilage was injured using a spring-loaded impactor. Force, displacement, cartilage thickness and impact area were measured to fully characterize the mechanical insult. Live-imaging with multiphoton microscopy (MPM) was used to quantify chondrocyte viability and matrix microcracks. Histology was performed to assess morphology and proteoglycan content.

For *in vivo* studies, horses were anesthetized and the impactor was positioned perpendicular to the talar articular surface under arthroscopic guidance. Three impacts were delivered per talus. Horses were euthanized at 6 or 12 weeks. India ink was used to mark matrix cracks. Impact/control osteochondral blocks and synovium were harvested for histopathology.

Varying impactor spring tension and tip geometry resulted in stresses from >160 to <40 MPa. Impact time averaged 1.7 ( $\pm 0.04$ ) ms. MPM of impacts revealed dead cells and microcracking. *In vivo*, cartilage injury resulted in joint inflammation, matrix damage and early histopathologic OA lesions within 6 weeks.

This model, which creates lesions similar to early OA in the human ankle, will allow investigation of early cellular events that initiate PTOA and preclinical testing of preventative and regenerative therapies to minimize OA development.

#### Cryogel Scaffolds: An Appropriate Scaffold Structure for Promoting Bone Regeneration

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This study compared cryogel and hydrogel scaffolds for the treatment of critical-sized bone defects. Cryogels are unique scaffolds which possess a sponge-like, macroporous structure and increased mechanical stability compared to hydrogels. Materials (silk, chitosan-gelatin and N-vinyl-2-pyrrolidone (NVP)) were tested as both hydrogels and cryogels in parallel for scaffold characterization (i.e. swelling, ultimate mechanical properties, cyclic loading, etc.).

Based upon our characterizations, silk cryogels proved to be superior in nearly every aspect when compared to silk hydrogels and the other cryogel materials. SEM imaging showed silk cryogels contained the highest average pore diameter ( $76.61 \pm 37.08 \mu\text{m}$ ), approaching published values of other bone engineering scaffolds. For uniaxial compression testing, the silk cryogel scaffolds exhibited an average peak load of  $1.61 \pm 0.68 \text{ kPa}$  which was significantly higher than the silk hydrogels, and 50% higher than NVP and chitosan-gelatin cryogels. The silk cryogels proved to be more mechanically stable than all other tested scaffolds and had a peak load close to that seen in new bone formation. Cyclic compression testing revealed all cryogels to have minimal stress relaxation, with silk cryogels having 5.95% over a 20 cycle span.

This study demonstrated that all materials evaluated were capable of being processed into a cryogel structure, and that these scaffolds have significantly different properties from traditionally formed hydrogel materials which make them appealing for bone engineering applications. Studies are ongoing to explore cellular infiltration, as well as doping the silk cryogels with osteoinductive materials to increase calcification potential, modify cellular response, and enhance mechanical strength.

#### Inverted Scaffold: Rapid Cell-Molding with a Bubbled Medium

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This study proposed an inverted-scaffold method for rapid molding of cells *in vitro*. A general scaffold is a cell-adherent structure with a desired shape for the fabrication of three-dimensional cellular tissue. On the other hand, in this study, the inverted scaffold is defined as a temporary cell-avoid structure, similar to a mold, which is maintained until seeded cells adhere. The goal of this study was to mold over-confluent-dense cells with the inverted-scaffold of bubbled medium. Firstly, Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum and 1% penicillin-streptomycin solution (168-23191) (Wako, Osaka, Japan) was prepared. Mouse skeletal myoblasts (C2C12) were suspended into the medium. The cell suspension with a cell density of  $5 \times 10^5$  or  $5 \times 10^6$  cells/mL was prepared and poured into a 35-mm cell culture dish (Falcon 353001) (Corning, Corning, NY). The poured suspension was bubbled by pipetting. After 2-h cultivation, the morphology of cells on the surface of dish bottom was monitored by a phase-contrast microscope. As a result, bubbles covering over the cell suspension was generated and aligned into a hexagonal pattern. The cells were trapped into the medium along the gap between bubbles. After the cultivation, the cells adhered on the bottom of dish with the hexagonal pattern. Especially, in case of high cell-density, the cells were stacked in vertical direction. In conclusion, the experiment demonstrated the molding of high-density cells by the inverted-scaffold of bubbled medium.

#### The Biopen for Direct Bio-printing of Stem Cells to Regenerate Articular Cartilage

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**Background:** Osteoarthritis (OA) is a progressive disease with no known cure. A significant proportion of OA arises from articular surface injuries experienced at an early age. Current surgical and biological techniques fail to demonstrate the ability to regenerate hyaline cartilage *in vivo* that maintains its property in the long term. 3D printing is an exciting new technology to deliver tissue-engineering techniques in orthopaedics.

**Methodology:** We have created a custom-designed hand-held extrusion ink-jet printing device (Biopen) that allows the simultaneous and co-axial extrusion of biomaterial scaffold (Bio-ink) and cultured cells directly *in vivo* into the defect that needs to be repaired. The co-axial printing allows the cell laden hydrogel Bioink to be printed as a core, encapsulated and protected by a photocrosslinkable hydrogel as a protective shell for 3D constructs. Metacrylated Hyaluronic acid hydrogel (HA-GelMa) has been chosen as Bio-ink, and Adipose Derived Stem Cells (ADSC) as cell source for cartilage regeneration. *In vitro* tests of survival (Live-Death stain), and differentiation toward chondrogenic pathway (Histology, IHC for Collagen type I and II, SOX9, Aggrecan) have been performed as preliminary data to support *in vivo* application.

**Results:** ADSC printed in HA-GelMa *in vitro* using the Biopen i) remain viable after printing and ii) are able to differentiate toward hyaline cartilage in chondrogenic differentiation media.

**Conclusion:** Live cell printing is a feasible means of delivering cells which retain the capacity to undergo functional differentiation, and is an innovative solution to deliver tissue-engineering techniques to the surgeon for their direct use *in vivo*.

#### Encapsulation of Simvastatin in Chitosan-Coated Alginate Microspheres

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The inflammatory response induced by implant wear particles still limits joint replacement longevity. Simvastatin was shown to have

anti-inflammatory properties, to stimulate bone growth, and to decrease bone loss. It may therefore be of interest for the modulation of the inflammatory response induced by implant wear particles. Alginate has been considered for drug delivery applications due to its biocompatibility, non-immunogenicity, and biodegradability properties. However, limitations include the difficulty to encapsulate hydrophobic drugs, as well as drug leakage due to the large porosity of alginate microspheres. Therefore, the objective of this study was to optimize simvastatin encapsulation in alginate microspheres by: 1. Increasing its solubility using hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ CD); and 2. Improving its encapsulation using dextran sulphate and chitosan. The highest complexation of simvastatin with HP- $\beta$ CD (98%) was obtained with the highest HP- $\beta$ CD:simvastatin molar ratio (10). Simvastatin complexed with HP- $\beta$ CD was then mixed with 3% (w/v) alginate solution with or without 1.5% (w/v) dextran sulphate, and was extruded in gelation medium made of 5% (w/v) CaCl<sub>2</sub> with or without 0.1% (w/v) chitosan. Results showed negligible simvastatin encapsulation in alginate microspheres with or without dextran sulphate. However, the encapsulation efficiency increased up to  $10.5 \pm 0.7\%$  when coating alginate microspheres with chitosan ( $p < 0.001$ ), and up to  $14.3 \pm 1.1\%$  when reinforcing chitosan-coated alginate microspheres with dextran sulphate ( $p < 0.001$ ). The latter increase was likely due to electrostatic interactions between dextran sulphate and chitosan, resulting in a denser coating. Future studies include testing higher polymer concentrations to possibly further increase the encapsulation efficiency.

#### Adhesion Prevention after Laminectomy using Silk-polyethylene glycol (PEG) Hydrogel

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Laminectomy is a common operation in spine surgery to reduce spinal cord and nerve pressure, but the effect can be compromised by scar tissue formed in the spinal canal and adhered to the dura, causing recurrence of low back pain. In the present study, biodegradable silk-PEG hydrogels were evaluated for adhesion prevention after laminectomy in New Zealand rabbits, using non-degraded expanded polytetrafluoroethylene (ePTFE) membranes and saline as controls. At 2,4,6,8,10 weeks post surgery, 10 rabbits in each group (totally 30) were sacrificed and the dural scar adhesion on the defects was examined by visual (Rydel-Baalaz) and histological scoring (modified Nussbaum). No significant difference among the three groups was observed within 2 weeks. Silk fully degraded within 6 weeks, leaving a gap separating the scar tissue and the dura mater. Severe dural scar adhesions formed in the saline control group after 8 weeks, while no or mild adhesion was observed in the ePTFE membranes and silk-PEG hydrogel samples, demonstrating the efficacy of silk-PEG hydrogels in preventing epidural adhesion after laminectomy. Human dermal fibroblasts (HS-865-SK cells) were cultured in the silk-PEG hydrogel extracts and on top of gel surfaces. Compared to tissue culture plate (no silk) controls and sonicated silk hydrogels (no PEG), the proliferation of fibroblasts in both conditions was significantly reduced initially due to the local release of the PEG, while cell growth resumed after 120 h. These results suggest that the surface properties of the hydrogels and local, temporal release of PEG account for adhesion prevention *in vivo*.

Withdrawn

#### Bridging the Osteochondral Gap in Mandibular Condyle Reconstruction with Multiphasic 3D Printing

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Individuals afflicted with temporomandibular joint disorders experience a reduced ability to perform the most basic human functions such as chewing and talking. In advanced disease states, total joint replacement is often necessary to improve range of motion and minimize pain. Current surgical treatments use either autogenous grafts or alloplastic implants to replace the condyle and ramus of the mandible. Although these interventions aid in improving joint function, a tissue-engineering strategy may be useful to expand the range of treatment options and offer an approach that not only restores functionality but also facilitates regeneration of the diseased tissue [1]. Toward this objective, 3D printing technology was used to fabricate patient-specific constructs with precise osteo- and chondroinductive regions to facilitate the formation of osteochondral tissue similar to that found in the mandibular condyle. The osseous region of the scaffold was composed of polycaprolactone and hydroxyapatite nanoparticles to promote bone formation and was manufactured via fused deposition modeling, while an extrusion-based method was used to print the chondral region, which was composed of hyaluronic acid hydrogel and decellularized cartilage. Patient data were obtained from computed tomography images to create implants with correct anatomical shapes, and pore architectures were designed with solid modeling software. Future work will be performed to observe the efficacy of the implants to promote osteochondral differentiation of human bone marrow stem cells *in vitro*.

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#### Generation and Functional *in vitro* Evaluation of Textile-templated Anisotropic Elastomeric Scaffolds for Cardiac Tissue Engineering

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Myocardial infarction and end-stage heart failure represent some of the major life threatening pathologies. Access to heart transplantation as a viable therapeutic modality is limited due to the shortage of donor organs and to the potential for rejection of the donated organ. Current studies focus on creating biomimetic cardiac patches to restore cardiac function, by repairing and/or regenerating the intrinsically anisotropic myocardium. In our studies, we used conventional textiles made of cotton or polyester yarns as anisotropic target templates for electrospinning anisotropic three-dimensional scaffolds from elastomeric polycarbonate-urethane (PCU, Bionate<sup>®</sup>). This approach yields surface topographies and mechanical properties that are significantly different compared to scaffolds electrospun from the same materials onto conventional 2-D isotropic flat-targets. These anisotropic textile-templated scaffolds exhibit mechanical properties comparable to those of a human heart and support the adhesion and proliferation of H9C2 cardiac myoblasts as well as primary cardiomyocytes, and guide the assembly of these cells into an anisotropic cardiac tissue-like conformation *in vitro*. On the polyester, but not the cotton- or flat surface-templated scaffolds neonatal rat cardiomyocytes exhibited prolonged and synchronous spontaneous contractility of the entire engineered construct for 10 days *in vitro* at a near physiologic frequency and strength. In summary, with this approach we demonstrate a simplified, straightforward approach for engineering bioactive anisotropic cardiac patches, based on a combination of bioengineering and textile-manufacturing techniques in concert with nano-biotechnology based tissue-engineering stratagems. This is an efficient and cost-effective approach to engineering 3D anisotropic, elastomeric PCU scaffolds that might serve as cardiac patches *in vivo*.

#### Fabrication of Silk Fibroin Cryogels with Osteoconductive Additives for Bone Tissue Regeneration

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Silk fibroin (SF) macroporous scaffolds were fabricated through cryogelation; a process involving controlled freezing to form ice crystals at subzero temperatures, resulting in a highly connected pore network upon thawing. SF was chosen because it is biocompatible, durable, and easily manipulated material. Cryogelation of aqueous SF solutions lead to the formation of a spongy scaffold with an interconnected pore network and average pore size of  $70 \pm 36 \mu\text{m}$ . The cryogels swelled to 68% of their total swelling capacity in 20 minutes while achieving equilibrium within 24 hours. The SF cryogels obtained by this process were highly elastic and could bear strains up to 50% without crack initiation, exhibiting an average modulus of  $101.3 \pm 34.9 \text{ kPa}$  (similar to that of new bone formation). Comparatively, SF hydrogel counterparts cracked at similar strains and had a significantly lower modulus of  $41.7 \pm 8.8 \text{ kPa}$ .

Additionally, Manuka honey (MH) and bone char (BC) were added as dopants (1 and 5% w/v) to SF cryogels to enhance bioactivity and scaffold calcification, respectively. MH, being more hydrophilic, increased the swelling ratio of SF cryogels at lower concentrations while BC decreased it. Both MH and BC decreased the mechanical strength of the SF cryogels with subsequent increases in pore size. Further studies are underway to investigate cellular infiltration and calcification activity in these scaffolds.

The excellent mechanical strength of SF cryogels, coupled with mild processing methods and highly porous structures, indicates their potential as scaffolds for bone tissue engineering, especially spongy bone.

#### Three-Dimensional Orientation Mapping and Quantification of Collagen Fibers in a Hormone-Sensitive Breast Tissue Model

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**Objectives:** There is a lack of metrics that is representative of localized collagen fiber organization in a three-dimensional (3D) manner. This study aimed at developing 3D orientation based biomarkers to better understand the matrix's role in breast epithelial morphogenesis.

**Methods:** Human breast epithelial T47D cells were seeded in a collagen gel, and treated with physiological concentrations of 17 $\beta$ -estradiol alone, and 17 $\beta$ -estradiol in combination with either promegestone or prolactin for two weeks. Two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) images were acquired to resolve the epithelial morphology and collagen fiber structure, respectively. A newly developed 3D weighted orientation vector summation technique was used to acquire the pixel-wise orientation of the collagen fibers. Based on its orientation, the 3D directional variance was extracted to assess the localized fiber organization for different treatments.

**Results:** There were significant differences in the 3D directional variance among the three treatment groups in regions surrounding epithelial structures ( $p < 0.05$ ). The 17 $\beta$ -estradiol plus promegestone treatment had the highest mean directional variance, and the 17 $\beta$ -estradiol alone group had the lowest one. These results revealed that hormones induced epithelial cells to reorganize surrounding collagen fibers and suggest that hormone-controlled changes of fiber arrangements contribute to the distinct morphology of epithelial structures (prolactin and budding, promegestone and branching).

**Significance:** The 3D directional variance is sensitive to subtle changes in the extracellular micro-environment and elucidates reciprocal cell-matrix interactions in the context of numerous applications involving the study of normal and/or diseased tissue development.

#### Liver-on-a-chip for *In Vitro* Alcoholic Liver Fibrosis Model

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Alcoholic liver disease (ALD) is the abundant liver failure in worldwide and it starts with steatohepatitis and fibrosis. Oxidative stress generated during alcohol metabolism activates hepatic stellate cells (HSCs) and extracellular matrix is over accumulated. Though the process is required for wound healing process, the liver may go to cirrhosis or hepatic cancer when the fibrosis is progressed to irreversible state. Meanwhile, *in vivo* disease models are widely used for the drug evaluations but they have ethical and economical problems remaining. Many *in vitro* models are developed for alternatives of animal models, but existing 2D cultured models have limitations in simulating *in vivo* system. Here, we devised spheroid-based microfluidic chip as the 3D liver-on-a-chip for *in vitro* alcoholic liver fibrosis model. We cultured primary hepatocytes HSCs in the fluidic chip integrating concave micro-wells and gave interstitial level of flow using osmotic pump. We did alcohol treatment to the spheroids with ethanol-containing medium (60  $\mu\text{l/ml}$ ) for 48 hours, observed the structural changes and conducted functional assessments during the ethanol treatment and recovery process after inducing injury. The injury group showed different morphologies and reduced liver specific functions indicated by CYP450 reductase expression and albumin secretion compared to the control group. Also the fibrotic structures of collagen type I covering the whole surfaces of the spheroids are observed in the injury group when they were not in the mono-cultured spheroids and the spheroids under static state culture.

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#### Application of Microfluidic Device for Neural Tissue Engineering

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In organ replacement design Controlled cellular orientation, proliferation and differentiation is of great importance. In the present study, we have focused on the production of microgrooves on the biodegradable polymer surfaces, the influences of physical guidance cues on directing alignment, and spatial control of neural differentiation of human Induced pluripotent stem cells (hiPSCs) *in vitro*. Scanning electron microscopy (SEM) imaging, quantitative real time RT-PCR (qPCR) and immunocytochemistry were used for the analysis of stem cell differentiation and the expression of neuronal-specific genes. Our observations confirmed the differentiation of hiPSCs to neuronal cells on microfluidic device and suggesting their potential applications in the regeneration of the damaged central nerve system. According to our findings, we may conclude that the microfluidic system reported could be used as a potential cell carrier for neuronal tissue engineering and a combination of this system and hiPSCs may have a potential application in nerve regeneration research and therapy.

#### Paracrine Effects of Human Urine-derived Stem Cells in Treatment of Female Stress Urinary Incontinence in a Rodent Model

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Human urine-derived stem cells (USCs) are a novel stem cell source that can be easily obtained from voided urine specimens. The objective of this study was to determine the efficacy of USCs in facilitating functional recovery via paracrine actions in a rat model of female stress urinary incontinence (SUI) induced by simulated childbirth injury. Female Sprague-Dawley rats were randomized into 3 groups and underwent vaginal distension followed by intraperitoneal delivery of USCs (VD+USCs), VD and saline, or sham VD and saline. One week after injury, treatment efficacy was assessed by measurement of leak point pressure (LPP) and histological analysis of the urethra. Quantitative data were analyzed by one-way ANOVA and Holm-Sidak posthoc tests with  $p < 0.05$  indicating a significant difference. After 1 week, LPP was significantly reduced after VD + saline compared to sham VD but not in VD + USC animals. On semiquantitative histological analysis, there was no significant difference between both the striated and smooth muscle components of the urethral sphincter of VD+USC animals compared to sham VD animals; VD+saline animals, however, demonstrated significant muscle fiber attenuation and disorganization. Additionally, elastin fibers in VD+USC animals were long, thickened, and mostly oriented compared to the short, thin, and disoriented fibers in VD + saline animals. No implanted USCs were found in the region of urethral orifice, suggesting recovery of urethral function in animals treated via paracrine effects of USCs. USCs facilitated recovery from simulated childbirth injury and represent an attractive, alternate stem cell source for the treatment of female SUI with potential for non-local delivery.

#### Bio-inspired Carbonate Apatite Bone Substitute for Alveolar Bone Regeneration

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In the area of dentistry, bone regeneration is very crucial issue to enhance quality of life. Inter-connective porous composite with Ca/P ratio closer to the original bone with the ability to promote bone formation and facilitate mass transfer management in the body is a critical aspect in the design of bone substitute. In this study, we aimed to investigate the clinical success rate of carbonate apatite (CHA) composite developed by dissolution-precipitation method to the

generation of alveolar bone. The drop-wise addition of ortho-phosphoric acid to aqueous suspension of calcium hydroxide was used to synthesize apatitic nanoparticles at room temperature inside denaturalized collagen system. Physical, chemical and mechanical properties were investigated before the series of *in vivo* and clinical studies. It was confirmed from the experiment that the reaction successfully resulted b-type carbonate apatite when it was analyzed from both XRD patterns and FT-IR spectra. The composite was also known to have Ca/P ratio 1.70 closer to human bone. The diametral tensile strength of the composite was 5.52 MPa. The results of clinical case study showed that carbonate apatite composite enhanced bone regeneration in the case of periodontitis with bone resorption and the case of intentional re-implantation during periodontal surgery. In conclusion, the carbonate apatite composite has been a promising technology to provide treatment to regenerate bone for periodontal as well as oral and maxillofacial surgery.

#### Silk Fibroin Structure Affected by Environment

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Silk protein is reported to have different crystalline structures such as Silk I and Silk II crystalline structures. The environment of silk fibroin solution, including the shearing strength, pH, concentration of metal ions, concentration of the silk fibroins and types of metal ions, were investigated before [1]. In this study, we aimed to understand the mechanism of the structure changes due to the hydrophilic-lipophilic environment.

To understand the mechanism, small molecules with different hydrophilic-lipophilic balance (HLB) were used to investigate the effect on the structures. The results show that with the hydrophilic-lipophilic balances changing high, the structure of silk fibroin would change to silk I structure while not to the silk II structure, which may be used to prepare a kind of liquid crystal state of silk fibroin.

Our results suggest the law of the interaction between the HLB values of molecule and the structure of silk fibroin protein. The rule may play a part in other protein interaction with different small molecule. The small molecule with low HLB value may induce protein to form more  $\beta$ -sheet while not  $\alpha$ -helix. The results indicated that this type of control may support to fine tune the requirement for engineering appropriate surface reactions, and providing desirable characteristic silk fibroin biomaterials in a specific situation.

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#### Design and Biosynthesis of Dynamic Protein Biomaterials

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Protein polymers provide a uniquely tunable family of functional biomaterials, that can mimic natural protein structure and function or be designed de novo for new, non native needs. Such polymer systems are fully degradable, towards the goals of regenerative medicine, biocompatible and tunable in terms of degradation rate and functions. With advances in genetic and protein engineering it is possible to construct genes encoding protein polymers composed of naturally occurring or non-natural amino acid sequences. However, the diversity of these types of protein polymers pursued for biomaterials-related needs remains limited. Therefore, robust high-throughput synthesis and screening methods are needed to expand options for protein-polymers for a range of applications. Here, I will present a combinatorial library approach combined with high throughput screening to select dynamic protein polymers with unique stimuli responsive properties, including tensile strength and



adhesion. Based on this study, new understanding of sequence-function relationships with this family of protein polymers is gained and can provide a guide to future library designs.

### Rapid and Economic Production of Bioprinted hMSC Constructs

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Bioprinting is a rapidly emerging biofabrication platform that is propelling the field of tissue engineering forward. A lack of advancement in cell expansion technology has created a bottleneck in tissue engineering processes, where access to high volume cell sources requires researchers to perform laborious cell expansion. We have optimized procedures for generating hMSCs at speeds and volumes that enable rapid experimental execution, yielding at least  $1 \times 10^8$  cells within 7 days, and up to  $1 \times 10^9$  cells in 2 weeks, thereby enabling a new era of billion-cell tissue engineering experimentation. Here, we show that "Ready-to-Use" hMSCs, i.e. cryopreserved cell stocks vialled at  $1 \times 10^7$  hMSCs/vial, can be directly thawed into Aggrewells<sup>TM</sup> to rapidly generate  $1 \times 10^4$  aggregates, or can be expanded for 1 passage prior to spheroid formation to obtain  $10 \times$  more aggregates within a week. hMSC spheroids generated in such a manner were more economical than conventional expansion and spheroid generation methods with  $>34\%$  reduction in cost of raw materials and  $>75\%$  reduction in culture time. Rapidly-formed hMSC spheroids maintained their critical quality attributes (CQA): the ability to fuse together into higher-order macroscopic tissue structures such as rings or rods. To bioprint hMSCs, spheroids were suspended in 2% alginate or PEGDA/4% alginate and 3D-printed using a BioBot 1 3D bioprinter to form complex 3D tissue architectures. The printed constructs demonstrated good viability, as assayed by live/dead staining. These feasibility studies open the door for rapid and economic biofabrication-based tissue engineering studies using a highly clinically-relevant stem cell source.

### Application of Fluorescent Materials using Silk Fibroin from Transgenic Silkworm

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Silk fibroin (SF) is a natural polymer widely used and studied for diverse applications in the biomedical field. Recently, genetically modified silks were reported to have been produced from transgenic silkworms. While these transgenic silkworms have served as an efficient method for producing fluorescent SF fibers, they are limited to certain industries, such as textile manufacturing, and to medical applications. To adapt transgenic silkworms for more practical purposes in other fields, it has been suggested that various forms of fluorescent SF should be developed. In the present study, we prepared various fluorescent materials using silk fibroin from transgenic silkworms. To investigate the biocompatibility of silk scaffolds *in vivo*, they were implanted subcutaneously in rats. The fluorescent color was observed *in vivo*, and the silk scaffolds were found to be biocompatible. These results indicate that fluorescent SF made by transgenic silkworms can be applicable as biophotonic biomaterials.

### Novel Biomimetic Bioreactor for the Recellularisation of Decellularised Hearts Under Synchronised Electrical and Mechanical Stimulation

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Cells and animal organs are currently used for drug testing. Unfortunately, neither of these fully recapitulate human physiology, as

would be ideal for drug testing. With the arrival of human induced pluripotent stem cells, the concept of manufacturing miniature human organs for pharmaceutical testing has surfaced. Cells are typically cultivated in flasks or dishes in CO<sub>2</sub>-incubators, which provide the cells with optimal temperature, humidity and pH for growth. However, for the development and culture of organoids, culture in passive flasks in incubators may be insufficient.

We have developed a bioreactor for the culture of cells seeded onto intact decellularised heart tissue under biomimetic mechano-electrical stimulation. The bioreactor is comprised of a custom designed fluid path, regulating electrical circuits and computer software. The heart is perfused through the aorta, mechanically stimulated by inflation and deflation of a balloon within the left ventricle, and electrically stimulated using platinum wires attached to the heart. The custom designed software allows the configuration of the device and records all data. The device monitors temperature and humidity within the culture chamber, the electrical activity within the organoid and both the perfusion and stimulation pressure of the heart.

Preliminary cell experiments have demonstrated the feasibility of growing cell-seeded decellularised hearts in the bioreactor. Currently, we are working on seeding human iPS-derived cardiomyocytes on decellularised hearts in the bioreactor to generate mature contracting cardiomyocytes in an appropriate 3D environment.

### Development of Tissue-engineered Cardiac Patch based on Silk fibroin

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A novel tissue-engineered cardiac patch composed of silk fibroin is under development using the electrospinning method. In this study, silk fibroin from *Bombyx mori* was combined with several functional materials such as polypeptides, polyurethanes and polycarbonates to enhance the functionality like elasticity and cellular affinity. Each silk-based cardiac patch was characterized by scanning electron microscopy, solid-state NMR, compressive elastic modulus and permeability. Moreover, these patches were implanted to inferior vena cava of the dog.

In the case of silk-polyurethane composite patches, it was found that the elongation ratio increased with the increase of polyurethane ratio. Moreover, no stenosis was confirmed around the implanted patches six month after the implantation. The histological stain evaluation revealed that the endosporium was formed on the implanted patches 10 months after the implantation. Furthermore, the patches were found to be gradually degraded by macrophages.

This study will give basic information on the development of new silk-like materials for tissue-engineered cardiac patches.

A detailed structural and physical analyses of the silk fibroin-polyurethane patches will be presented.

### A Comparative Study between Nonmulberry Silk Fibroin Blended PVA and PCL Nanofibrous Matrices: Potential ECM for Bone Tissue Engineering

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Nanofibrous scaffolds of mulberry silk fibroin are widely accepted as bone scaffolds. Nonmulberry silk protein fibroin of *Antheraea mylitta* has better mechanical properties over mulberry silk with improved cell proliferation due to built-in RGD sequences, which could unveil new horizons for its application in regenerative

medicine. Both poly-vinyl alcohol (PVA) and poly ( $\epsilon$ -caprolactone) (PCL) are reported as osteogenic materials. In this work, electrospun nanofibrous nonmulberry A. mylitta fibroin silk (2 wt%) matrices of PVA and PCL (10 wt%) are evaluated biophysically and for cyto-compatibility using osteoblast like cells (MG 63). The nanofibrous scaffolds of pure PVA and PCL serve as control. FTIR reveals effective blending of silk fibroin with PVA and PCL. Thermal stability of the nanofibrous matrices are measured from melting temperature ( $T_m$ ) using DSC. The average fibre diameter - from SEM images - of PVA (2SF/PVA) and PCL (2SF/PCL) reveals  $177 \pm 13$  and  $152 \pm 18$  nm respectively. Mechanical strength of the nanofibrous matrices increases with addition of fibroin; 2SF/PCL (4.9 MPa, 29.4%) exhibits greater plasticity over 2SF/PVA (4.8 MPa, 14.5%). Confocal microscopy of the constructs after 14 days *in vitro* culture exhibits more intensive and homogenous distribution of actin filaments for 2SF/PCL along with better ALP activity over 2SF/PVA over 3 weeks of cell culture. Taken together, the results indicate that both the blends of PVA and PCL with nonmulberry fibroin are suitable as scaffold for bone tissue engineering; while 2SF/PCL blends hold a statistically significant advantage over 2SF/PVA.

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### Transplantation of Adipose Tissue Stem Cells and Olfactory Ensheathing Cells: A Combinatorial Approach for Spinal Cord Injury Regenerative Medicine

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Spinal Cord Injury (SCI) is a highly incapacitating condition for which there is still no treatment. Cellular therapies are seen as a promising tool for SCI repair. From the myriad of cells currently being tested, Adipose Tissue Stem Cells (ASCs) secrete factors that promote neuronal proliferation; in addition, Olfactory Ensheathing Cells (OECs) are characterized by promoting neuronal regeneration and guidance. Interestingly, our group has previously shown that OECs present positive paracrine interactions with ASCs [1]. In this sense, we intend to combine ASCs and OECs, testing their effects on: 1) an *in vitro* model of axonal regeneration, using dorsal root ganglia explants (DRGs); and 2) an *in vivo* hemisection rat model of SCI. DRGs were co-cultured with ASCs/OECs during 4 days, after which axonal outgrowth was measured. *In vivo*, 80,000 cells of a mixture of ASCs and OECs was injected rostral and caudally to the injury site. Animals without treatment were used as controls. The behavioral evaluation included the BBB test, activity box test and average velocity on water. *In vitro* results revealed that OECs alone or in coculture with ASCs promote the highest increase of neurite outgrowth. Moreover, rats treated with ASCs/OECs presented improved locomotor scores on the BBB scale. Histological analysis revealed a lower inflammatory profile of animals treated with the transplantation of cells. No major alterations were observed in astrogliosis and axonal organization. Overall, these results support the use of these cells as a therapeutic approach for SCI.

### Correlations between Physical-chemistry Properties and *In vitro* Characterization of Nanoliposomes (based on Natural Lecithins)/chitosan Blend Scaffolds for Tissue Engineering

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Biomaterials-based scaffolds combining bioresorbability and bioactivity are often used in tissue engineering to optimize cell adhesion and growth proliferation. Due to their functional properties, chitosan-based scaffolds are one of the most promising materials in the field of regenerative medicine.

In this work, we developed for the first time, in our knowledge, nanoliposomes functionalized chitosan films based on soya and salmon lecithins and determined the physical-chemistry characteristics and their effect on human mesenchymal stem cells (hMSCs) in function of lecithin concentration in chitosan films.

The physical-chemistry properties were characterized by various measurements, such as water contact angle, swelling kinetic test, and Torsional Harmonic Atomic Force Microscopy analysis. For further underlying cell-matrix interactions, the effects of nanoliposomes compositions and concentration on cell adhesion, metabolic activity and proliferation were investigated using hMSCs obtained from fresh human umbilical cords.

In this work, a correlation between the modified physical properties (surface wettability, swelling ratio, roughness and local stiffness) of the chitosan thin films modified by adding of "green" nanoliposomes and cell adhesion/proliferation will be established. It's worth to note that none of the films induced any cytotoxic effect.

We will demonstrate that nanoliposomes /chitosan blend thin films, on certain concentrations, could offer good cyto-friendly functionalization for hMSCs, and can be used for tissue engineering applications.

### Thermoresponsive Drug Delivery Systems for Medical Devices with Shape Memory Effect

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Biodegradable polymers with shape memory effect present exciting possibility for development of less invasive smart medical devices. However, techniques used to fabricate the devices often require thermal processing, which prevents incorporation of thermosensitive bioactive agents.

The goal of the present study was to develop thermoresponsive drug delivery systems in form of coatings and microparticles, which could be utilized together with scaffolds exhibiting shape memory effect.

The polymers were composed of blocks of L-lactide (LA) and caprolactone (CL) with varying CL-block lengths. To study the release of growth factors, we decided to use Bovine Serum Albumin (BSA) as a model protein. BSA was incorporated into the polymers at concentration of 5 wt/wt% using water in oil emulsion technique. The emulsion was then utilized to fabricate thin polymer films and microparticles. Thermal properties were characterized using Differential Scanning Calorimetry and wettability using sessile drop technique. The release study was performed in Phosphate Buffered Saline (PBS) at 37 °C for a period of 35 days.

Presence of BSA increased crystallinity of the CL-fraction of the polymers. There was no burst release of BSA. After first day of incubation, the elution of BSA was faster in case of the polymer containing shorter CL-blocks and was equal to approximately 1% of initial load per day during first week of the experiment. This difference could arise due to lower crystallinity of the polymer and its higher hydrophilicity. Current work focuses on investigation of the effect of FGF and BMP release on biological response of MSC.

### Engineering Stem Cell Niche using Cell-interactive Alginate Hydrogels

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*In vitro* engineering of stem cell niche, a specific microenvironment where stem cells are located *in vivo*, has been recently spotlighted in tissue engineering approaches. One critical factor to engineer stem cell niche may include proper design of a scaffold that mimics *in vivo* microenvironments. In this study, we hypothesized that a control of cell-cell interactions within synthetic extracellular matrices (ECMs) could be essential to create hematopoietic stem cell (HSC) niche *in vitro*. Angiopoietin-1 (Ang1) plays a critical role in the maintenance of HSCs in bone marrow through binding to the

tyrosine kinase receptor (Tie2). Thus, alginate hydrogel was modified with Ang1 to induce interactions with Tie2 of HSCs. Alginate was chosen as a base synthetic ECM, as it has been often used as a tissue engineering scaffold. HSCs were cultured on Ang1-modified alginate gels without using feeder cells, and Ang1/Tie2 interactions appeared to be critical for maintenance of HSC quiescence. This approach may provide useful means to develop biomimetic systems in tissue engineering applications.

#### Modification and Characterization of Novel Di-functional Hyaluronic Acid using Michael's Addition and Click Chemistry

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Native extracellular matrices (ECM) has a complex structure, therefore supportive materials need to possess multiple functional groups in order to adequately mimic the ECM. Artificial ECM with multiple bioactive peptides will improve attachment and function of encapsulated cells in scaffold. Hyaluronic acid (HA) is an important component of the ECM in many tissues, and is a regulator of cellular differentiation, migration, proliferation and angiogenesis. To widen its biological effect, we modified hyaluronic acid with 2 independent functional groups (thiol and azide) for Michael's addition and click chemistry to promote the independent binding of 2 bioactive peptides. The functionalization HA backbone was characterized by FT-IR, <sup>1</sup>H-NMR, and TNB assay. The ability of the modified HA to gel by Michael's addition, and the mechanical properties were tested. The thiol group peak was detected at 2.8 ppm by NMR and TNB assay on HA with thiol and thiol/azide the azide peak was detected at 2096 cm<sup>-1</sup> by FT-IR in di-functional HA. Gelation occurred using a 1:1:2 ratio of 1%/2% di-functional HA with polyethylene glycol dimethacrylate. These results indicated that thiol and azide functional groups were presented on the novel modified HA, and reactive under certain conditions. Furthermore, those functional groups on HA are available to bind with different peptides to better mimic the native ECM on scaffold.

#### Non-invasive Longitudinal Evaluation of Bone Regeneration by Hybrid SPECT/Micro-CT Imaging for Tissue Engineering

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Recently, grand strides were made in the advancement of novel biomaterial-based implants for the treatment of bone fractures. The growing complexity of such implants requests for simultaneous progress in means to assess their efficacy as well as the course of induced bone regeneration in preclinical models. Micro-computed tomography (micro-CT) excelled as efficient technology for non-invasive evaluation of newly formed bone tissue with high resolution. However, micro-CT fails to reveal information on biological processes taking place during bone fracture healing on a spatiotemporal level. Single photon emission computed tomography (SPECT) on the other hand, due to its broad applicability and cost-effectiveness, would be a promising technique for following such biological processes with for nuclear imaging comparably high resolution (<1 mm) and with high sensitivity. Here, we apply multi-pinhole SPECT/micro-CT hybrid imaging to assess bone regeneration induced by modularly designed poly(ethylene glycol)-based hydrogels matrices that deliver bone morphogenetic protein to murine critically sized calvarial bone defects. By combining longitudinal micro-CT and multi-pinhole SPECT evaluations we determine within this synthetic hydrogel implant non-invasively the spatiotemporal progress of bone formation as well as bone remodeling. End point assessments by histological evaluation and high resolution mi-

cro-CT endorse the performance of this method for following as well as optimizing bone-forming biomaterials for tissue engineering approaches.

#### Composite Membranes for Treatment of Cutaneous Mold Infection

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Necrotizing invasive mold infections in soft tissues present a clinical challenge to treat and result in significant morbidity and mortality in an increasing population of patients with severe immunosuppression or trauma. Systemic antifungal therapy is often limited by fungal destruction of the local vasculature, preventing delivery of therapeutics to the site of infection. We have developed an injectable, biodegradable membrane to serve as both a local antifungal delivery vehicle as well as scaffold for regenerating cutaneous tissues. Previously, we had demonstrated that poly(glycerol sebacate) acrylate (PGSA) can be used for the controlled delivery of antimicrobial compounds. However, by introducing gelatin microparticles (GMPs), hybrid natural/synthetic material composite membranes can be synthesized that allow for pore formation upon GMP degradation. Uncrosslinked PGSA was loaded with the antifungal medication voriconazole (VRC) and mixed with gelatin microparticles (GMPs) to produce a liquid composite. This injectable solution was crosslinked *in situ* by exposure to blue light from a conventional dental lightemitting diode (LED) system. The physicochemical properties, degradation rate, VRC release rate, and its bioactivity against a pathogenic fungal isolate (*Aspergillus fumigatus* strain Af293) were evaluated. Pores within PGSA/GMP composite membranes were formed and increased in diameter over time after incubation in collagenase-containing media. VRC was successfully released at physiologically-relevant concentrations over 15 days, a clinically-relevant time span. The released VRC was capable of mitigating the growth of *A. fumigatus*. As an injectable membrane capable of the local release of bioactive antifungals, this therapy represents a paradigm shift in the treatment of cutaneous fungal disease.

#### Nylon 680 Scaffolding for Msc Colonization as Prior Step to Bioprinting in Dentistry

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The aim of our research was to 3D print scaffolds with different porosities from FDA approved nylon 680 using FDM conventional 3D printer and test these scaffolds for cytotoxicity and biocompatibility with human adipose tissue-derived stem cell which underwent osteogenic differentiation *in vitro*. Standard nylons have been tried in the past. The nylon 680 polymer is a pure polymer that is FDA approved. There are no additives used in the chemical manufacturing or extrusion processes. Print temperature is 238C and this nylon is also compatible with ethylene oxide and flash steam post processing. Its translucent and somewhat transparent properties allowed us visual inspection for defects.

For controlled porosity of scaffolds for FDM 3D print was used a free software - Google Sketch-up that was used for 3D modeling of the scaffolds of different porosity and STL export to our FDM machine (Felix 3.0) with highest accuracy of 50 micron printing. 3D printing with highest-50 micron accuracy often resulted in opaque material appearance. For slicing was used free software Slic3r. Morphology of scaffolds was evaluated with inversion microscope Zeiss Axiovert 100 and SEM (for which the scaffolds were gold-plated with JFC 1100 ions and analyzed by JSM 840

JEOL). Test of cytotoxicity and biocompatibility was evaluated according to ISO norm 10993-5. Obtained results proved that nylon 680 scaffolds were non-toxic and biocompatible with used stem cells with optimal porosity around 300 microns. After further testing they might be considered to be used in tissue engineering and regenerative medicine of hard-tissues.

#### Successful Clinical Application of a New Allograft Material for Delayed Fracture Healing in Two Dogs

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A 3-year-old, castrated male Pomeranian (case 1) and a 1-year-old male Pomeranian (case 2) were referred for delayed union fracture of left radius and ulna in both patients. In these cases, the fractures had been treated using external skeletal fixator in local animal hospital. But, they had been failed to normal fracture healing. Therefore, re-operation was performed and the fracture sites were fixed with 2.0 plate and screw. The fracture gap was filled with a new allograft material (C350C) obtained from beagle's femoral cancellous bone and 200 ul of matrigel containing 60 ug of recombinant human bone morphogenetic protein-2 (rhBMP-2). Radiographic assessment was performed after surgery. In case 1, on the radiographs, the fracture of radius and ulna were united at 7 weeks after re-operation and fracture lines were not observed at 12 weeks. In case 2, radiolucent lines between radius bone segments were remained and proximal segment of radius was beginning to bridge the distal ulnar segment at 2 weeks after re-operation. The fracture gap was more narrowed at 7 weeks. The proximal and distal radius bone segments were united completely at 12 weeks. In these two cases, the use of C350C, a new allograft material, promoted new bone formation, moreover, matrigel and low dose of rhBMP-2 enhanced new bone formation, too. Thus, it is thought that these materials are useful for successful delayed union healing.

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#### Peripheral Nerve Targeted Non-Viral Gene Transfer for Endogenous Stem Cell Recruitment

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**Objective:** To assess the feasibility of non-viral gene transfer for stem cell targeting in peripheral nerve regeneration in the rat major pelvic ganglion (MPG).

**Material and Methods:** Male Sprague Dawley rats (350 g, n = 12) MPGs were injected with 75 µl of plasmid expressing firefly luciferase driven by an enhanced CMV promoter (1 µg/ml). At 3, 7, 14, 21, and 35 days post-MPG injection, luciferin was administered prior to imaging with the Xenogen *In Vivo* Imaging System. Erectile function (EF) was assessed by intracavernosal pressure (ICP) in response to cavernosal nerve stimulation 2-weeks post-injection. Luciferase mRNA expression was assessed using qPCR. Histology was assessed with immunofluorescence.

**Results:** *In vivo*, trans-dermal MPG luminescence was detected by 3 days post-injection with continued expression up to 3 weeks in all 3 animals evaluated at that time point. No luminescence was detected by 5 weeks post-injection. qPCR of luciferase mRNA demonstrated expression in MPGs from animals injected with the luciferase plasmid but not in control animals with detectable amplification by 30 cycles. ICP measurements demonstrated no negative effect in EF in animals undergoing plasmid injection. Histology demonstrated luciferase expression in MPGs.

**Conclusions:** MPG directed non-viral gene transfer therapy is feasible and safe. Durable expression is achievable without injury to

the MPG as assessed by EF. Expression of plasmids can be monitored serially in real-time with non-invasive imaging. This study supports the investigation of non-viral gene therapies for nerve regeneration.

#### Uniform Microspheres with Antibiotics Fabricated by a Tapered Fluidic Device for Sustained Release

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Based on solid-in-oil-in-water (S/O/W) emulsification, we fabricated uniform poly( $\epsilon$ -caprolactone) (PCL) microspheres containing water-soluble antibiotics, such as tobramycin, vancomycin and gentamicin, using a fluidic device with a pristine or tapered glass capillary. Three types of antibiotic were dispersed in an organic solvent by ball-mill process earlier than microspheres preparation. The PCL organic solution containing the three types of antibiotic was served as the discontinuous phase into the fluidic device, where an aqueous phase containing surfactant introduced as the continuous phase. The release behavior revealed that the vancomycin and gentamicin were released from the uniform PCL microspheres up to approximately two months in a more sustained manner than tobramycin, which is because of the solubility difference of the antibiotics in water. The antimicrobial activities of three type of antibiotic released from the uniform PCL microspheres were evaluated using *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*).

#### Robust Myelin Formation in 3D Microengineered Hydrogel Co-cultures of Dorsal Root Ganglia Neurons and Schwann Cells

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The interaction between Schwann cells (SC) and neurons during axonal development and regeneration affects growth direction and myelination capability. Although the importance of utilizing 3D biomimetic microenvironments has been demonstrated, few studies have investigated SC/neuron interactions and myelination in 3D hydrogel co-cultures. We developed a novel *in vitro* 3D co-culture model that allows SCs and neurons incorporation in a unique constrained, polarized geometry by micropatterning cell-restrictive and cell-permissive hydrogels using digital projection photolithography. Utilizing this design, we encapsulated DRGs and SCs into a cross-linked dextran 3D culture system and investigated factors which led to the formation of myelin. We analyzed two culturing processes along with the influence of collagen on neuronal growth and myelination. We demonstrated that our 3-D co-culture setting provided us with aligned, highly fasciculated neuronal growth robustly ensheathed with compact myelin. The compact myelin structure was confirmed using transmission electron microscopy (TEM), while the volume of myelin formation and the ratio of myelin production to neuronal growth in 3D hydrogels were quantified with confocal microscopy of tissue constructs stained for P0 protein, myelin basic protein (MBP) and myelin associated glycoprotein (MAG). Ascorbic acid (AA) has been shown to be necessary for promoting SCs to generate myelin by enabling them to form a basal lamina in 2D cultures. We demonstrated that in 3D cultures, longer exposure to AA promoted a larger degree of myelination and, along with collagen I and III, resulted in maximum myelin generation. This platform provides a robust tool for drug development and myelination studies.

#### Photocrosslinkable Hyaluronan Hydrogels Incorporating Platelets Lysate for Periodontal Tissue Regeneration Exhibit Mitogenic and Anti-microbial Properties

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Guided Tissue Regeneration, as well as various combinatory therapies have been employed in the repair of periodontal defects with some success; however an optimal strategy capable of restoring the anatomy and functionality of the lost periodontal tissues is still to be achieved.

Platelet Lysate (PL) has great potential for tissue regeneration, as source of growth factors involved in essential stages of wound healing and regenerative processes such as chemotaxis, cell proliferation and differentiation<sup>1</sup>. Herein we report the development an injectable photocrosslinkable hydrogels, prepared from methacrylated Hyaluronic Acid (me-HA) and incorporating PL.

The photocrosslinking reaction, triggered after UV excitation of the photoinitiator Irgacure 2959, produced stable and versatile hydrogels. The viscoelastic properties and resilience to degradation by hyaluronidase of the hydrogels were enhanced with the incorporation of PL. Moreover, human Periodontal Ligament Fibroblasts (hPDLFs), either seeded or encapsulated into the hydrogels, showed enhanced biological performance, proportionally to the amount of PL incorporated. Additionally, the hPDLFs seeded on the surface, tend to migrate into the hydrogels incorporating PL. Remarkably, the PL provided antimicrobial properties against methicillin-resistant *Staphylococcus aureus*.

The unique mitogenic, chemotactic and prophylaxis-maintenance properties displayed by the photocrosslinkable HA-PL hydrogels here reported, makes them outstanding materials for tissue engineering approaches targeting various tissues, namely the periodontium.

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## 3D Bioprinting of Human Pluripotent Stem Cells for Tissue Engineering

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In recent years, the use of a simple inkjet technology for cell printing has triggered tremendous interest in the field of 3d biofabrication or organ printing. A key challenge has been the development of printing processes which are both controllable and less harmful, in order to preserve cell and tissue viability and functions. In this talk, we will present the development of a valve-based stem cell printer that has been validated to print highly viable and functional human embryonic stem cells (h-ESCs) and induced pluripotent stem cells (hiPSCs). 3D bioprinting based on both scaffold based (using different printable hydrogel materials) and scaffold-free (using hanging droplet technique) approaches will be discussed. This work demonstrates that the valve-based printing process is gentle enough to maintain stem cell viability, accurate enough to produce spheroids of uniform size and cell-containing hydrogel structures, and that printed cells maintain their pluripotency. In addition, our recent work on printing human stem cell derived liver tissues for animal-free drug testing applications will be presented.

## Reversible Swelling Step during Tissue Decellularization and its Effect on Cell Infiltration and Biodegradation

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Cells removal from xenogeneic-planar tissues has resulted in biologic templates with properties that promote regeneration of diverse tissues 1–2. The decellularization of bovine pericardial tissue (DBP) by nonionic detergent 4-octylphenol polyethoxylate (Triton X100, Tx) and Tx combined with reversible alkaline swelling (STx) produces gradients in the residual composition but preserves the anisotropy and the tensile properties<sup>3</sup>. In addition, STx method maintains the fibrous and laminar structure of the native pericardial tissue whilst opens the interfibrillar spaces<sup>3</sup>. This work compares the outcomes of the subdermal implantation (on Wistar rats) of biologic templates manufactured by the Tx and STx methods by means of histological analysis, in addition it explores the infrared thermography as noninvasive tool in the study of the performance of implants. H&E and Masson's trichrome staining revealed a lower density of mononuclear cells around STx-DBP than around Tx-DBP after 1-day implantation concurrently showing a higher infiltration of cells. Infiltrated cells into Tx-DBP showed a fibroblastic morphology after 7-days implantation. Focal vascularization was observed after 7-days implantation in STx-DBP, whereas for Tx-DBP, it was observed after 14-days. Degradation at 14 days was more pronounced in STx-DBP than Tx-DBP. A highest density of immune cells was observed around non-decellularized tissue and peracetic acid treated-tissue, which was maintained after 14 days. On the other hand, IR photographs revealed differences in emissivity and temperature as a function of implantation time. Finally, the increase of the interstitial spaces into decellularized pericardial templates produces an increased cell infiltration and accelerated biodegradation in a rat subdermal implantation model.

## Fabrication of 3d Porous Silk Scaffolds by Particulate (salt/sucrose) Leaching for Bone Tissue Reconstruction

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Silk fibroin is a biomaterial being actively studied in the field of bone tissue engineering. In this study, we aimed to select the best strategy for bone reconstruction on scaffolds by changing various conditions. We compared the characteristics of each scaffold via structural analysis using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), the swelling ratio, water uptake, porosity, compressive strength, cell infiltration and cell viability (CCK-8). The scaffolds had high porosity with good inter pore connectivity and showed high compressive strength and modulus. In addition, to confirm bone reconstruction, animal studies were conducted in which samples were implanted in rat calvaria and investigated by micro CT scans. In conclusion, the presented study indicates that using sucrose produces scaffolds showing better pore interconnectivity and cell infiltration than scaffolds made by using a salt process. In addition, *in vivo* experiments showed that hydroxyapatite accelerates bone reconstruction on implanted scaffolds. Accordingly, our scaffold will be expected to have a useful application in bone reconstruction.

## The Development of Large Scale and Medium Exchange System for Pluripotent Stem Cell Stirred Suspension Culture

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**Introduction:** The stirred suspension culture using a bioreactor system is an efficient method for the large scale expansion of human iPS cells. We showed the design of bioreactor equipped with delta shaped paddle impeller capable of cell aggregates formation at TERMIS-NA 2103. Two types of single use bioreactors (working volume 30 mL and 100 mL) were launched last year in Japan. At this conference, we show the results of culture by using the newly developed large scale bioreactor (working volume 500mL) and medium exchange system.

**Material and method:** The single cell of iPS cells suspension were prepared from 2D culture and inoculated into 100mL reactor. The cell aggregates in stirred suspension were collected and

dissociated into the single cells by using enzymes. The obtained single cells were re-inoculated into 500 mL bioreactor as scale up. We used the medium exchange system which equipped for stirring, temperature control and the tilting of reactor vessel for daily medium exchange operation.

**Results and discussion:** The large scale bioreactor using the delta shape paddle impeller enabled to create a lot of aggregates 200 to 300 micrometer diameter in the cultivation for 4 to 5 days. Furthermore the number of cells increased 5 to 10 times to the number of inoculated cells. Medium exchange process in large scale culture is labor-intensive and involves risk of contamination. We have developed a system capable of medium exchange safely and reliably in large scale culture.

### 3-D Bioprinting of Bone Constructs for Craniomaxillofacial Reconstruction

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The face is composed of a complicated underlying bony/cartilaginous framework that supports muscle, secretory organs, and skin/subcutaneous structures. In the restoration of large defects, autologous techniques (i.e. locoregional and free flaps) are limited in terms of the total tissue coverage they can provide and are associated with donor-site morbidity. In this study, we developed a new system based on computer-aided design/computer-aided manufacturing (CAD/CAM) and rapid prototype construction. An engineered bone construct was fabricated using our novel 3-D bioprinting system which simultaneously delivers a structural biodegradable polymer and a tissue-specific cell-laden hydrogel. Human-derived stem cells were mixed with the composite hydrogel along with poly( $\epsilon$ -caprolactone) (PCL) and printed on a sacrificial structure. At 1 day of culture, over 90% of cell viability within the printed bone structure was measured (n=3). After osteogenic differentiation for 28 days (n=5), Alizarin Red S staining indicated calcium deposition on the 3-D bone structure. However, the 3-D constructs without differentiation failed to show any calcium deposition when stained with Alizarin Red S. 3-D bioprinting system could generate 3-D freeform shapes with multiple cell types and biomaterials, resulting in various architectures that have the potential to replace human tissues. We demonstrate that the bioprinting can simultaneously deliver a supporting polymeric template and 3-D patterned deposition of cell-laden hydrogels in a precise manner. The printed tissue constructs are able to organize into mature tissues of their specific characteristics *in vitro* and *in vivo*.

### Bioluminescence Imaging for Live Cell Tracking in Tissue Engineering Applications

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Tissue engineered organs require effective and validated cell seeding protocols when translated clinically. To adequately design these protocols, simple, reliable and reproducible cell-tracking techniques are required. Techniques already available are often either invasive, overly labour intensive, not entirely specific or limited to analysis of a small segment of the graft. Bioluminescence imaging (BLI) is a well-established method of *in vivo* imaging that is commonly used in real-time analysis of disease and efficacy of drugs.

We determined the applicability of BLI as a method to track cells in bioreactor cultures and eventually *in vivo*, in the development of tissue engineering applications. Lentiviral transduction of various

cell types was performed using a transfer vector that constitutively expresses a fluorescent protein and luciferase. The BLI system was characterised through comparison to pre-established techniques (Alamar Blue<sup>®</sup> and CyQuant<sup>®</sup>). The potential of the system as a cell tracking method in tissue engineering applications was examined by tracking cells on both synthetic and biological tubular scaffolds in a closed bioreactor system and *in vivo*.

BLI was comparable to well established techniques for cell tracking *in vitro*. Viable transduced cells could be accurately detected and tracked when seeded in all conditions tested. In addition, BLI was shown to deliver information on cell distribution on the scaffold and could provide a comprehensive assessment of the cells over the entire duration of the experiment.

This is an effective, non-invasive and simple cell tracking method that is proven to be a valuable tool in tissue engineering of bioartificial complex organs.

### Effect of Silk/ $\beta$ -Tricalcium Phosphate Scaffolds for the Bone Tissue Engineering

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Silk is a potential material for biomedical applications because it has several attractive properties such as biocompatibility and biodegradability. However, it lacks certain of mechanical properties for bone replacement as a graft material. To increase osteoconductivity and osteoinductivity, we modified the mechanical properties and osteoconductivity of silk with  $\beta$ -tricalcium phosphate ( $\beta$ -TCP).  $\beta$ -TCP consists of good degradability, osteoconductive and minimal cytotoxicity. For this study, silk100, silk75/ $\beta$ -TCP25, silk50/ $\beta$ -TCP50 and silk 25/ $\beta$ -TCP75 scaffolds were chosen provided by CG-bio. We checked the physical and chemical characteristics of silk/ $\beta$ -TCP scaffolds using SEM, EDS, compressive strength, X-ray diffraction and FTIR, etc. Also, osteogenic differentiation of rabbit bone marrow mesenchymal stem cells (rBMSCs) cultured in scaffold was evaluated by using alkaline phosphatase (ALP), MTT, RT-PCR, SEM and alizarin red S red staining as an assay.

These results showed that silk25/ $\beta$ -TCP75 scaffold increased cell proliferation and differentiation of rBMSC *in vitro* for 1 to 4 weeks. Also, histology result from implantation showed consistency with the above results. Thus, silk25/ $\beta$ -TCP75 scaffold can be envisioned as an useful biomaterials for future bone regeneration applications.

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### Preparation of Keratin Nanofibers for Biomedical Application

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Keratin is the major structural protein contained in mammalian hair and nail. Because keratin molecules include specific amino-acid sequence (RGD, LDV, etc.) involved with cell adhesion, it has been attracted attention as a new material which can be applied to the scaffold for cell culture. In this study, keratin protein extracted from wool fibers was processed into nanofiber sheets having different fiber diameter and pore size in order to mimic the structure of extracellular matrix. The potential of keratin nanofibers as a scaffold material has been evaluated in terms of its physical and cytophilic properties.

Keratin protein in wool fibers was effectively extracted by oxidation method in about 40% yield. Spinning solution was prepared by dissolving keratin and PEG(MW:500000) to formic acid. Keratin/

PEG mixed spinning solution could be spun to the nanofiber in diameter range between 100–300 nm by electrospinning. Physical properties of keratin nanofibers such as fiber diameter and hydrophilicity were greatly affected by the content of PEG in the mixture. The rate of biodegradability and solubility of keratin nanofibers in aqueous solution were possible to control by moderate exposure to vapor glutaraldehyde. In order to evaluate cytophilic properties of keratin nanofibers, cell culture on them was carried out using mouse fibroblast. Fibroblast seeded on keratin nanofibers was well attached and proliferated. From these results, we conclude that keratin nanofibers have a potential to be able to apply to scaffold materials.

### Enhanced Cryosurvival of Human Red Blood Cells Mediated by the Application of Biomimetic Inorganic Nanoparticles

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Long-term storage of red blood cells (RBC) at ultra-low temperatures (cryopreservation) is needed to ensure a readily available, safe blood supply for transfusion medicine. As cryopreservation is traditionally accomplished using high concentrations of toxic agents (dimethyl sulfoxide), significant interest exists in the development of alternative biocompatible cryo-protectants, such as trehalose. In order to provide maximum protection, trehalose must be present on both sides of the cell membrane, yet it has very low permeability to the cell membrane; therefore research has focused on methods for delivering trehalose into cells; i.e. via the use of liposomes, biopolymers, electroporation, etc. So far, there has been no report on the utilisation of inorganic compounds for delivery of trehalose into cells.

The aim of the current work was to investigate for the first time the possibility of using inorganic compounds to load mammal cells with trehalose and to use colloidal suspensions of biomimetic apatite nanoparticles as trehalose carriers.

Results of the study showed that cryosurvival of RBC incubated in the apatite-trehalose suspensions ( $70 \pm 2\%$ ) was dramatically enhanced compared to the ones incubated in PBS-Trehalose ( $47 \pm 3\%$ ) and PBS only ( $10 \pm 5\%$ ). Results indicate that biomimetic apatite colloidal nanoparticles increased the uptake of trehalose in RBC, leading to increased cell cryosurvival after freeze-thawing. This is the first report on the application of biocompatible inorganic compounds for cryopreservation applications. Demonstrated results show great promise for future applications of biomimetic apatite nanoparticles in the cryopreservation of blood and mammal cells in general.

### Alginate Nanofibers with Tunable Biodegradability for Regenerative Medicine

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The electrospinning technique has the unique capability to create nanofibrous scaffolds that mimic the native tissue structure and are attractive for regenerative medicine [1,2]. Here, we show the use of sodium alginate, a natural polymer, for the production of electrospun nanofibers with adjustable biodegradability rate. Controlling the biodegradation of alginate nanofibers under physiological conditions is an indispensably important step in tissue engineering and drug delivery. Sodium alginate fibers are in fact readily soluble in aqueous environments, and therefore cross-linking procedures are necessary to increase their stability and structural integrity. Several approaches have been proposed to this aim, in particular, reactions with glutaraldehyde (GTA) and Ca<sup>2+</sup> ion. For instance, the use of GTA poses numerous problems due to the release of toxic compounds during the degradation of the nanofibers in the body. Our approach is based on the modification of the chemical structure of the alginate nanofibers by acidification of carboxylate groups and formation of poly(alginic

acid). The resulting nanofibrous scaffolds exhibit high biocompatibility, as proved by *in-vitro* assays. Therefore, we developed a novel strategy to increase the water stability of alginate nanofibers, preserving their morphology and no cytotoxicity [3]. In this way, we tuned the biodegradation rate of the nanofibers, making them promising for biomedical applications.

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### Combining NanoHydroxyapatite/Chitosan Hydrogels: Correlation between Nanostructure and Physico-Chemical Properties

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Tissue engineering is an interdisciplinary research field that is emerging as an alternative approach to traditional surgical strategies in repairing damaged tissues. A current challenge is the development of scaffolds with structural organization seeded with multiple cell types for replacing more complex tissues.

In a first approach, our aims are to establish a simple process for elaboration of nanostructured hydrogel and to propose a better way to appreciate the interactions between the organic (chitosan matrix) and inorganic (hydroxyapatite HA nanoparticles) sublattices in this system. Hydrogels are high-water content materials prepared from cross-linked polymers. Chitosan is a natural polymer with a great potential as scaffold material thanks to the polymer's biocompatibility, low toxicity and biodegradability. In this context, we achieved a nanostructure of chitosan hydrogel by incorporation of HA crystallites as a biomaterial model.

HA nanoparticles were prepared by coprecipitation of calcium chloride and sodium hydrogenophosphate in aqueous solutions for biocompatibility. The influence of synthesis time on the size, morphology and crystallinity nanoparticles was characterized by Transmission Electron Microscopy (TEM) and X-Ray Diffraction (XRD). XRD and TEM results showed that the morphology and crystallinity evolve with the synthesis duration. The relevance of a thermal treatment (calcination) to improve the crystallinity of the nanoparticles was investigated.

A one-step process of elaboration of nanoHA/ Chitosan hydrogel was optimized to obtain a homogeneous dispersion and a controlled structuration. The resulting scaffold was characterized by InfraRed Spectroscopy and Scanning Electron Microscopy to determine a correlation between nanostructure and nanoHA properties (size, concentration, dispersion).

### An Extracellular Matrix Mimicking Hydrogel Based on an MMP Cleavable Elastin-Like Recombinamer

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Specific tissue engineering approaches currently aim to mimic the ECM environment and are limited by an excessively slow degradation rate of the scaffolds applied. This work addresses that issue by presenting a biocompatible and biodegradable elastin-like recombinamer-based hydrogel, cleavable by matrix metalloproteinases (MMPs), enzymes that are well known for their role in ECM remodeling. It also displays a cell adhesion capability, due to the presence of RGD amino acid sequences in the structure backbone.

The two recombinamers composing the hydrogel were obtained through iterative-recursive recombinant technology, production in *E. coli* and purification by inverse-transition cycling. Eventually, they were chemically functionalized on the lysine residues, respectively, with an azide group on one of the recombinamers and an activated alkyne on the other, in order to permit the biocompatible catalyst-free click chemistry reaction, for the hydrogel cross-linking.

The material features have been confirmed by NMR, HPLC, FT-IR, MALDI-TOF and DSC. The expected fragmentation has been asserted by degrading the dissolved cleavable recombinamer and the hydrogel in an aqueous solution containing MMPs. Rheological studies showed a storage modulus ranging from 1 kPa to 4 kPa, proportional to the recombinamer concentration. Cytocompatibility, cell adhesion and proliferation, have been proved by culturing HFFs and HUVECs in the hydrogel.

The presented covalently bound scaffold favors its substitution by newly formed tissue, as it is being gradually degraded by the MMPs secreted by the cells.

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### Indirect Monitoring during Perfusion based Bioreactor Culture of Large Hypertrophic Cartilage Constructs

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Perfusion-based bioreactors are becoming a standard for the creation of large tissues without the formation of a necrotic core. However, biological 3D processes in a bioreactor are difficult to observe and in-process controls, criteria for graft quality and efficacy are challenging to establish. As a proof of concept we have cultured 6x3 mm hypertrophic cartilage constructs derived from rabbit bone marrow mesenchymal stromal cells of 7 donors in a perfusion bioreactor. Non-invasive monitoring of glycosaminoglycans (GAG), alkaline phosphatase activity (ALP) and calcium content (CA) in the culture medium was performed during 3 weeks of chondrogenesis and 2 weeks of hypertrophy. GAG and ALP represented matrix deposition and cell activity, whereas CA represented matrix calcification. Cumulative GAG was the best predictor for the amount of cartilage formed *in vitro*. Surprisingly, addition of a pan-metalloproteinase inhibitor increased the cumulative GAG by 2.1 fold and the tissue size accordingly, leaving the correlation undisturbed. Samples were implanted subcutaneously in nude mice. Bone formation after 8 weeks was measured by microtomography and histology. Bone volume negatively correlated with cumulative GAG during culture ( $r = -0.77$ ) and ALP at 2 weeks ( $r = -0.68$ ). However, bone marrow correlated positively with both values ( $r = 0.95$  and  $r = 0.61$  respectively). Calcium did not show any correlation. In conclusion, cumulative GAG during culture and ALP at 2 weeks can be used as non-invasive tools to monitor bioreactor culture and predict endochondral ossification potential of hypertrophic cartilage constructs. Based on these results, graft quality and efficacy criteria could be established to improve standardization and regulatory compliance.

### Monitoring Skeletal Myotube Formation on Opaque Scaffolds using Optical Imaging

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There is a growing need to monitor cellular organization non-invasively during *in-vitro* tissue maturation. In this work we applied

a novel optical imaging technology to visualize muscle progenitor cells (MPC) proliferation and myotube formation through a scattering opaque scaffold. Our new imaging system consists of a hollow core fiber (HCF) embedded in polycaprolactone (PCL) electrospun scaffold, close to the surface, for insertion of optical fiber for local laser excitation. Fluorescent Emission from labeled elements is detected by Electron Multiplying Coupled Charged Device (EMCCD) camera. Multiple EMCCD images are then processed to give a reconstructed image of fluorescence distribution. We first analyzed the effect of physical parameters on image quality using fluorescent beads embedded in PCL and PCL-Collagen scaffolds. Results showed that scaffold thickness had a minor effect, collagen content increased image background noise, and far-red fluorescent emission gave a better signal when compared with green fluorescent emission. Using the results obtained with fluorescent beads, we optimized the image reconstruction process and applied these conditions to visualize fluorescently labeled MPC during transition from single cells to multi-nucleated myotube organization.

In conclusion, our novel imaging system provides non-destructive means to visualize MPC proliferation and differentiation through an opaque tissue construct with a relative large working distance (~10 cm) and at cell level resolution (~20 μm), which can have a wide use in a variety of tissue constructs. Furthermore, quantification of fluorescence emission can be used in the future to dynamically monitor complex cellular and molecular events.

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### Design and Fabrication of a Well-set, In-house Prepared 3D Bioprinter

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Nowadays, the bioprinters are used to print cells and scaffolds simultaneously. In this study, a home-made bioprinter is designed and fabricated to print 3D structures. To achieve a 3D work-space for printing, Cartesian coordinates were used. The mechanisms of the X and Y axes movement are placed independently from the mechanism of Z axis movement to minimize the vibration applied to the printed material. This bioprinter has 3 nozzles which are connected to a syringe pump to print different materials at the same structure. The belt and pulley mechanism is used to control the position of the end-effector in the XY axes. This mechanism can decrease the time of printing as a critical parameter in the printing process. For the Z direction movement, the ball-screw mechanism is selected which enhances the load bearing and the precision of vertical movement. Although it leads speed limitation in Z direction, it has not an impact on final printing time as the time is a function of XY axes movement speed.

Some calibration tests, like displacement measurement, speed, and repeatability were conducted to measure the accuracy of the system. Also, different types of hydrogels including Alginate, Fibrin, and dECM were printed to approve the function of the printer. The final printed structures investigate that the process of printing is accurate and coinciding with the pattern designed in device software.

In conclusion, we have designed and fabricated a 3D-bioprinter with appropriate accuracy and function which is cost-effective, well-set and applicable for using in *in-vitro* researches.

### Agent-Based Approach to Simulate Vascularized Bone Formation in Biodegradable Porous Hydrogels

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The success of engineered bone tissue growth in biomaterial scaffolds is determined by the rich vascular supply and high



mineralization. Blood vessel formation through angiogenesis from host tissue is the common mechanism for scaffold vascularization, while bone growth can be induced by either external growth factor supply or ceramic incorporation to stimulate cell differentiation and growth. Calcium phosphates, such as tricalcium phosphate (TCP), and hydroxyapatite (HA) are widely used materials due to their high biocompatibility and osteoinductivity. Interactions between blood vessel formation, bone cell growth and modified biomaterial structure are often difficult to study with experimentation alone. Computational models are powerful tools to test different hypothesis and strategies to explore promising alternatives in an organized manner. Agent-based models (ABM) are an attractive option to describe cell behavior and emerging tissue characteristics. We have previously developed an ABM to study angiogenesis and osteogenesis (Bayrak *et al.*, 2014) in porous, growth factor loaded biomaterials. The current work extends and modifies the model to investigate bone tissue regeneration and study the effects of HA and TCP incorporation to degradable, porous, biomaterial structure. The model showed bone regeneration can be induced using HA- and TCP-loaded scaffolds and suggested the optimum ratio between these materials.

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### Gellan Gum-coated Gold Nanorods as Nanotools for Bone Tissue Engineering

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Gold nanorods (AuNRs) have been widely studied for several biomedical applications. Their optical properties, ease of synthesis and their high surface area make AuNRs an interesting tool for drug delivery and tissue engineering applications, namely bone tissue engineering. However, before biomedical usage, the modification of their surface chemistry is required, in order to improve their biocompatibility and stability under biological conditions. Natural-based polymers are good candidates due to their biocompatibility and gel-like structure, allowing controlled release of drugs/bioactive agents. Herein, we report, for the first time, the successful preparation of a core-shell nanostructure using low-acyl gellan gum (GG) for the coating of AuNRs (AuNRs-GG). This method implies the successive deposition of anionic and cationic polyelectrolytes, followed by the formation of a GG shell. Transmission electron microscopy (TEM) images revealed that AuNRs were individually embedded around a GG shell of 7 nm thick. UV-vis spectroscopy showed that AuNR-GG were stable in a wide range of pH and ionic strength and for several months in an aqueous solution. *In vitro* studies using rabbit adipose stem cells and a pre-osteoblastic cell line (SaOS-2) showed that AuNRs-GG are non-cytotoxic after 14 days of culture. TEM analyses have confirmed that internalization occurs on both cell groups, with aggregation of nanoparticles within cytoplasmic vesicles. Additionally, the mineralization of SaOS-2 will be studied in presence of nanoparticles, in order to assess their effect on osteogenic capacity. The proposed system has shown noteworthy features and a great potential for further use in drug intracellular delivery for osteogenic purposes.

### Induction of Coordinated Ciliary Beating of Respiratory Epithelial Cells by Laminar Flow in a Custom-Made Bioreactor

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Directed mucociliary clearance is the cornerstone of airway tissue engineering. It obviates mucus plugging, which is a major problem of artificial tracheal replacements. A link between mechanical stimulation and ciliary beating as short-term response of an intact epithelium was already shown *in vitro* and *in situ*. However, in tissue engineered tracheal constructs, coordinated ciliary beating has yet to be achieved.

We developed a bioreactor which allows culture of respiratory epithelial cells at air-liquid interface with laminar air stimulation. The cells in the bioreactor can be microscopically visualized without disassembly. A diaphragm pump allows physiological stimulation with the same shear stresses as in the trachea. When culturing primary epithelial cells in the bioreactor, a differentiated epithelial layer with cilia built up within 4 weeks.

We were able to induce directed and coordinated ciliary beating after long-term stimulation with laminar air flow visualized by particle tracking of fluorescent microbeads. Culture without laminar flow stimulation did not induce directed ciliary movement.

The induction of directed ciliary beating in tissue engineered respiratory epithelium not only provides a means for the study of the underlying physiologic processes of mucociliary clearance in the human lung and a research tool to investigate the effects of mechanical ventilation on the airways but might also promote attempts in airway tissue engineering.

### Exosome Expression and Role in Extracellular Matrix Mediation of Macrophage Phenotype

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**Introduction:** Biologic scaffolds derived from mammalian extracellular matrix (ECM) have been used for the repair and reconstruction of a variety of tissues. Exosomes, nano-sized extracellular vesicles, can mediate cell-cell communication and are involved in different cell processes. These biologic effects are possible due to exosomes' ability to transport microRNAs (miRNAs), mRNAs, and proteins. Little is known about the effect of ECM derived signaling molecules on exosome secretion and exosome signaling, specifically in the context of macrophages polarization.

**Objective:** To investigate the potential role of exosomes in macrophages polarization (M1 to M2) triggered by ECM.

**Methodology:** THP1 derived macrophages were treated with ECM degradation products such as: Porcine Urinary Bladder Matrix (UBM) and small intestinal submucosa (SIS). Exosomes were extracted from the supernatant for western blot and electron microscopic evaluation. Protein and miRNA markers which are associated with M1 and M2 macrophages were examined. The effect of exosome treatment alone upon macrophage phenotype is being investigated.

**Results:** We have identified 16 of 26 proteins that are typically present in exosomes (61.5%) via Mass Spectrometry. CD-9, CD-63, CD-81 and HSP-70 markers were confirmed via western blot for both UBM and SIS treatments.

**Significance:** Our results show that ECM can induce macrophages to secrete exosomes. These exosomes may play a significant role in macrophages polarization and downstream cell signaling. Our goal is to identify a unique signature of miRNA, mRNA and protein cargo in exosomes after ECM treatment and develop an exosome-mediated therapeutic strategy.

### 3d Construct Hydrogels Functionalized by Nanoliposomes for Tissue Engineering Application

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The hydrogels are 'soft' microscopic particles consisting of cross-linked polymeric molecules. They are valued for their functionality and ability to tune physical properties in industrial applications including controlled drug delivery, cosmetics, pharmaceuticals and tissue engineering. The overall goal of the proposed abstract is to functionalize the 3D hydrogel constructs from alginate and GelMA by nano-liposome. The nanofunctionalization will be improved the physicochemical, mechanical and biological properties. The mechanical properties of IPN disc before and after nanofunctionalization were studied at two scales (nanoscale by AFM and mesoscopy scale by rheometer). The results showed after adding the nanoliposomes, the mechanical properties increased at two scales. The morphological property changed by adding the nanoliposome, the presence of the pore with regular size after nanofunctionalization was observed. This new morphology will be improve the transfer of nanoliposome and the surface contact. The results confirmed a high cellular viability after nanofunctionalization. The IPN discs possess with nanoliposome has stronger mechanical characteristics in comparison to the hydrogel without nanoliposome. We showed by adding the nanoliposome from natural sources, the cells proliferation of NIH 3T3 increases. These new properties are interesting to develop the new matrix used in tissue engineering.

### Dihydrofolate Reductase Levels Provide a Mechanism for Stem Cell Resistance to Chemotherapy

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Effective chemotherapy treatments kill cancer cells but also can induce significant, short- and long-term damage to otherwise healthy tissue. A common chemotherapeutic drug, methotrexate (MTX), is known to damage cells, both normal and cancerous, by binding to dihydrofolate reductase (DHFR) and inhibiting DNA biosynthesis. One long-term consequence of standard, high-dose MTX treatment is loss of healthy tissue. Our experimental objective was to investigate adipose-derived stem cell (ASC) resistance to MTX compared with somatic cell sensitivity, assessed using normal human fibroblasts (NHFs). We also explored DHFR protein levels as a possible mechanism of resistance. Results showed that ASCs retained proliferative and differentiation potential after MTX treatment while NHFs senesced. Western blot showed greater upregulation of DHFR in ASCs than NHFs during MTX treatment. To determine whether this was the primary mechanism of MTX resistance, NHFs were either transfected with a DHFR plasmid or given exogenous purines and pyrimidines during MTX treatment. Both rescue approaches were effective, with NHFs showing no loss of proliferation following exposure to MTX. Based on these findings, ASC resistance is due to the cell's ability to dramatically increase DHFR levels. Purine and pyrimidine production, through either DHFR overexpression or exogenous supplementation, is capable of rescuing normal somatic cells from the negative effects of MTX. The natural resistance of ASCs to this chemotherapeutic raises the possibility of using ASC-based therapies to ameliorate tissue loss during MTX treatment. Likewise, understanding the mechanism of resistance provides options for rescuing targeted cells in a tissue and identifying more effective chemotherapy strategies.

### Double Delivery of Bone Morphogenetic Proteins (bmps) and Epigenetically Active Small Chemicals by Calcium and Titanium Based Scaffolds Produced by Additive Manufacturing

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The treatment of large bone defects still poses a major challenge in orthopaedic and cranio-maxillofacial surgery. The aim of this study was to determine the osteoconductive potential of titanium and calcium phosphate based implants generated by additive manufacturing and the application of these scaffolds with osteoinduction by BMP

and epigenetically active small chemicals via an *in situ* forming degradable implant.

Defined implants were produced by additive manufacturing and tested in calvarial defects in rabbits and compared to untreated defects. In the last series a critical size defect treated with SLA produced titanium based implants as such or doped with BMP or BMP and epigenetically active small chemicals.

Analysis by  $\mu$ CT and histomorphometry revealed that all generatively produced structures were well osseointegrated into the surrounding bone. The histomorphometric analysis, based solely on the middle section, revealed that bone formation was significantly increased in all implant treated groups compared to untreated defects. In the critical size defect, the scaffold alone was sufficient to yield defect bridging after 16 weeks. Addition of BMP and epigenetically small chemicals was able to increase the area of osseous regeneration when delivered with a faster degrading polylactide-glycolide polymer used to generate an *in situ* forming implant.

Designed porous, lightweight structures have potential for bone regeneration and augmentation purposes, especially when complex and patient-specific geometries are essential. The combination of the osteoconductive scaffold with osteoinductive BMP and epigenetically small chemicals show effect solely in a fast degrading composition of the *in situ* forming implant.

### An Aseptic Processing Assessment of Autologous Cell-based Manufacturing for Immunotherapy: Experiences of a Facility Operating under the Medical Practitioners Act in Japan

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**Objective:** Although there are only a few cell-based medicinal products under the Pharmaceutical Affairs Law, many numbers of cell therapies using cultured cells are treated in clinical under the Medical Practitioners Act in Japan. We focused a cell processing facility for autologous cancer immunotherapy, and assessed.

**Methods:** The producing period was divided into three (early, medium, and late). In the early period, peripheral blood mononuclear cells, which were isolated from blood, were cultured in BINKIT<sup>®</sup> initial medium (BIJ, Japan) added with 5% heat-inactivated autologous plasma and BINKIT<sup>®</sup> Initial cocktail. After 3 days of cultivation for activation, the cells were transferred to a culture flask in BINKIT<sup>®</sup> subculture medium. In the medium period, a fresh culture medium was added to the flask according to the cell number every 2–3 days. In the late period, the cells were transferred to a culture bag until the end of culture when the number of cultured cells started to logarithmically increase. All the processing was operated in safety cabinets (in ISO class 7 room), and no antibiotic was used.

**Results:** The production of 29858 cases had been carried out by the facility in 5 years (2010–2014), and 18 contamination cases (0.06%) were observed. 10 cases were occurred in the early period, and 8 cases were in the medium period. No distinct contamination was observed in the late period.

**Conclusion:** The results suggested that usage of the sealed-vessel culture systems, which lid-opening were minimized, showed low contamination risks in operation at safety cabinets.

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### Chitin-based Biomaterials Facilitate Structure Formation of the Glandular Organs by Regulating The Dynamics of Basement Membrane

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Tissue structure is important for inherent physiological function, and also required for organ regeneration when tissue is engineered. Many essential organs responsible for secretion, nutrition supply, or metabolite exchange, are benefited by ramified tissue architecture

because of enlargement of biological reaction surface. For the purpose of tissue engineering, complex tissue structures are still challenging to be recapitulated. The salivary gland is a typical glandular organ important for saliva secretion and regulation. The salivary glands develop from epithelial-mesenchymal interaction, and accordingly depend on the support of basement membrane (BM). Chitosan-based biomaterials had been demonstrated to be competent in facilitating tissue structural formation of the salivary glands. However, the underlying mechanism accounting for chitosan morphogenetic effect has still remained elusive. Using the developing submandibular gland (SMG) as a model, it was found that chitosan effect diminished when BM components were removed from cultured SMG explants. With chitosan, BM components and receptors increased, and expressed in tissue-specific manners beneficial for SMG branching. Chitosan effect decreased when either BM components or receptors were removed, and reduced as well when downstream signaling of BM components and receptors was blocked. Our results revealed that the morphogenetic effect of chitosan on salivary glands branching is through BM. By regulating BM components and receptors, chitosan efficiently stimulated downstream signaling to facilitate salivary gland branching. This study revealed the underlying mechanism accounting for chitosan effect in engineering of SMG branching, which paves ways for further optimization and application of chitosan-based biomaterials in tissue engineering of the salivary glands.

#### Osteogenesis on Elastomeric Biomaterials: Mimicking the Bone Development Microenvironment

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In this study, we challenge the notion about the selection criteria for bone engineering scaffolds based chiefly on their relative mechanical comparability to mature bone. We investigated the contribution of mechanical stimulation on osteogenesis within an elastomeric substrate. *In vitro*, under cyclic compression, we report FAK overexpression on the elastic scaffold compared to on a stiffer substrate as well as upregulation of Runx2, osteocalcin and bone sialoprotein. *In vivo*, while implanted in a semi-loaded rabbit ulna critical size bone defect, osteoprogenitor cells recruited on the elastomer contributed to complete gap bridging with new bone while minimal bone formation was seen in the empty controls. We conclude that, as load-transducing substrates, elastomers transmit mechanical signals to osteoprogenitor cells to promote differentiation and matrix maturation in a mechanically similar microenvironment to osteoid tissue where native bone-forming cells differentiate to develop load-bearing bone.

#### Platelets as “Micromachines” for Sensing and Actuation of Targeted Drug Delivery for Hemostatic Agents

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Hemorrhage remains the major cause of trauma-related fatalities for both civilians and soldiers. The few clinically available treatments to abate non-compressible hemorrhage can lead to uncontrolled thrombosis and are biological products, which can cause life-threatening immune reactions and blood borne infections - all are major concerns for first responder treatment. Clearly, a need exists for targeted delivery systems for hemostatic agents that avoid side effects of current therapies, increase drug efficacy, and are effective for non-compressible injuries.

We have developed a paradigm-shifting targeted drug delivery system that hybridizes with the patient's platelets to deliver hemostatic agents specifically to sites of vascular injury. Targeted delivery is contingent on platelet contraction, occurring only upon activation at sites of clot formation and is mediated by the platelet biochemical and biomechanical microenvironment. Vehicles are biochemically and

mechanically fine-tuned to induce adhesion to circulating platelets and rupture upon platelet contraction, enabling spatiotemporally controlled release of hemostatic agents strictly to sites of vascular injury.

The targeted delivery vehicles are polyelectrolyte capsules fabricated via layer-by-layer deposition of poly-L-lysine, poly-L-glutamic acid, and fibrinogen. These capsules successfully incorporate into forming clots and bind to platelets via fibrinogen displayed on the capsule exterior. Our recent data demonstrates that capsule walls rupture during clot formation and platelet contraction, resulting in spatiotemporally controlled platelet-actuated payload delivery. Employing the chemomechanical action of activated platelets to function as both the sensor and actuator of the system ensures drug release occurs only in environments requiring hemostatic augmentation - a new paradigm of targeted drug delivery.

#### A Novel Design of Wound Dressing Matrix with Electrospun Porous Fibers and Antibiotic Loaded Chitosan Microbeads

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Skin is the largest organ that controls temperature, water loss and extraneous insults from chemicals and microorganisms. Clinical intervention might be essential for a full thickness skin loss including epidermis and dermis and with a size more than 4 cm diameter. Large size skin loss demands advanced regenerative therapy employing *in vitro* generated tissue engineered substitutes. Three dimensional scaffolds with properties like biocompatibility, biodegradation and mechanical strength is required to generate bio-artificial tissues. The primary criteria in designing the scaffold are its similarity to the native extracellular matrix. Electrospinning technology allows fabrication of three dimensional nanofibrous scaffolds that closely mimic natural extracellular matrix. In this study, poly  $\epsilon$ -caprolactone (PCL) polymeric scaffolds was fabricated and modified for wound dressing application. PCL mats with porous fibres (PPCL) were fabricated and modified by surface hydrolysis and characterized by Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy and water contact angle analysis. The cytotoxicity, cell adhesion, viability and cytoskeletal distribution analysis using fibroblasts and keratinocytes confirmed that the scaffolds are non-cytotoxic and promotes cell adhesion and proliferation. The scaffold thus proved as a potential scaffold for skin tissue engineering was further improved by entrapping antibiotic loaded chitosan microbeads within porous PCL mats for use as wound dressing matrix. The cytotoxicity, cell adhesion, proliferation, hemocompatibility and antimicrobial activity tests confirmed its suitability as wound dressing matrix. Incorporation of biomolecules to promote healing and *in vivo* studies by implantation will reassert this new design of bead entrapped fibrous mat as a viable matrix for skin tissue engineering.

#### Microfiber-reinforced Electrospun Scaffolds with Structure and Composition Gradients for Enhanced Ligament-bone Integration

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Most of the existing tissue-engineered scaffolds for anterior cruciate ligament (ACL) regeneration are unable to recapitulate the structural, compositional and mechanical properties in native ligament-bone interface and were found to induce poor osteointegration. The rupture at insertion site is still the most common concern for the failure of ligament grafts. Here we present a unique electrospinning strategy to fabricate microfiber-reinforced poly (L-lactide-

co-glycolide) acid (PLGA) mesh with both structure and composition gradients, which was further rolled into a cylindrical ligament scaffold. Morphology observation showed that the orientation of the electrospun nanofibers gradually changed from random to highly aligned which aims to mimic the variable orientations of collagen fibers from bone to ligament tissues. Nanoscale hydroxyapatite particles and bone morphogenesis protein were successfully encapsulated into the electrospun PLGA nanofibers and their contents gradually altered in a similar gradient manner to the structural organization. The structure and composition gradients were found to regulate cellular morphology as well as direct the biomineralization and differentiation of bone-specific cells. The incorporation of microfibers into the electrospun meshes significantly improved the mechanical strength of the rolled ligament scaffolds, which can withstand the maximum tensile force of  $129.08 \pm 18.85$  N after implantation *in vivo* (rabbit), close to that of native ligament-bone fixation ( $118.14 \pm 14.04$  N). The microfiber-reinforced gradient scaffolds could potentially induce osteointegration and realize long-term biological ligament-bone fixation.

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### Degradation of 3D Printed, Highly Porous, Bone Tissue Engineering Scaffolds

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Controlling pore architecture is crucial for developing bone tissue engineering scaffolds with desirable properties. Porogen leaching methods produce stochastic porosities that lack repeatability in fabrication, potentially contain disjoint void spaces, and likely result in irregular degradation, mass transport, and stress-distribution. 3D printing affords us the ability to fabricate structures with systematically designed porosity. In this work, we utilize the triply periodic minimal surface, Schoen's Gyroid, as the basis for our pore geometry. Gyroid-derived porous scaffolds feature an exceptional range of achievable porosity (approx. 2 to 98%), continuous curvature, and uniform mass distribution. Furthermore, the pore and strut dimensions can be easily tailored on a per case basis. We have physically realized Gyroid scaffolds with struts as thin as 125 microns by 3D printing poly(propylene fumarate) (PPF). In a pilot degradation study, 88% porous cylindrical scaffolds ( $r = 5.0$  mm,  $h = 5.0$  mm, strut size = 200  $\mu$ m, pore size = 700  $\mu$ m) submerged in 0.1 M NaOH at 37°C lost  $18.5 \pm 1.3\%$  and  $34.3 \pm 7.8\%$  mass after 7 and 14 days, respectively. At the same time points, complex modulus dropped by an average of  $26.0 \pm 6.0\%$  and  $50.1 \pm 4.8\%$  with a frequency sweep from 0.5 Hz to 3.0 Hz. The yield stress of these scaffolds reduced by  $45.7 \pm 3.2\%$  at day 7 and by  $59.1 \pm 9.8\%$  at day 14. This design methodology elucidates a high level of CAD-driven surgical planning. We design patient-specific porous implants beginning with a 3D CT scan that ensures an accurate fit which in turn promotes osseous integration and outstanding aesthetic restoration.

### Computational Modeling and Quantitative Multivariate Analysis of Pluripotent Stem Cell Morphogenesis

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Pluripotent stem cells (PSCs) capable of differentiating into all somatic cell types offer a unique platform to study dynamic morphogenic processes that recapitulate developmental events *in vitro* to form primitive tissue structures. However, the relative contributions of various environmental factors in guiding PSC fate decisions and morphogenesis, such as growth factors and intercellular signaling, are difficult to assess experimentally due to the complex interactions and spatiotemporal dynamics of such cues. To complement existing experimental approaches, a 3D computational model of PSC differentiation was developed by

combining partial differential equation diffusion models, cellular agent-based approaches, and physical models of morphogenesis. The resulting multiscale modeling framework was used to investigate how spatial patterns of PSC differentiation arise due to microenvironmental cues governing complex morphogenic behaviors within 3D ESC aggregates. Agent-based modeling captured complex spatiotemporal patterns of PSC aggregate differentiation depending on local rules of interaction between neighboring cells. In addition, a novel spatial pattern recognition system was developed using network theory to extract meaningful spatial metrics from networks of cells in order to make direct quantitative comparisons between experimental and computational modeling results. A stochastic ODE-based model of FGF4 signaling and inherent stochastic Nanog expression levels by PSCs could explain observed spatial patterns of differentiation. These results demonstrate that emergent patterns of PSC differentiation result from integration of multiple cues governing 3D morphogenesis and provide a novel network approach for quantitative pattern classification based on multiple cell phenotype parameters.

### Synergy of Endothelial and Neural Lineage Cells from Adipose-Derived Stem Cells to Preserve Neurovascular Structures in Hypoxic-Ischemic Brain Injury

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Neonatal hypoxic-ischemic (HI) brain injury cause disruption of neurovascular integrity and lead to life-long functional deficit in human development. However, there is no effective therapy to prevent brain damage and its consequences. The purpose of current study is to evaluate the benefit of cell-based therapy on HI injured brain and to investigate the underlying mechanism of cell therapy. Our lab demonstrated the possibility to differentiate the adipose-derived stem cells (ASCs) into endothelial lineage cells (ELCs) or neuronal lineage cells (NLCs) by using chemical or mechanical stimulations, and studied the therapeutic effect of the cells by transplanting ASCs, ELCs, NLCs, or combination of ELCs and NLCs (E+N) into neonatal HI brain injured rats. The E+N treatment showed significant decrease of infarction and apoptotic area, and preservation of the neurovascular structure. The long term recovery of cognitive and motor function were improved in E+N treated group. The beneficial effect of the treatment may through the cell-cell interaction via neurophilin-1 signal in ELCs and C-X-C chemokine receptor 4 and fibroblast growth factor receptor 1 signal in NLCs promoted the migration ability of combination ELC and NLC. Moreover, the transplanted ELCs and NLCs were able to engraft into host tissue and promote of endogenous neurogenesis and angiogenesis. In summary, the results in current study suggested that synergistic effect between ELCs and NLCs can facilitate the therapeutic effect on preserving brain integrity and function after brain HI injury.

### Molecular-level Surface Analysis Demonstrates that Detergent Selection Impacts Extracellular Matrix Proteins

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Decellularized matrix scaffolds, such as porcine urinary bladder matrix (UBM), may be prepared through a range of decellularization techniques, commonly using ionic, zwitterionic or nonionic detergents. Whilst removal of cellular material is regularly assessed, the impact of

detergent selection on ECM structure and composition is less commonly investigated. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a powerful surface analysis technique to probe biological structures with high mass resolution and surface specificity. We report the use of ToF-SIMS to distinguish the basement membrane complex of UBM prepared by treatment with 1% SDS, 4% deoxycholate, 8 mM CHAPS or 3% Triton X-100 for 24 hours. Principal components analysis (PCA) reveals spectral differences between treatment groups. In addition to insights into remaining cell debris and traces of residual detergent, we can further probe these data sets to investigate how detergent selection impacts proteinaceous ECM components. Using a reduced peak list of known characteristic amino acid fragments, PCA distinguishes native bladder tissue from decellularized UBM. Additionally, PCA highlights spectral differences between UBM treated with ionic and charge-neutral (zwitterionic and nonionic) detergents. Notably, the basement membrane surface of UBM prepared with ionic detergents SDS and deoxycholate yield less intense characteristic peaks from hydrophobic amino acids than UBM treated with charge neutral detergents CHAPS and Triton X-100. Harsher ionic detergents may denature protein structure and break protein-protein interactions through binding of their hydrophobic tail to hydrophobic amino acid residues. Such damage is hypothesized to cause sub-optimal *in vitro* and *in vivo* responses.

### Promotion of Salivary Gland Regeneration Following Radiation Damage by Adipose-derived Stem Cells-Seeded Small Intestinal Submucosa Gel

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**Objectives:** Adipose-derived stem cells (ASC) have been known to contribute to SG tissue repair/regeneration. The purpose of this study was to investigate whether locally transplanted ASC within small intestinal submucosa (SIS) gel could participate in restoration of SG hypofunction after radiotherapy with long-term engraftment.

**Materials and Methods:** Human ASC-seeded SIS gel was prepared and injected into the SGs of mice after local irradiation (IR). Survival of transplanted ASC was tracked by fluorescence *in situ* hybridization (FISH) analysis for Y-chromosome-specific sequences. Gross morphological and structural changes were evaluated at 8 and 16 weeks after treatment in each experimental group: sham, PBS, SIS, ASC, and ASC/SIS group. Functional studies of measurement of salivary protein contents were performed. *In vivo* mode of actions of ASC including paracrine effect was also explored.

**Results:** Local injection with the ASC and ASC/SIS resulted in less fibrosis and acinar cell loss than the PBS group. Functional restorations such as increased salivary capacity and levels of salivary proteins relative to the PBS and SIS group were also observed. The microscopically improved tissue remodeling of salivary epithelial (AQP-5), endothelial (CD31), myoepithelial ( $\alpha$ -SMA) and SG progenitor cells (c-Kit) were noted in the ASC/SIS group. The ASC within SIS showed prolonged survival relative to the ASC group and most engrafted cells were found in acinuctal structures with expression of paracrine mediators such as VEGF and FGFs.

**Conclusion:** These results show that ASC-seeded SIS may participate in tissue remodeling following radiation damage by releasing paracrine mediators into radiation-inflicted surrounding SG tissues.

### The Effect of Adding Dental Implants to the Reconstructed Mandible Comparing the Effect of using Ti-6al-4v and Niti Hardware

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**Background:** Adding dental implants to those patients who undergo mandibular segmental defect surgery is made more difficult where the grafted bone would be stress shielded and resorbed due to the use of highly stiff Ti-6Al-4V hardware. Failure to maintain

grafted bone would place dental implants at risk. In order to minimize the stress shielding effect caused by stiffness mismatching, we have recently proposed the use of porous NiTi hardware as a substitute for Ti-6Al-4V fixation hardware.

**Methods:** We simulated boundary conditions of maximum occlusion of 526N at the left M1. All of the mandible components including cortical and cancellous bone, teeth, periodontal ligament, and fixation hardware and screws were imported into ABAQUS for further simulations. The purpose of this study is to determine the effect of adding dental implants (diameter range: 3–5 mm, Length range: 8–16 mm) to the following cases: 1) a healthy mandible (control), 2) a reconstructed mandible using Ti-6Al-4V hardware and a double fibular barrel graft, and 3) a reconstructed mandible using NiTi hardware and a double fibular barrel graft. The resection site is considered to be at Left M1-3.

**Results:** Using FEA studies, we have shown that stress concentration on the dental screw increased as the diameter decreased and its length increased. We have found that the stress distribution on these dental implants was more similar to a healthy mandible in group 3 in group 2.

### A Method to Create Collagen Scaffolds with Elastic-like Characteristics for Regeneration of Dynamic Tissues

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Type I collagen is a biomaterial that has been extensively applied in constructs for tissue repair. However, mechanical properties of these constructs are often not in compliance with tissue that needs to be regenerated. In this study, we report a technique to alter the mechanical characteristics of collagen scaffolds and induce elastic-like properties. Tubular scaffolds were prepared from insoluble collagen fibrils by a casting, molding, freezing and lyophilization process. Next, a unique combination of compression, corrugation and carbodiimide crosslinking was applied. Morphology of the scaffold was assessed using light- and electron microscopy and mechanical characteristics were examined by force monitored stretching experiments. After compression, corrugation and chemical crosslinking, scaffolds displayed reduced porosity and showed a crypt-like structure comparable to the bellows of an accordion. In addition, elastic-like characteristics were introduced; when the corrugated structure was stretched in an aqueous environment and the applied force was released, the scaffolds instantly returned to their corrugated (native) state. This phenomenon was not observed in non-polar solvents, indicating that hydrophobic interactions are important in this respect. Mechanical testing demonstrated that corrugated scaffolds were stronger and more extensible than porous scaffolds. In conclusion, with the applied technology collagen scaffolds were prepared which possess elastic-like characteristics, likely to the introduction of hydrophobic links. This technique may be used to prepare tailor-made scaffolds that resemble the mechanical properties of the tissues with elastic tissues such as ligament, intestine, lung, and bladder.

### Periodontal Ligament Stem Cells Suppress the Immunogenicity of Dendritic Cells by Down-regulation of Non-classical MHC-like Glycoprotein CD1b

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Mesenchymal stem cells (MSCs) have been utilized as a promising stem cell therapy for their capability of tissue regeneration and immunomodulatory function. Recently, CD146+STRO-1+MSCs from periodontal ligament stem cells (PDLSCs) were identified, but their immunological role in dendritic cells (DCs) has been completely unknown. In the presence of lipopolysaccharide isolated from *Porphyromonas gingivalis* (LPS-PG), a major bacterium in the oral cavity, we discovered that CD146+STRO-1+PDLSCs as well as bone marrow mesenchymal stem cells (BMSCs) significantly decreased non-classical MHC glycoprotein CD1b on DCs, resulting in defective T cell proliferation. This study unveiled an immunomodulatory role of PDLSCs in regulating DC mediated T cell immune responses, demonstrating potential of PDLSCs for new promising stem cell therapy.

### Human Immune Response to Xenogeneic Bio-engineered Tooth Root

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The urgent shortage source of donor organs for allotransplantation has become another challenge for tissue engineering based regenerative medicine. As meet many physiological and biochemical requirements, pigs are considered to be potential donors for discordant xenotransplantation of several organs. Our previous study showed that regeneration of periodontal ligaments (PDL), cementum-like tissues, and pulp-dentin complexes has been achieved using human treated dentin matrix. However, limited source of human tooth may hampered the clinic application. In the present study, we constructed bio-engineered tooth root by combination of porcine derived treated dentin matrix (pTDM) with human dental follicle derived stem cells (hDFCs), and the immunogenicity of xenogenic bio-root was also investigated. Using ELISA test, partial dentinogenesis related proteins were released by pTDM more than hTDM. Furtherly, qRT-PCR and western blot results indicated that pTDM could induce hDFCs to odontoblast differentiation. Slightly proliferation of immune cells was observed when xenogeneic bio-root was co-cultured with human peripheral blood mononuclear cells (hPBMCs) *in vitro*. The results above indicated pTDM as an alternative to hTDM for the inductive function of pTDM and acted as a guidance for further specific immunoregulation research. Transplantation *in vivo* will be studied for xenogeneic matrix based tissue engineering. pTDM showed a promising potential in tooth regeneration.

### Tissue Response and Antibacterial Activity of Azithromycin Coated Polymeric Membranes

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**Introduction:** Periodontitis is an inflammatory disease resulting in the progressive loss of alveolar bone and periodontal ligament attachment around teeth which may ultimately lead to tooth loss [1]. Guided Tissue Regeneration (GTR) - a widely utilized surgical technique to promote periodontal regeneration is clinically unpredictable. This project aims to develop and evaluate the dose dependent antibacterial activity and tissue/cell response of azithromycin (antibiotic) coated polymeric membranes for improving the clinical outcomes of GTR.

**Experimental Methods:** PCL membranes are fabricated via solution electrospinning. The PCL membranes are then subjected to a calcium phosphate (CaP) coating and later with different doses of azithromycin. Azithromycin encapsulation, release, antibacterial and

cellular/tissue response are assessed both *in-vitro* and *in-vivo* on different doses of azithromycin coated PCL/PCL-CaP membranes.

**Results:** Azithromycin was successfully loaded onto PCL/PCL-CaP membranes using a novel ethanol evaporation technique. *In-vitro* release profile of azithromycin from the membrane demonstrated a controlled release from the membranes over seven days of incubation with PBS at 37°C. Antibacterial activity of azithromycin coated membranes on staphylococcus aureus suggest that the electrospun membranes loaded with azithromycin are capable to inhibit bacterial growth.

**Significance:** This research addresses a topical issue in periodontal regeneration and can significantly impact the patient's life quality. This project will lead to the fabrication of a novel class of polymeric fibrous membrane for the local delivery of azithromycin in a controlled and sustainable manner, which is unprecedented in the literature.

**Reference:** [1] Armitage GC. *Annals of Periodontology*. 4, 1-6, 1999.

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### In-Situ Label-Free Biosensing of Epithelial Differentiation Markers for Salivary Gland Tissue Engineering

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Label-free electrochemical impedance sensing shows great promise in the realm of tissue engineering for studying the effects of engineered biomimetic scaffolds on the differentiation by detecting secreted proteins. Using highly specific antibodies as the sensitive element and attaching to a conductive surface, the high sensitivity of alternating current in a conductive solution isolates the targeted protein. This work seeks to enable a new type of *in-vitro* bioassay, one which enables combination of a live cell culture environment including tissue scaffold constructs, with electrochemical biosensing for secreted cell products. This enables more rapid and less expensive access to direct information about cell behavior and differentiation than prevailing ELISA-type assays. SABPA (salivary androgen binding protein alpha) is produced from secretory acinar cells in mouse gland tissue at postnatal day one, and exhibits characteristics making it an ideal target for indicating differentiation. Recombinant SABPA protein and purified antibodies targeting SABPA from antiserum have been derived, and are being used to develop the sensing performance of the biosensor. Affinity film immobilization is achieved by applying a self-assembled monolayer of heterogeneous crosslinker on 250 um pitch gold electrodes, followed by an activated antibody solution. Alternating electrical bias is then applied across the two identical gold electrodes, varying frequency from 100 to 50,000 Hz, and the impedance is measured. Several elements of the Randle's equivalent circuit for EIS Spectroscopy exhibit the necessary sensitivity for biosensing, especially the "charge-transfer resistance". This platform will enable effective biosensing of epithelial differentiation markers for advanced tissue engineering scaffolds.

### Scale-Up of Immune Cell Therapies for Organ Transplants

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Organ transplants require the recipient to take immunosuppressant drugs. An alternative to immunosuppressant drugs is the administration of a large dose of natural regulatory T cells (Tregs)<sup>1</sup>. A therapeutic number of cells (suggested to be 30 x 10<sup>6</sup> cells/kg<sup>2</sup>) need to be made available at the right time. The current *in vitro* expansion process for Tregs is well established at bench scale using static multi-well plate culture but this is labor intensive and not conducive to wide-spread supply. A robust and cost effective expansion bioprocess would enable

Treg therapy to replace immunosuppressant drugs, so improving the post-transplant outcome for the patient's quality of life, reduce or eliminate additional NHS care due to complications, and reduce the cost of transplantation. The objective of this work was to experimentally and numerically design a semi-automated cost-effective process for Treg cell therapy manufacture, based around a fluidized bed bioreactor (FBB). We have demonstrated that a FBB is suitable for CD4+ cell expansion (of which Tregs are a sub-set) by operating the FBB at the minimum fluidization velocity of  $6 \times 10^{-8} \text{ ms}^{-1}$ . The maximum shear stress a cell experienced was  $6.7 \times 10^{-4} \text{ Pa}$ , at the terminal velocity. Furthermore the system required no human intervention for 1 week so significantly reduced labour costs, and used comparable media volumes to plate culture thereby verifying the process's potential for Treg cell therapy scale-up.

1. Issa, F. *et al.* Trends Immunol. 34, 2013.
2. Brunstein, C.G. *et al.* Blood 117, 2011.

### Bone-like Apatite Coated Antibacterial Guided Tissue Regeneration Membranes

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Guided tissue regeneration therapy by using a barrier membrane is a commonplace strategy in treatment of periodontal defects. The first-generation membranes do not exhibit a truly optimal design, with showing no biological activity. Besides having acceleration properties for periodontium regeneration, membranes with antibacterial properties seems to be essential when considering the high infection risks in the oral environment. To avoid the antibiotic use, natural extracts may be a good alternative for that. Herein, we reported a new barrier membrane with two different functions, namely antibacterial effect and bone bonding ability. The membranes with different PLLA/PCL ratio (100/0, 90/10, 80/10, 70/10, 0/100 w/w) were prepared with the pre-optimized ethanol extracted propolis (EEP) amount by solvent casting technique. SEM micrographs showed that the surface topography of the scaffolds was completely changed in the presence of propolis, having a self-patterning honeycomb-like structures. The presence of EEP also affected the mechanical characteristics of the blend films as well as their thermal behaviors. In the presence EEP, the films become more pliable with an elastic modulus in the range of 122–336 MPa. The antibacterial properties of the samples were evaluated by zone inhibition tests against *S. aureus*. All the film with propolis showed zone inhibition between 13–17 mm while there was no in control samples. To obtain bone-like apatite layer on the membranes, one side of the membranes were treated with UV-ozone and subsequently immersed in simulated body fluid at 37°C for 7 days. Bone-like apatite layer formation was observed all treated samples

### Bovine Microcrystalline Hydroxyapatite Calcium as a Potential Bone Graft Substitute

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**Introduction:** Globally, over two million bone grafting procedures are performed annually, with an increase of 13% per year. Despite the rise in demand, current bone grafts are becoming increasingly limited due to safety concerns, escalating costs and supply issues. Microcrystalline Hydroxyapatite Calcium (MCH-Cal™) is a bovine material with potential to be osteoconductive and osteoinductive due to the presence of hydroxyapatite and growth factors, respectively.

**Methods:** The aim of this study was to assess the therapeutic potential of MCH-Cal™ as a bone graft substitute. A number of growth factors and the bone matrix protein, osteocalcin, were ex-

tracted from MCH-Cal™ and quantified using immunoassays. The effect of MCH-Cal™ on human osteoblast growth and differentiation was evaluated in a 3D collagen gel over a 14-day culture period using alamarBlue® assay (Invitrogen) and real-time PCR, respectively.

**Results:** MCH-Cal™ contained a number of osteoinductive growth factors including IGF-1, IGF-2, total TGF-β and osteocalcin. The alamarBlue® assay demonstrated significant increases in the number of viable osteoblasts over time ( $p < 0.0001$ ). The gene expression results suggested that MCH-Cal™ did not alter the expression of Runx2, Alpl, Bglap, RANKL and OPG over the 14-day culture period.

**Conclusions:** In light of its cytocompatibility, retained growth factors and low manufacturing costs, MCH-Cal™ has the potential to be used as a sustainable biomaterial for bone regeneration. Further research is warranted to explore the role of the retained growth factors in MCH-Cal™ as well as the safety profile of this product.

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### "Regeneration of the Dental Pulp in Adult Patients using Autologous Mesenchymal Stem Cells : A Proof of Concept"

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The combination of tissue engineering and stem cell-based therapies advances can be applied in endodontics for the replacement of damaged tooth structures, and the regeneration of the dental pulp. The purpose of this study is to evaluate the benefit of autologous human dental pulp stem cell, with platelet rich fibrin like scaffold for the regeneration of the dental pulp.

For this, 5 patients diagnosed with irreversible pulpitis following a clinical evaluation and radiographic diagnostic tests were enrolled for the study. Following, an ethical committee approval and a signed consent, the dental pulp (DP) of the patients was harvested, and mesenchymal stem cells (MSCs) were isolated. All patients' root canals were treated with calcium hydroxide to avoid the development of opportunistic infections. Dental pulp stem cells (DPSC) were then expanded under strict GMP conditions. At 20 days post-culture, DPSC were collected, tested for the absence of microbial contamination and then resuspended in an injection solution. Patients were implanted in the root canal with either autologous platelet rich fibrin alone or in combination with 1x10<sup>6</sup> DPSC. The clinical and radiographic evaluation was performed at 1 and 12 weeks post-implantation.

All the patients showed no sign of infection, pain, swelling or apical lesion. However, no significant difference between the two treated groups was detected at this level. Additional vitality tests using laser doppler and longer follow-up are required for a complete evaluation of this treatment. This proof of concept show the feasibility of a translational application of new regenerative procedure in endodontics.

### Non-invasively Refilling Drug-Eluting Devices

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Local drug delivery depots have significant clinical utility, but there is currently no noninvasive technique to refill these systems once their payload is exhausted. Inspired by the ability of nanotherapeutics to target specific tissues, we hypothesized that blood-borne drug payloads could be modified to home to and refill drug delivery systems. To address this possibility, we have developed bioorthogonal- and nucleic-acid-based methods for selective disease targeting by small molecules and nanoparticles. We demonstrate the utility of this system through repeated drug refilling and release in a cancer model as well as selective drug homing in lower limb ischemia and osteomyelitis. These results suggest a new paradigm for drug delivery: repeated and controlled release of drugs at a local site and this advance is expected to have

applications in refilling drug depots in cancer therapy, wound healing, and drug-eluting vascular grafts and stents.

### Glycocalyx Integrity Influences Nanoparticle Uptake by Endothelial Cells

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Atherosclerosis is a precursor of cardiovascular disease, a leading cause of global mortality. Mechanisms of endothelial cell (EC) dependent atherosclerosis are not fully understood. The EC surface sugar coat—the glycocalyx (GCX)—may play an important role, since it is shed in atherosclerosis. GCX may be a possible therapeutic target if it can be regenerated. Nanoparticle-based regeneration to treat cardiovascular disease is becoming very popular, and we wish to study how GCX alterations may impair or amplify the effect of regenerative agents delivered to sites of atherosclerosis via nanoparticles. To demonstrate how nanoparticle-based drug delivery is impacted by GCX conditions, we expose rat fat pad EC (RFPEC) with intact GCX to ultra-small PEGylated gold nanoparticles. The RFPEC with intact GCX do not exhibit any nanoparticle uptake. In contrast, RFPEC with protein deficient and collapsed GCX retain some nanoparticles. RFPEC with enzymatically degraded GCX heparan sulfate, the most abundant component of the GCX, retain a more substantial number of nanoparticles. In another case, after enzymatic heparan sulfate degradation, we induce GCX regeneration by adding heparan sulfate to the culture media for its incorporation into the GCX. HS regeneration results in restoring blockage of nanoparticle entry into RFPEC. This work indicates that the GCX integrity and composition does influence nanoparticle uptake by EC. We look forward to further elucidating how glycocalyx mediates the action of nanoparticle drug carriers, especially those that are under development for cardiovascular disease treatment. Funding: Northeastern University and IGERT Nanomedicine Science & Technology Program at Northeastern University (NSF/DGE-096843).

### Differences in Fetal Bovine Serum Affect the Responsiveness of Cells to Mechanical Loads

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Nowadays, the end-point of a cell culture in bone tissue engineering (BTE) is the acquisition of a well mineralized extracellular matrix. The biological performance of BTE relies on evaluation of the cell capacity to proliferate and to produce extracellular matrix by quantification of gene expression and by histology or calcium quantification assays. Micro-computed tomography (micro-CT) allows monitoring of BTE mineral constructs in a non-destructive manner. Although fetal bovine serum (FBS) is commonly used as supplement in cell cultures, its high composition variability between different brands and batches leads to differences in the experimental outcomes. Nevertheless, only few studies have focused on a systematic investigation of the differences. While we have recently reported the influence of FBS type on matrix mineralization under static culture conditions, it is still unknown how FBS affects cells in dynamic cultures. Different FBS types were used to differentiate human mesenchymal stem cells down the osteogenic lineage under dynamic spinner-flask bioreactors. Opposite to static culture conditions, differences in FBS affected the responsiveness of cells to differentiate under mechanical loads. Although all FBS types up-regulated the expression of bone-specific genes, differences in the osteogenic differentiation stage were observed among the different FBS. Accordingly, micro-CT analysis only showed mineral deposition for cultures in an advanced differentiation stage.

Thus the selection of the FBS type is crucial for the success in the acquisition of BTE constructs. The combination of micro-CT with molecular biology techniques will benefit efforts to optimize scaffold design and cell culture conditions for scaling-up the BTE constructs.

### 3D Reconstitution of Brain Stromal Microenvironment

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Neural stem cells (NSCs) reside in a specialized microenvironment termed the “Neural stem cell niche”, which maintains the capacity of both self-renewal and differentiation of NSCs through various microenvironmental cues involving growth factors, small molecules, extracellular matrix (ECM), cell-cell and cell-ECM interactions, and brain vasculature. To increase our knowledge of the mechanisms governing the behaviors of NSCs, it should be required to comprehensively understand their instructive stromal microenvironment.

To investigate microenvironmental regulation on NSCs' behavior and homeostasis, vascular niche on ECM was formed in 3D. The 3D reconstitution dramatically presents NSC behavior under various microenvironments. For example, brain vasculature enhanced NSCs' self-renewal and at the same time, also regulated their differentiation fate. The microfluidic study enabled various types of interactions, on spatial proximity, chemical/physical interactions and interstitial molecular transport.

### Multiscale and Multidisciplinary Analysis of Rat Bone Health Following In Utero Vitamin D Deficiency

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Bone mechanical competence is derived from a number of size, material and structural components, which are directed by the bone biology environment. Previously we have described an integrated approach to interrogate these components within a bone sample to predict strength. In this investigation, we utilise this approach together with computational modelling to analyse a rat model of *in utero* vitamin D deficiency (VDD). Femora from 21 day old male rats were analysed ( $n=5$  for control and VDD groups) for osteogenic gene expression by RT-qPCR, microarchitecture and bone mineral density by  $\mu$ CT scanning, fracture toughness by notched bend testing and overall bone strength by three-point bend testing.  $\mu$ CT scans were used to generate FE models to predict bone strength computationally. Femora from VDD background rats were found to have reduced midshaft area when compared to controls ( $p=0.03$ ) despite no detected difference in bone volume. FE modelling predicted lower compressive failure forces for the VDD femora ( $p=0.04$ ). This was confirmed experimentally in the three-point bend data, where VDD femora failed at lower loads compared to controls ( $p=0.04$ ). No differences were found within osteogenic gene expression, BMD, fracture toughness or cortical thickness. These results show how *in utero* VDD causes reduced bone health in male rats at 21 days of age and indicates how mechanical function of bone can be predicted. These data and approach can be used to inform and target bone regenerative therapy and scaffold tissue formation to key components of bone health.

### Micro-RNA Plasmid Loaded Nanoparticles Efficiently Modulate Transforming Growth Factor-beta1 Expression in Healing Intrasynovial Flexor Tendons: An In Vitro and In Vivo Study

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**Aims:** Formation of adhesion around the healing tendon is a major cause of poor function of the extremities. Because transforming growth factor (TGF)- $\beta$ 1 is highly expressed in the adhesion tissues and is considered responsible for adhesion and scar formation, we propose to deliver micro-RNAs (miRNAs) to down-regulate expression of this key gene in order to prevent adhesions in the healing digital flexor tendon.

**Methods:** Nanoparticles made of polylactic-co-glycolic acid were prepared and were then incorporated with plasmids expressing miRNAs for inhibiting TGF- $\beta$ 1 gene expression. The cultured tenocytes from chicken toes were used to test efficiency of transfection of the cells by plasmid released from nanoparticles. Then 30 long toes of 15 chickens were divided into 6 groups (5 tendons each). The 6 groups were 2-, 4- and 6-weeks after nanoparticle-plasmid (mock miRNA)-injected groups and 2-, 4- and 6-week nanoparticle-plasmid (miRNA TGF- $\beta$ 1)-injected groups. Expression levels of TGF- $\beta$ 1 in the tendons were evaluated with Western blotting. Tendon healing histology was also evaluated.

**Results:** The tenocytes could be effectively transfected by the nanoparticle-plasmid complex. The TGF- $\beta$ 1 is significantly down-regulated after with nanoparticle-plasmid treatment at all three time-points after surgery ( $p < 0.05$  or  $p < 0.01$ ). Histology analysis did not demonstrate any significant inflammation or necrosis in tendons injected with nanoparticle-plasmid. The tendon healing was not affected by use of nanoparticle-plasmid.

**Conclusions:** Our results show that delivery of micro-RNA loaded plasmid through nanoparticles can efficiently modulate genes associated with adhesion formation, which may be a promising method to limit adhesion formations.

#### Human Adipose Stem Cells Differentiated on Braided Poly lactide Scaffolds is a Potential Approach for Tendon Tissue Engineering

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The main objective was to find an efficient strategy to produce *in vitro* a potential tendon tissue engineering construct. The preliminary tests included the optimization of the tenogenic differentiation medium (TM) for the human adipose stem cells (hASCs), and the screening of biomaterials and tissue engineering scaffold structures. The optimized TM enhanced significantly the tenogenesis of the hASCs. The braided poly-L-D-lactide 96/4 (PLA) filament scaffolds in the TM condition supported hASC adhesion, proliferation and tenogenic differentiation compared to the porous poly(L-lactic-co- $\epsilon$ -caprolactone) 70/30 (PLCL) scaffolds. The braided PLA scaffolds supported the formation of a uniform layer of the hASCs when cultured in the TM compared to the maintenance medium (MM) condition after 2 weeks of culture. The total collagen content and the gene expression of tenogenic markers of the hASCs was significantly higher in the TM condition after 14 days of culture. The elastic modulus under tension of the braided PLA scaffold was approximately in the same order of magnitude as that reported for the native human Achilles tendon tissue. Our study showed that the optimized TM is needed for the efficient and rapid *in vitro* tendon-like matrix production of the hASCs. The braided 8-filament PLA scaffolds combined with the TM significantly enhanced tenogenic differentiation of the hASCs. The proposed tendon tissue engineering applications represent a novel and feasible application with wide applicability.

#### Compression Perfusion Bioreactor for Articular Cartilage Engineering

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We have recently developed an elegant and easy to assemble bioreactor that can apply both shear and compressive force to engineered tissues. In our system, alginate hydrogel beads with encapsulated human mesenchymal stem cells (hMSCs) were cultured under dynamic conditions and with mechanical compression to mimic the *in vivo* mechanical environment. A customized pressure sensor was developed to track the pressure fluctuations when shear forces and compressive forces were applied. Results showed increased pressure with compression, where peak pressure was  $10 \pm 3$  MPa, but no significant pressure change caused by perfusion. We previously demonstrated that the perfusion bioreactor itself can improve the proliferation of hMSCs. In this present system with mechanical compression, hMSCs exhibited similar proliferation rates compared to the non-compression control group. However, chondrogenic differentiation was enhanced with compressive forces. For example, after 14 days, alcian blue staining showed more extracellular matrix formed in the compression group. Our sensor and bioreactor provides a new and more accurate way to describe the pressure within engineered cartilage, particularly in regard to the pressure that cells are experiencing. As mechanical stimulation can affect receptors on the surface of cells, and therefore affect downstream gene and protein expression, this method is a critical development in the investigation of how mechanical stimulation affect chondrogenesis of progenitor cells and therefore the future direction of engineering cartilage tissues.

#### Quantitative Evaluation of Human Culture Operation for the Design of Automated Cell Production System

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For the industrialization of regenerative medicine, it is necessary to establish an automated cell production system which contribute to operational stability for cell manufacturing. However, culture operations are required special care and rough culture operations often cause to decrease cell growth and viability, especially for cells which have very sensitive external stimuli like iPSC cell. Therefore, it is important to minimize damage to the cells during culture process and if we want to prepare high quality cells, it is necessary to acquire well-trained culture operation expert technique. However, when we intend to prepare specification of culture operation to design automated cell production system, it is difficult to determine a design of culture operation because expert technique has not been quantified. In this study, we have developed a new device to quantify the human culture operation and compared experts with trainees by measuring vibration and tilt during culture operations (moving of dish, cell seeding, medium exchange, etc.). As results, we enabled to confirm differences in the variation of the maximum acceleration and rhythm of acceleration change between experts and trainees. Thus, we have demonstrated that it is possible to quantify the expert culture operation using this device. Furthermore, it is considered this device enable to support technical acquisition to quantify operator's culture operation for a design of the automated cell production system.

#### GMP-compliant MSC-isolation and Expansion from Sternum of CABG Patients

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**Objectives:** Mesenchymal Stromal Cells (MSC) emerge as attractive candidates for myocardial regeneration. Clinical use of MSC

from sternum (sMSC) is not established yet. We tested the suitability of sMSC from CABG patients by comparing their characteristics with those of MSC from iliac crest of healthy donors (icMSC).

**Methods:** MSC were isolated using a GMP compliant system supplemented with 5–8% platelet lysate for *ex vivo* expansion. Aspirate was seeded in culture medium for 10 or 14 days at a density of 50,000 leukocytes/cm<sup>2</sup>. After 3 days, non-adherent cells were removed. Medium was exchanged twice per week. MSC were harvested and re-seeded at a density of 4,000 cells/cm<sup>2</sup> for 5 or 7 days.

**Results:** Sternum aspirate had a lower content of CD34+ cells and colony-forming unit-fibroblasts (CFU-F). Compared to icMSC, significantly lower yield (MSC/ $\mu$ l aspirate) of sMSC was observed after 15 days, but not after 21 days. Both sMSC and icMSC fulfilled release criteria of MSC for currently ongoing clinical trials in tissue regeneration. Quality controls included microbial and endotoxin testing, karyotyping, flow cytometry analysis (CD3–, CD34–, CD45–, CD73+, CD90+, CD105+) and differentiation capacity (adipo-, chondro- and osteogenic). While sMSC tend to differentiate to adipocytes, icMSC prefer the osteogenic pathway. No difference was observed in the number of cumulative population doublings (sMSC: 39.3 $\pm$ 10.8 vs. icMSC: 44.6 $\pm$ 7.2;  $p=0.299$ ) in long term cultures for more than 60 days.

**Conclusion:** MSC can be isolated and expanded from sternum in quantities sufficient for clinical application. Thus, sMSC could be used for autologous myocardial cytotrophy.

### Influence of Electron Beam Irradiation Dose on Bone Regeneration by Octacalcium Phosphate Collagen Composites

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Octacalcium phosphate and collagen composite (OCP/Col) achieved efficient bone regeneration with excellent resorbability 1. After confirmation of safety and efficacy by preclinical animal studies and clinical trial, the present study investigated the influence of electron beam irradiation dose on bone regeneration by OCP/Col to secure the commercialization of OCP/Col.

OCP/Col was prepared as previously described<sup>1</sup>, and the packed OCP/Col was subsequently sterilized using electron beam irradiation (15 kGy or 40 kGy). A standardized defect, 9 mm in diameter, was made in the rat calvarium. Then, a disk of OCP/Col (15 kGy or 40 kGy; 9 mm diameter 1.5 mm thick) was implanted into the defect. Five defects of each group were treated and fixed 4, 12, and 24 weeks after the implantation. Then, the specimens were decalcified and stained with hematoxylin and eosin. For histomorphometrical analysis, the percentage of newly formed bone in the defect (n-Bone%) was calculated.

In OCP/Col (15 kGy), newly formed bone was nucleated by the implanted OCP/Col. And it was enhanced and occupied throughout the defect with the resorption of OCP/Col. Also, vigorous bone remodeling and bone maturation was observed. In OCP/Col (40 kGy), newly formed bone was unnoticeable rather than OCP/Col (15 kGy). Histomorphometric analysis showed nBone% of OCP/Col (15 kGy) was significantly higher than that of OCP/Col (40 kGy) at 4 and 24 weeks.

These results suggest that the difference of electron beam irradiation dose would influence on bone regeneration by OCP/Col.

1. Kamakura S, *et al.*, J Biomed Mater Res B Appl Biomater 79, 210, 2006.

### Generation Of Induced Neuronal And Glial Cells From Adipose-derived Stem Cells

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Tissue engineering approaches for central and peripheral nervous system injuries maybe enhanced by *ex-vivo* generation of neuronal and glial like cells by direct reprogramming of adult stem cells. Numerous transcription factors are instrumental during development for the for-

mation of neurons, and the forced expression of selected factors *in vitro* can direct neuronal conversion of somatic cells. Adult stem cells have also been shown to differentiate into Schwann cells by chemical induction alone. Here we describe the generation of induced neuronal and glial cells from adipose-derived stem cells, by lentiviral vector expression of novel transcription factors combined with 3D culture.

To produce induced neuronal cells ADSCs were transduced with lentiviral vectors expressing, Brn2, Ascl1, Myt1l and NeuroD1 (BAMN), and to produce schwann cell like cells, Krox20, Oct6, Sox10 and Brn2 (KOSB) were used with expression controlled by doxycycline induction. Transduced cells were seeded in 2D as monolayers or in 3D a porous polystyrene based substrate. Cells were cultured in induction media for up to 3 weeks, prior to immunostaining for neuronal-specific  $\beta$ III-tubulin, or glial-specific S-100 $\beta$ . 3D culture significantly enhanced neuronal reprogramming by two fold compared to monolayer cultures with a corresponding increase in MAP2 gene expression and extensive neuronal networks. For conversion to Schwann cell like cells, the KOSB cocktail of transcription factors resulted in spindle shaped morphology with enhanced expression of S-100 $\beta$  and Myelin Protein zero (MPZ). Direct reprogramming of adult stem cells results in specific neuronal and glial like cells which may contribute to *ex-vivo* engineered myelinated cultures.

### A Facile Synthetic Extracellular Matrix Approach for Functional 3D Co-culture of Endometrial Stromal and Epithelial Cells

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Co-culture models of epithelia and a supportive stroma contribute to a fuller understanding of intercellular signaling underlying epithelial pathologies. Here we present a synthetic extracellular matrix polyethylene glycol (PEG) scaffold capable of supporting 3D cultures of multi-lineage cells (i.e., epithelial and fibroblast cells) through functionalization with peptides that enhance cellular adhesion through 1) integrin-mediated cellular attachment and 2) extracellular matrix (ECM) capture from cell deposition. A semi-empirical approach was used to design and screen a panel of polyethylene glycol (PEG)-based hydrogels incorporating cell and matrix adhesion peptides and proteolytic (matrix metalloproteinase) degradation sites. Features were chosen to foster cell attachment, epithelial polarization, and stabilization of cell-secreted ECM proteins by Ishikawa and tert-immortalized human endometrial stromal cells (tHESCs) and primary endometrial cells isolated from endometrial biopsies. Identification of a branched fibronectin peptide containing both the RGD and synergy domains from the fibronectin III 9,10 domains along with peptides that bind fibronectin, collagen IV and laminin were sufficient to support long-term (weeks) endometrial co-cultures that displayed hormone mediated decidualization and matrix metalloproteinase mediated tissue breakdown.

### Nanostructured Selenium Coating for Anti-infective Medical Implants: *in vitro* and *in vivo* pilot studies

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**Bacterial infection** and associated biofilm formation remain leading complications following implantation of any foreign materials into the body to repair or regenerate tissue [1]. Standard antibiotic therapy often requires very high, toxic doses leading to impaired healing [1,2].

Built upon our experience with nanoparticles of selenium (Se), here we aimed to investigate the cytotoxicity and antimicrobial properties of Se nanoparticles and their coatings against common drug resistant bacteria.

*In vitro* assays were conducted to study the nanoparticles' antimicrobial activity against methicillin-resistant staphylococcus aureus and epidermidis and their toxicity toward fibroblasts and blood cells. *In vivo* studies were implemented to investigate the ability of the nanoparticle coatings applied on titanium (Ti) plates and screws to restrict biofilm formation in a rat femur model.

The Se nanoparticles exhibited strong growth-inhibitory effects toward both bacteria at Se concentrations as low as 0.5 ppm *in vitro*. A concentration of 128 ppm Se showed no toxicity toward fibroblasts and low haemolysis. The nanoparticle coating was applied to Ti for the animal studies. Results clearly showed reduction in biofilm formation on coated plates and decreases in the numbers of viable bacteria retrieved from coated screws.

We demonstrated here a versatile non-drug based, non-toxic antimicrobial coating that could restrict biofilm formation to implants. This coating platform therefore has a strong potential for a wide range of tissue repair and regeneration applications.

1. Huh, A.J., *et al.* Journal of Controlled Release, 2011. 156(2):128.
2. Hidayat, Levita K., *et al.* Archives of internal medicine, 2006.166.(19): 2138.

#### Effects of Charge, Size and Physicochemical Properties of Polypeptides on their Transport into Cartilage: Implications for Drug Delivery

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Intra-tissue drug delivery into avascular tissues like cartilage remains a challenge due to the dense meshwork of collagen and aggrecan that prevent drugs and drug-carriers from penetrating into the tissue. Furthermore, after the drugs are administered in the intra-articular (i.a.) joint space, they clear out rapidly via lymphatics or vasculature. The high negative fixed charge density of cartilage provides a unique opportunity to utilize electrostatic interactions for augmenting transport rate, uptake and binding of drug-carriers inside the tissue. We showed that Avidin (Av), a positively charged protein, exhibits ideal characteristics for targeted i.a drug delivery into cartilage due to its small-enough size (66 kDa, 7 nm diameter) and optimal net charge (+7, estimated using Donnan theory). We used this knowledge to design four polypeptides with MW ~ 3,000 Da to investigate the interplay of physicochemical properties, size, and charge on their transport and binding within cartilage: (i) Polylysine-hydrophilic (AKAKAKAKAKAKANANAN, +7), (ii) Polylysine-hydrophobic (AKAKAKAKAKAKAFAPAF, +7), (iii) Polyglutamic-hydrophilic (AEAEAEAEAEAEANANAN, -7), (iv) Polyglutamic-hydrophobic (AEAEAEAEAEAEAFAPAF, -7)

*In-vitro* transport experiments revealed that diffusion times for cationic peptides into cartilage explants were longer than that for anionic peptides. Cationic peptides penetrated through full-thickness cartilage within 3 h, partitioned upward, and had long retention times within cartilage due to weak-reversible electrostatic binding with intra-tissue negatively charged aggrecans. Hydrophilic peptides demonstrated higher diffusivities and uptake ratios compared to their hydrophobic counterparts. To understand the effects of size-charge interactions, we compared transport of lysozyme (+7, MW ~ 14.2 kDa), which has similar net positive charge but larger MW. These findings have implications in designing drug-carriers and understanding their transport in connective tissues.

#### Repair of Dog Sciatic Nerve Defeat by using an Electrospun Silk Fibroin based Nerve Graft

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Peripheral nerve repair represents a common clinical challenge, and the current gold standard for treating large nerve defects involves the implantation of nerve autograft that is limited by graft availability, secondary deformities, and potential differences in tissue structure and size. Silk fibroin (SF) based biomaterials possess favorable physicochemical and excellent biocompatibility, and own increasing applications in biomedical fields. Several studies have detailed its suitability as a template for peripheral nerve regeneration. To accelerate translation from preclinical experimental studies toward clinical application, here we selected the dog sciatic nerve injury model that is more clinically relevant and enables the inter-species scaling up to humans. The electro-spinning was performed for preparing a nerve graft, which was composed of a SF nerve guidance conduit inserted with oriented SF filaments. The 30-mm-long sciatic nerve gap in dogs was bridged by this SF based nerve graft. At 12 months after nerve grafting, behavioral investigation, functional and histological assessments showed that SF based nerve graft group yielded an improved outcome, which in many aspects, was closer to that yielded by autograft group than that by non-grafted group. The electrospun SF based nerve graft help to promote peripheral nerve regeneration and functional restoration. All the data increase the feasibility of translation to clinical trials using an electrospun SF based nerve graft for peripheral nerve applications.

#### Safety and Effects of Hydroxyapatite Nanoparticles in Mesenchymal Stem Cells

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Natural bone encompasses nanosize blade-like crystals of hydroxyapatite developed in adjacent connection with collagen (Col) fibers. The cells included the Nanoparticle spheres of hydroxyapatite (N-HA) in the cytoplasm through phagocytosis. There is no literature report of cytotoxicity and genotoxicity in bone marrow mesenchymal stem cells (MSCs) whose nuclear membrane status is cultured in a N-HA medium.

N-HA, dispersed in reconstituted fibrous Col, were prepared in three weight ratios of 75:25, 65:35 and 50:50 (N-HA:Col). MSCs from rabbits were seeded and cultured on the N-HA/Col and HA/Col microbeads and characterized. The cultured MSCs on the N-HA were prepared for the cytoplasm and nuclear membrane examination by Transmission Electron Microscope (TEM).

N-HA were distributed evenly throughout the Col matrix and aggregated to microbeads as determined by scanning electron microscopy. Electron and confocal microscopy showed that the MSCs spread and attached to microbeads via focal adhesions, while staining for F-actin and DNA revealed the presence of stress fibers. The phenotype of the MSCs in the flow cytometry was remained as CD11a-, CD44+, and CD90.1+. The optimal weight ratio was 65:35 of N-HA for the normalized alkaline phosphatase activities. The cytoplasmic and nuclear membranes of MSCs were intact while the blade-like N-HA was phagocytosed in endosomes of the cytoplasm.

MSCs are capable of proliferating and differentiating in appropriate combinations of N-HA/Col. The osteogenic ability of MSCs was enhanced by N-HA. There is no cytotoxicity or genotoxicity found. Thus it is a promising composite for future clinical applications.

#### Biocompatibility and Drug Binding and Release Properties of Milled Silk Particles

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Silk particles are often prepared by the bottom up approach of liquid solid phase transfer from the silk solution obtained from

dissolving silk fibres. We have used the alternate top down approach of milling to prepare micron and sub-micron silk particles. These particles are far more stable than those prepared by bottom up approaches as the natural crystalline structure is retained during the fabrication process. Moreover, the process is environment friendly and scalable. This study investigated the potential use of milled silk particles as a biocompatible carrier for drug delivery applications. Loading of model drugs Orange G, Azophloxine, Rhodamine B, and Crystal Violet on particles shows that equilibrium loading can be achieved in less than 10 min at room temperature compared to 2–3 days needed for fibres. There is no evidence of burst release and release rate is reduced as particles become finer. Silk particles can be coated with a barrier layer during their fabrication which can be potentially used to provide additional control over drug release behaviour. Particles prepared from different silk varieties are able to support the adhesion and migration of mouse fibroblasts. The particles of silk from the semi-domestic silkworm *Anthrea Assamensis* supported the highest cell growth of all varieties tested, significantly higher than silk particles from *Bombyx mori*. These studies indicate the potential of milled silk particles as a stable, biocompatible platform for drug delivery and other biomedical applications.

#### Selective Control of Cell Adhesiveness on Elastin Like Recombinamers-Biofunctionalized Gold Surfaces

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The aim of this work is to obtain selective cell adhesiveness on gold surfaces functionalized with two different Elastin Like Recombinamers (ELRs). The ELRs are synthesized through genetic recombination techniques allowing us to introduce into the polymers new functionalities such as bioactive domains thus conferring a smart behaviour and a high potential for several application including biomedical devices. Those contain cysteines at the amino-terminal region in order to graft on the gold substrate through a redox reaction. Two different ELRs, showing different bioactivity, were designed and produced. One of the ELR contains a cell adhesive RGD sequence, that allows biospecific adhesion of mammalian cells and, the other one, is synthesized as a control polymer without any bioactive sequence and showing anti-fouling character. This method avoid the need of an intermediate linker before the immobilization of the protein. The reaction between the gold surface and the SH groups were assessed by contact angle, X-ray photoelectron spectroscopy (XPS), atomic force microscope (AFM), scanning electron microscope (SEM) and Fourier-transform infrared spectroscopy (FTIR). Certain areas of the surfaces having a width of 200  $\mu\text{m}$  have been therefore cleaned by Maldi-Tof Ultraflexترم laser ablation in order to functionalize these areas with the ELR that contain cell adhesive RGD sequence. Subsequently we cultured human foreskin fibroblast (HFF1) cells on surfaces to investigate the bioactivity in terms of cell adhesion on regions which are functionalized with the ELR containing RGD sequence.

#### Aligned Nanofibers Direct and Enhance Axonal Regeneration after Spinal Cord Injury

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Spinal cord injuries (SCI) result in the extensive loss of nerve functions, and thus lead to life-long disabilities. Unfortunately, SCI treatment is challenging due to the complex cascades of biological events at the injured sites that is followed by the secondary phase of tissue damage. The inhibitory microenvironment at the injured sites also inhibits the regeneration of damaged axons. Therefore, an emerging therapy focus is the development of biomaterials combined with neurotrophic factors to induce nerve regeneration *in situ*. In our study, we report for the first time the use of aligned nanofibers to provide contact

guidance cues for *in vivo* regrowth of axons. The scaffold design consists of aligned poly ( $\epsilon$ -caprolactone-co-ethyl ethylene phosphate) nanofibers that are supported within a collagen hydrogel that is further endowed with neurotrophin-3. A hemi-incision model at C5 level in the rat spinal cord was chosen to evaluate the efficacy of this scaffold design. At three months post-injury, this scaffold elicited the longest attainable neurite length of  $1039.53 \pm 264.99 \mu\text{m}$ , which was significantly longer as compared to the untreated group ( $371.78 \pm 194.83 \mu\text{m}$ ,  $p \leq 0.01$ ) and the isotropic hydrogel-treated group ( $287.42 \pm 100.11 \mu\text{m}$ ,  $p \leq 0.001$ ). Importantly, the regenerated axons and blood vessels followed the direction of the aligned nanofibers, regardless of their orientation. Cavity formation, which was reported in the untreated group, was not observed in the presence of this scaffold. Taken together, we envision that our aligned nanofiber scaffold could serve as a promising platform for SCI treatment.

#### Bioreactor-Based Manufacturing of Engineered Cartilage Grafts: Paradigm Validation in a Large Animal Study

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Manufacturing-related limitations associated with conventional techniques are central challenges for the successful translation of tissue-engineered products into widespread clinical use. The automated bioreactor-based manufacturing of autologous grafts in a standardized process, from cell harvest to therapeutic application, was successfully implemented here for the first time with the generation of functional large-scale cartilage grafts tested in a challenging orthotopic animal model.

Chondrocytes isolated from small nasal or articular biopsies by an automated digestion unit were seeded onto a bilayer collagen/hydroxyapatite scaffold in a disposable perfusion bioreactor. In a streamlined process, cells were expanded and re-differentiated onto the scaffold under perfusion. Non-invasive monitoring of pH and dissolved oxygen using chemical-optical sensors were used as in-process controls, while extracellular matrix proteins were measured in spent medium to define appropriate release criteria. The engineered cartilage grafts were implanted in chronic femoral condyle defects in adult sheep, with cell-free scaffolds and empty defects as controls. Biochemical and histological analysis confirmed extensive cell proliferation and abundant deposition of glycosaminoglycans and collagen type-II during production. Constructs generated in bioreactor from nasal chondrocytes had significantly higher ICRS II scores than other groups at 3 months after implantation, and had completely filled the defects with hyaline repair tissue and integrated into the surrounding cartilage after 12 months.

The large animal study demonstrated feasibility of the manufacturing paradigm, and safety and effectiveness of the bioreactor-produced grafts. The findings establish a solid foundation for the automated, regulatory compliant and cost-effective production of human nasal cartilage grafts for broad clinical use.

#### Acellular Gellan-gum based Bilayered Structures for the Regeneration of Osteochondral Defects: A Preclinical Study

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In orthopaedics, treatment of osteochondral (OC) defects remains a clinical challenge. Autologous osteochondral mosaicplasty has been used for OC treatments although with donor site morbidity

present. Engineering a structure capable of mimicking different tissues (cartilage-subchondral bone) could be an approach to regenerate OC defects. We have been proposing bilayered structures to regenerate osteochondral defects [1]. This study investigates the pre-clinical performance of bilayered hydrogels and spongy-like hydrogels in *in vivo* models, in subcutaneous and orthotopic models. Bilayered structures were produced from Low Acyl Gellan-Gum (LAGG) from Sigma-Aldrich, USA. Cartilage-like layers made of 2 wt% LAGG. Bone-like layers made of 2 wt% LAGG with hydroxyapatite incorporation (20% and 30%) (w/v). Hydrogels and spongy-like were subcutaneously implanted in mice to evaluate the inflammatory response and OC defects were induced in rabbit knee to create a critical size defect: 4 mm diameter and 5 mm depth, then hydrogels and sponges were implanted. Hydrogels were injected allowing *in situ* crosslinking and spongy-like were pre-formed by freeze-drying. Subcutaneous implantation (2 weeks) and critical size OC defect (4 weeks) studies were performed. Cellular behavior and inflammatory responses were assessed by histology staining and biochemical function and matrix deposition by immunohistochemistry. Both OC structures stability and new cartilage and bone formation were evaluated by *vivo*-computed-tomography (Scanco 80). No acute inflammatory response was showed. New tissue formation and integration in adjacent tissues were observed. A novel strategy for regeneration of OC defects can be designed encompassing both, hydrogels and spongy-like structures and cellular approaches.

1. Oliveira JM. *et al.* Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells. *Biomaterials* 27, 6123, 2006.

#### Vascularized Bone Engineering using Rapid Vascular Fabrication Technique

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Fabrication of three-dimensional vascularized bone graft is a major challenge in bone tissue engineering. Here, we propose a fabrication strategy of osteon-like tissues in which a microvascular was located at the center and surrounded with calcium phosphate layers. Considering that cells consume oxygen and nutrients during fabrication processes, a rapid micromolding technique of perfusable vascular structure was established using electrochemical cell transfer. To this end, we designed a zwitterionic oligopeptide which forms a self-assembled molecular layer on a gold surface and is detachable from a gold surface by applying a negative electrical potential. Human umbilical vein endothelial cells (HUVECs) adhering on a gold needle via the oligopeptide layer were transferred to the hydrogel within 5 min, along with electrochemical desorption of the oligopeptides. Reproducible fabrication of the perfusable endothelialized microchannels can be achieved using needles of any diameter ( $\phi$ 200–700  $\mu$ m). To apply this approach to the fabrication of osteon-like structures, bone marrow derived mesenchymal stem cells (MSCs), HUVECs, and osteoblast-laden microgels containing octacalcium phosphate powder were encapsulated into the hydrogel. During perfusion culture, encapsulated HUVECs formed microvascular, MSCs and osteoblasts produced calcium substrate in the hydrogel. This can be a fundamental technique for engineering osteon-like tissues.

#### Endometrial-engineered Cell Sheets for Resumption of Fertility

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Women with endometriosis or experience of abortion surgery may suffer from endometrial stromal injury, and this injury can sometimes

cause female infertility. In view of this situation, we studied the use of autologous endometrial cells as a source of cells for the reconstruction of endometrium. We proposed a method to engineer cell sheets as an alternative therapy of endometrial reconstruction. Briefly, we harvested endometrial cell sheets on a transparent polyvinylidene fluoride (PVDF) membrane grafted disulfide bond-containing amino acid and biopolymer in order, and the disulfide bond can be cleaved by adding amino acidic reductant so that the cell sheet can be detached from the surface of the membrane. In this study, the endometrium from the mouse carrying a luminescence gene was used as a cell source. After tissue separation, DMEM/F12 medium with 10% charcoal-stripped serum were used to culture endometrial cell on the disulfide-bonding containing membrane as mention above. After cultivation, the reductant were added, and the multi-layered cell sheets were detached and were transferred onto electrospun poly L-lactide (PLLA) carrier. After that, vital assay, haematoxylin staining and immune-histochemical staining assay were used to evaluate engineered cell sheet.

In summary, engineered mouse endometrial cell sheet were successfully fabricated by a cultured cell sheet system that we proposed. The PLLA carrier was applied to hold the cell sheet for operation-ready, and the cell sheets were at survival state confirmed by the vital assay. Based on these results, follow-up experiments and assessments in clinical practice are needed to evaluate this method.

#### Influence of Longitudinal *In Vivo* Micro-Computed Tomography on Fracture Healing in a Femoral Defect Model

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Characterization of the healing process is crucial in preclinical fracture healing studies. One recent approach to non-invasively capture the entire healing process of each animal is the repeated application of *in vivo* micro-computed tomography. However, as adverse effects of longitudinal imaging on the development of bone properties have been reported in the literature in some cases, there is a need to also assess radiation-associated impact on fracture healing. Female 20-week-old C57BL/6J mice (control group: n=6; scan group n=7) received a femur osteotomy. The scan group received weekly scans of the osteotomy area (weeks 1–5), whereas controls were only scanned at week 5 (ScancoMedical, Brüttisellen, Switzerland; isotropic-voxel-resolution: 10.5  $\mu$ m; 2 stacks of 211 slices; radiation dose: 1.3 Gy/stack). Weekly micro-CT measurements during the fracture healing period did not significantly alter structural callus properties as assessed by standard parameters for control (TV: 8 mm<sup>3</sup>, BV/TV: 35%, I<sub>x</sub>: 1.8 mm<sup>4</sup>) and scan groups (TV: 8 mm<sup>3</sup>, BV/TV: 37%, I<sub>x</sub>: 1.7 mm<sup>4</sup>). Cortical bridging occurred in 86% of the control and 83% of the scan group ( $\mu$ CT evaluation program V6.5-1). In conclusion, longitudinal *in vivo* micro-CT imaging up to five times can be considered suitable for monitoring fracture healing in adult C57BL/6 mice. However, further safety evaluation should be considered when applying the scanning protocol to other mouse models, e.g. different strains, age and osteoporotic animals.

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#### Comparing PLGA and PLGA/Chitosan Nanofibers Seeded by Msc: A Cell-scaffold Interaction Study

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Recently a great deal of attention has been paid to scaffolds made from mixtures of synthetic and natural polymers. Such

polyblends include the desired characteristics of both polymer types, i.e., the increased strength and durability of the synthetic polymer and the specific cell affinity of the natural one. Among different cell types, mesenchymal stromal cells (MSC) are the most widely utilized source of autologous cells in clinic. In this respect, studies including scaffolds with appropriate interaction with MSC can be of great value.

In the present study, poly(lactic-co-glycolic acid)/chitosan (PLGA/CH) nanofibers were produced through emulsion electrospinning and their interaction with human MSC was compared to PLGA nanofibers fabricated through conventional electrospinning.

The morphological, physio-chemical and mechanical properties of above-mentioned scaffolds, as well as their cell interaction characteristics were studied using techniques such as electron and confocal microscopy, tensile testing, MTT assay and gene expression.

It was seen that the hybrid construct (PLGA/CH) represented more hydrophilicity compared to PLGA, whilst its tensile strength and porosity were similar to those of PLGA. Both the scaffold types supported MSC adhesion and gene regulation over the 2-week culture time. However, MSC cultured on PLGA/CH represented increased metabolic activity and proliferation in all time-points. According to quantitative PCR analysis, both PLGA and PLGA/CH nanofibrous scaffolds supported the gene regulation of MSC into bone, fat and neuronal cells, with no significant difference. Herein, our comparison has demonstrated that PLGA/CH is a favorable substrate for tissue engineering studies as compared to PLGA nanofibers.

#### **μCT Imaging of Polymeric Scaffolds for Bone Tissue Engineering**

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Polymeric scaffolds are of great value for bone tissue engineering (TE) as their structure, composition and shape can be modified according to the specific needs. Micro-computed tomography (μCT) is a popular non-invasive tool to determine their qualitative as well as quantitative properties such as pore diameter and interconnections. However, for cell studies, these scaffolds are used in a physiological environment and do not provide enough contrast to be segmented from fluids after taking up water. In this study, hydroxyapatite (HA) was incorporated into porous silk fibroin (SF) scaffolds to determine their morphological parameters with μCT imaging in a physiological environment. The homogenous incorporation was confirmed by histological analysis and scanning electron microscopy. μCT imaging demonstrated that with low HA concentrations, the scaffold structure could not fully be outlined in the wet state. However, higher concentrations increased the scaffold volume/total volume from the invisible SF scaffold to 4.46 ± 0.82% in scaffolds with a SF:HA ratio of 17:5 (w/w) which not only allowed to distinguish the scaffold from the surrounding fluid but also to visualize a continuous scaffold structure similar to the dry state. Knowing these structural parameters opens up several new possibilities for TE such as: i) evaluating the effects of pore size and geometry on cell growth and differentiation, ii) calculating fluid flow and nutrient transport, and iii) predicting mechanical competences of scaffolds while taking degradation processes into account.

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#### **Optimized Structural Design and Experimental validation of 3D Printed Bone Replacements**

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With the dawn of 3D printing technology, patient-specific implant designs are set to have a paradigm shift. This work presents a topology optimization method to design craniofacial bone replacement implants and perform mechanical testing on the designed implants to check its feasibility under masticatory forces. A topology optimization method in designing patient-specific craniofacial implants has been developed to ensure adequate load transfer mechanism and restore the form and function of the complicated craniofacial defect such as the mid-face. Validation of these topologically optimized finite element models using mechanical testing is a critical step. Instead of inserting the implants into a cadaver or patient, we embed the implants into the computer-aided skull model of the patient, fuse them together to 3D print the complete skull model with the implant. Until recently, strain gages have been used to measure strains for validation. However, digital image correlation (DIC) method for full-field strain measurement provides a continuous deformation field data. A digital image correlation (DIC) strain measurement technique was used to capture the full field strain on the surface of the loaded skull model. The finite element model of patient-specific craniofacial implants is validated against the strain data from the DIC obtained during the mastication simulation. The principal maximum and minimum strains were compared. The mechanical testing shows that the design can withstand the masticatory force of 534N. This work demonstrates innovative designs of bone replacement implants for patient-specific craniofacial anatomical defects using a multi-disciplinary approach involving imaging, computer aided design, and 3D printing.

#### **Time-dependent Deformation or 'Creep' of Viscoelastic Hydrogels Leads to Rac1-dependent Enhanced Spreading, Proliferation, and Differentiation in Human Mesenchymal Stem Cells**

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Whilst 'stiffness' or 'rigidity' is a recognised physical property of substrates (and tissue microenvironments) that influences many cellular behaviours, tissues and their extracellular matrices are not purely rigid but viscoelastic materials. This viscoelasticity results in such materials displaying increased deformation with time under the imposition of a defined force, that is time-dependent deformation or 'creep'. We created three viscoelastic polyacrylamide hydrogels of equivalent stiffness but that displayed varying rates of creep (over three orders of magnitude) under an imposed 3 kPa shear stress (as applied by adhered cells). hMSC fate choices were assessed in basal or induction medium for up to 7 days. We observed that with increasing substrate creep (at a constant stiffness), hMSC spread area significantly increased, FA length significantly decreased, the proliferation rate of the hMSCs increased, and myo-, adipo- and osteogenic differentiation significantly increased. Blebbistatin and Y-27632 (ROCK inhibitor) treated hMSCs on purely elastic, low creep substrates resulted in equivalent morphologies and FA lengths to untreated hMSCs on high creep substrates. Creep-induced energy loss at adhesions clearly inhibits cells building tension through actin-myosin activity. Using a real-time Rac1-FRET biosensor, we observed significantly increased Rac1 activity in the MSCs on high creep substrates, at 4 h and 24 h time points. Increased Rac1 activity facilitates spreading (to build cytoskeletal tension) and is also known to drive many other cell fate choices. Tuning substrate viscoelasticity to introduce varying levels of creep provides a new tool with which to tune and direct stem cell outcomes.

#### **Peptide Insulin for Tendon Differentiation of Human Mesenchymal Stem Cells**

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Strategies for the regeneration of tendon tissue include the use of biomimetic scaffolds in combination with physical and/or biochemical stimulation. Physical cues may arise from the topography of the scaffold, whereas biochemical signals can be delivered via exogenous growth factors. Electrospun fiber matrices are attractive tendon scaffolds as they mimic collagen fibers naturally found in the extracellular matrix and are easily fabricated. Growth factors such as GDF-5 have been observed to drive tendon differentiation, however, clinical use of growth factors proves to be a challenge, as high doses are often required for effective use. Peptide insulin has been shown to differentiate bone marrow derived MSCs after 24 hours of treatment on TCP towards tendon lineage with increased expression of tendonogenic markers (1). The current study further explores the use of insulin treatment on electrospun fiber matrices. Cells seeded on fiber matrices received a single treatment of 10 pM, 0.1 nM, 1 nM insulin, or 1 ng/ml, 10 ng/ml, 100 ng/ml of GDF-5, or control. Cell proliferation and increased RNA expression of tendonogenic markers were observed to be higher on all treatment groups when compared to control. Confocal imaging also showed a more organized ECM on cells treated with 1 nM insulin. The data suggest that a single 24-hour treatment of insulin was sufficient to drive the differentiation of MSCs, grown on fiber matrices, towards tendon. Implications for an effective single treatment of insulin may negate the need for high dose and continuous release of growth factors.

#### Potential Micro/nanoparticles/films from Nonmulberry Silk Fibroin for Biomedical Use

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Silk especially from *Bombyx mori* silkworm has been explored for various tissue engineering applications due to its excellent biocompatibility, non-toxic nature, low or minimal immune responses within host systems, tunable biodegradability, robust mechanical properties and versatility in processing based on tissue specific requirements. The present study discusses the possibilities of silk fibroin from fiber and liquid silk of two indigenous non mulberry silkworms as a potential biomaterial in biomedical application. Fibroin was extracted as liquid silk from the posterior silk gland of larvae and 2-D film was fabricated using standard methods. The films were characterized using SEM, Dynamic contact angle, FT-IR, XRD, DSC and TGA. The films of both the silkworms showed similar properties with the presence of  $\alpha$  helical conformation, hydrophobic in nature and unaffected by organic solvents. This study provides significant insight into a new arena of research in biomaterials. Silk fibroin nanoparticles have been prepared using desolvation procedure. Size of the nanoparticles were determined using zeta potential and was found to be in the range 90–185 d/nm and average surface charge of the particles were determined to be around  $-23$  mV. FTIR results have shown the presence of amide I and amide II structure at peaks  $1628\text{ cm}^{-1}$  and  $1519.40\text{ cm}^{-1}$  respectively indicating the persistence of its structural properties. SEM results have confirmed the formation of nanoparticles of above mentioned size range. XRD results have shown both amorphous and crystalline nature of the silk fibroin nanoparticles indicating peaks at 16.5 and 22.4 respectively.

#### The Use of Extracellular Matrix (ECM) Hydrogel for the Treatment of Ischemic Stroke

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Ischemic stroke as a result of middle cerebral artery occlusion affects approximately 800,000 patients annually. Treatment of this devastating disease has largely focused upon methods for living with neurologic deficits. Although cell based approaches for replacement of functional brain tissue have been attempted, none have been notably successful nor has cell based therapy been accepted as a standard of care.

Biologic scaffold materials composed of extracellular matrix (ECM) have been shown to facilitate replacement of functional tissue in many body systems and recruitment of endogenous stem cells has contributed to these positive outcomes (1–4). ECM degradation products have been shown to promote central nervous system (CNS) stem cell mobilization and differentiation *in vitro* (5, 6). Hydrogel forms of ECM have provided a viable method for effective and minimally invasive delivery to a variety of “difficult to reach” anatomic locations including the brain. The present study investigated the delivery of an ECM hydrogel into stroke regions using a rat MCA occlusion model. Method of delivery involved catheter-based, transcranial replacement of necrotic CNS debris with ECM hydrogel. Within 24 hours, the hydrogel was populated by a mixture of cell types including macrophages, and nonmacrophage mononuclear cells. Phenotypic characterization of this cell population is currently being conducted. The potential for an ECM hydrogel to create a favorable microenvironmental niche within injured CNS tissue is demonstrated in this study.

#### Fabrication of Functionally Hepatic Tissue by Bottom-up Method using Spheroids Covered with an Endothelial Layer

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Liver regenerative medicine has attracted attention as a possible alternative to liver transplantation. The major challenge of this approach is to construct implantable hepatic tissue with a high cell density and high functionality. In this study, we try to construct a highly functional three-dimensional hepatic tissue by stacking endothelial cell-covered hepatocyte spheroids (Bottom-up method). We prepared endothelial cell-covered hepatocyte spheroids in the following way. Hepatocyte spheroids were formed by rotation culture of primary rat hepatocytes. The surfaces of spheroids were coated with collagen. Then, collagen-coated hepatocyte spheroids and human umbilical vein endothelial cells (HUVEC) were co-cultured to form HUVEC-covered hepatocyte spheroids. The spheroids were collected and inoculated into hollow fibers. In this way, we fabricated hepatic tissue with high cell density. The hepatic tissues were evaluated by cell survival, liver-specific functions, and histology. As a result, we found an improvement in liver-specific functions (ammonia removal and albumin secretion) and cell survival. In addition, HUVECs were distributed regularly at every 100  $\mu\text{m}$  within the tissue, and living cells were present in the whole tissue throughout the culture period. In summary, we successfully fabricated highly functional hepatic tissue by bottom-up method using HUVEC-covered hepatocyte spheroids.

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#### Combinatorial Approach to the Creation of a Self-assembling Amphiphilic Materials Library for Drug Delivery

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Self-assembled materials made from amphiphilic molecules form lyotropic liquid crystalline nanoparticles in water with distinct internal nanostructures (i.e. mesophases). These materials have shown potential as drug delivery vehicles for a wide range of drugs including hydrophobic, hydrophilic, and amphiphilic drugs. Previous studies revealed that the mesophases and lattice size of the nanoparticles directly affected the encapsulation and release of the drugs. The aim of this work was to create a library of self-assembled nano-materials using a combinatorial approach. Mesophases of lipid nanoparticles of

monoolein, monopalmitolein, and phytantriol were modified by the addition of saturated fatty acids with carbon chain-length from C7 to C16. The nanoparticles were then characterized using high throughput synchrotron small angle X-ray scattering (SAXS). The results showed that both the fatty acid content and chain-length affected the nanoparticle mesophases and lattice parameters. The increasing fatty acid content resulted in a higher negative surface Gaussian curvature and triggered phase changes with a sequence from a primitive cubic, to a diamond cubic, a hexagonal, and an emulsified microemulsion phase. The influences of fatty acid chain-length to lipid mesophases were more complicated with the expansion and reduction of regions with hexagonal phase and diamond cubic phase respectively in samples with longer fatty acid chain-length. Additionally, longer chain fatty acids did not incorporate well with lipids at high concentrations, causing phase separation and the appearance of a lamellar crystal phase. In summary, the materials library provided valuable information for the selection of self-assembled amphiphilic delivery vehicle for a specific drug.

### 3-D Bioprinting of Skeletal Muscle Constructs for Reconstruction and Restoration of Muscle Function

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Rapid progress has been made in the development of a bioengineered muscle tissue by combining cells and biomaterials. However, the conventional tissue engineering methods are limited by the ability to build volumetric tissue constructs with cellular organization, which are inadequate for replacing extensive muscle defects. Additionally, innervation is critically important following implantation for recovery of contractile function. In this study we fabricated 3-D skeletal muscle constructs by using fibrinogen-based hydrogel containing human muscle progenitor cells and investigated whether they could be robust enough to maintain structural and functional characteristics *in vivo*. Implanted bioprinted muscles into the muscle defects developed oriented myotubes or muscle fibers with integration of host vascular and nerve tissues, and muscle mass and muscle function were increased. Our results demonstrate that creation of innervated volumetric engineered muscle tissues using the 3-D bioprinting system is feasible and that the functional muscle construct can contribute to restoration of muscle functions.

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### Silk Fibroin Nanofiber Scaffolds by Cold-plate Electrospinning for Artificial Skin Reconstruction

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Silk fibroin nanofibers fabricated by traditional electrospinning were considered an excellent candidate due to their desirable biocompatibility. However, it is generally difficult for cells to infiltrate the nanofibers due to its small pore size and sheets-like appearance. To overcome this challenge, a key parameter is the control of the pore size of silk fibroin electrospun such that cells can easily infiltrate into the nanofibers and proliferate internally. In the present study, a facile and efficient strategy have successfully been introduced that can produce 3D silk fibroin nanofibers, obviating an intrinsic limitation of traditional and salt-leaching electrospinning by introducing cold-plate electrospinning. The cell attachment and infiltration studies indicated the use of 3D nanofiber scaffolds by cold-plate electrospinning as a potential candidate to overcome intrinsic barriers of electrospinning techniques. The 3D nanofiber scaffolds using this technique presented a high porosity with controlled full-thickness and an easy way to deposit on facial curvature; the resultant nano-

fibers scaffolds laid best attributes is the ideal candidate for artificial skin reconstruction.

### Encapsulation of Protein Drug using Electrospayed Porous Poly(D,L-lactide-co-glycolide) Microparticles

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Porous poly(D,L-lactide-co-glycolide) (PLGA) microparticles were demonstrated to be used for sustained delivery of protein by entrapping protein inside the inner pores. The porous PLGA microparticles were prepared by electrospaying. Bovine serum albumin (BSA) solution were penetrated into the inner pores and then the outer surface pores were closed using a dimethyl sulfoxide (DMSO) for sustained release. BSA were studied as a model protein drug and secondary structure of encapsulating protein can be retained. The encapsulation efficiency of FITC-DEX and effects of closed pore on the properties of sustained protein drug release are also demonstrated. The porous PLGA microparticles can potentially be employed for the biomedical application to the protein encapsulation.

### Bioprinting of Mechanically Strong Cell-laden Tissue Engineering Constructs

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3D bioprinting includes various technologies that can spatially dispense materials and cells according to scanned or pre-designed shapes into 3D structures. Among them is extrusion-based bioprinting that has been widely used to print polymer melts, microparticles and hydrogels with encapsulated cells. The extrusion bioprinting technology requires the materials to be within a certain range of viscosities and a homogeneous status during printing so the material exits the print head nozzle as a continuous and uniform strand which exhibits sufficient mechanical properties to form a 3D structure without collapsing. This requirement has restricted the use of many materials during the process. For example, extracellular matrix (ECM) components have relatively long gelation time (minutes to hours) and low mechanical properties even after gelation, which make the printing of them difficult in terms of forming 3D structures that do not deform under their own weight and are robust during handling.

We have developed a novel extrusion-based bioprinting platform technology that allows the extrusion of a core-shell continuous strand that is mechanically strong and also supports intrinsic cellular morphologies and functions. The shell of the fibre consists of a mechanical strong hydrogel which is robust during handling. The core consists of decellularised ECM or ECM components that are superior to synthetic materials with regard to supporting intrinsic cell morphologies and functions. This technology could be used for fabricating load-bearing tissue engineering constructs.

### Fabrication of Bioabsorbable Bone Fixation System using Silk Fibroin

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Bioresorbable fixation systems(PLGA, PLLA etc) have been popular for the treatment of bone fractures. However, their mechanical properties are not appropriate and its price is very expensive. To overcome these problems, we fabricated a bioresorbable silk fibroin fixation plate/screw by using centrifugal force casting method. We investigated its regenerative effect on femur defects in rats. The silk fibroin plate/screw had a compressive pressure similar to that of a polylactic acid plate, and a tight, pore-free microstructure. Bilateral



segmental bone defects (2-mm length) were created in the femur of 12 adult rats. One side was fixed with the silk fibroin plate/screw, and the other side was bioresorbable plate/screw (Biosorb™) fixation. Gross inspection revealed no specific complication. At 1, 2, 4, and 8 weeks postoperatively, the femur were explored by micro-computed tomography and histological examination. New bone formation and osteoblast activity were observed in sides treated with the silk fibroin plate/screw, and bony defects were completely healed within 8 weeks. These results suggest that the silk fibroin plate/screw is a potential candidate for a new bioresorbable fixation system.

#### **Dreamer: An Innovative Bone Filler Paste for the Treatment of Periodontitis**

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Periodontitis is a bacterially induced chronic inflammatory disease that destroys the connective tissue and bone that support teeth. It is nowadays recognized that uncontrolled inflammatory response of cells from the host defense system to oral bacteria, mostly based on genetic predisposition, is at the basis of periodontal tissue loss. Existing bone filler materials work through so called scaffolding effect (osteoconduction) and sometime through selected biological molecules that stimulate bone regeneration (osteinduction). Innovative bone fillers specific for bone regeneration of patients affected by periodontitis should be endowed also with the capability to operate as a moderator in inflammatory response and oxidative stress damage. We developed an innovative paste for bone regeneration in periodontal patients by the exploitation of polyphenol molecules. Polyphenols, present in grape and winery by-products, are since long known for their antiinflammatory properties and literature suggests they also stimulate bone regeneration. This new biomaterial contains polyphenols extracted from winery by-products, phosphate ceramic particles, collagen type I. Laboratory and preclinical evaluation show that: it promotes a high antioxidant effect, not present on commercially available product, it releases polyphenols such as: gallic acid, proanthocyanidins, catechins, epicatechins, epigallocatechins in excess of 1 week; it shows, by RT-PCR of macrophages culture, a significant reduction of iNOS gene fold expression, compared with the material without polyphenols; it enhances bone regeneration in a 56 days model of bone defect in the medial condyle of rabbit femur with respect to the control. Test show that Dreamer could be an efficacy solution for periodontitis.

#### **Generation of an Autologous Oral Mucosa Substitute for Cleft Palate Repair**

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**Objective:** Cleft palate is a prevalent cranio-facial malformation and its current treatment has numerous limitations such as altered palatal bone growth and development. The objective of this work is to analyze the potentiality of a bioengineered oral mucosa in an *in vivo* model of cleft palate.

**Methods:** 4-mm palate defects were created in sixteen 3-week-old New Zealand white rabbits. Animals were categorized as: positive controls without palatal defect (PC), negative controls in which the palatal defect was not repaired (NC), animals in which the injury was repaired using an acellular scaffold (AS) and animals with the palate injury repaired with a complete bioengineered oral mucosa generated from autologous oral mucosa biopsies (BOM). Morphometric and histological analyses were performed by CT-scanning, H&E, picrosirius, Schiff Periodic acid -PAS- and alcian blue staining.

**Results:** Our results demonstrated that BOM animals showed similar palatal growth and development than PC animals. Collagen

fibers compaction and orientation and the amount of proteoglycans and glycoproteins were also similar. In contrast, NC and AS animals showed significant palatal growth alterations with differences in the composition and amount of extracellular matrix elements.

**Conclusions:** Our results confirm the *in vivo* potential of oral mucosa substitutes for the treatment of cleft palate.

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#### **The Quality Stability of Human Epithelial Cell Sheet after Transportation by Air**

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Cell sheet engineering makes it possible to harvest confluent cells as a sheet-shaped structure without trypsin digestion, which allows us to transplant intact cell sheets with extracellular matrix to a patient. In this concept, we succeeded in the transplantations of human autologous cell sheet for the purpose of those regenerations for cornea, esophageal mucosa, cardiac muscle, periodontal tissue, and articular cartilage. As a next attempt toward the practical use of the cell sheet engineering-based therapy, the preclinical study which assessed quality stability for human autologous oral mucosal epithelial cell sheet after transportation by air has been conducted. The seven hours transportation from hospital to cell-processing facility caused neither microbial contamination nor deterioration of human oral mucosal tissue and serum for fabricating a cultured cell sheet. We successfully fabricated the epithelial cell sheets by the same fabrication method prescribed in standard operating procedures. Those cell sheets were packed in the hermetically-sealed container to prevent microbial contamination and then carried back to the hospital in contact with the cell culture insert by the special container whose inside had been kept heat in 37 °C. The quality testing for the transported cell sheet showed that the quality had been maintained after the transportation. These results indicate that this transportation provides the useful epithelial cell sheet for clinical research.

#### **Stimulation of Adult Limb and Tail Regeneration with Lizard Spinal Cord Implants**

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Lizards exhibit the remarkable ability to regenerate amputated tails, making them the closest relatives of mammals to display enhanced healing abilities as adults. Yet, like mammals, lizards are unable to regrow lost limbs, distinguishing lizards as the only adult vertebrates to combine regenerative tissues (tail) and non-regenerative tissues (limbs) in the same animal. These attributes specify lizards as extremely relevant model organisms for studying and manipulating adult regeneration [1]. We hypothesize that lizard tail regeneration is induced by signals originating from the spinal cord, and transplanting spinal cord pieces into amputated lizard limbs will improve healing. Lizard spinal cord pieces were removed and/or added to the stumps of amputated lizard tails and limbs. Spinal cord removal resulted in loss of regeneration in amputated tails, and transplantation of exogenous spinal cord pieces to tail stumps lacking endogenous spinal cords restored tail regeneration. Similarly, exogenous spinal cord pieces implanted as autografts within dorsal muscles of original tail portions induced normally structured ectopic regenerates at implantation sites, resulting in multi-pronged "forked" tails. Finally, exogenous spinal cord pieces implanted into normally non-regenerative amputated hind limbs of lizards induced enhanced, yet tail-like, regenerates. In conclusion, this study has identified the lizard spinal cord as necessary and sufficient for inducing regeneration. Furthermore, the signals produced by the lizard spinal cord are remarkably robust, capable of inducing regrowth in otherwise non-regenerative limbs. Future studies will test the abilities of lizard spinal cord-derived signals to enhance healing in mammals.

## Reference

1. Lozito and Tuan. 2015. Developmental Biology.

### Effects of Mesenchymal Stem Cells on the Healing Process of Osteoporotic Bone after Biomaterial Implantation

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The main key question is influence of only stem cells or only the material on the surrounding tissue in case of combined usage of biomaterials and stem cells. The aim of work was detection of growth factors, bone resorption inhibitors and ground substance proteins in osteoporotic bone of experimental rabbits after 3 months of hydroxyapatite/tricalcium (Hap/TCP) phosphate implants supported by mesenchymal stem cells (MSC).

**Methods:** Tissue were obtained from 6 New Zealand rabbit who underwent ovariectomy to develop experimental osteoporosis, but 1.5 months later their hip bones were implanted by Hap/TCP under support of MSC. Control bone contained only Hap/TCP implants. 3 months later animals were euthanized and blocks with implants removed for detection of BMP2/4, OPG, OC and OP immunohistochemically.

**Results:** Bone demonstrated numerous osteocalcin-containing osteocytes in distance from the granulati in experimental side. Osteopontin-containing cell number was up to moderate and more prominent than in control. OPG positive osteocytes were numerous to abundant in control, but less distinct in experimental bone. BMP2/4 showed the most abundant appearance with notable domination in the experimental side.

**Conclusions:** Hap/TCP implants in combination with MSC shows inhibition of resorption, stimulation of growth and mineralization 3 months after implantation in osteoporotic bone. However, OP is more slowly renovating bone protein in comparison to the osteocalcin in 3 months time period of osteoporotic bone.

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### PDGF-BB-Loaded Injectable Gelatin Matrix in Conjunction with Extracorporeal Shock Waves for Periodontal Tissue Regeneration: *In-Vitro* Assessment

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Periodontal disease, resulting in the loss of cementum, periodontal ligament, and alveolar bone, remains an important clinical problem. Strategies using biomaterials, cell and/or growth factors for periodontal tissue regeneration, particularly at the later stages of the disease, continue to undergo investigation. We are evaluating the feasibility of an injectable form of gelatin, conjugated to hydroxyphenyl propionic acid (Gtn-HPA) and loaded with PDGF-BB, to target the endogenous cell population present on the tooth root, for treatment of the early stage of the disease process. Moreover, a simple pre-operative treatment with extracorporeal shock waves (ESWs) has the potential to increase the endogenous population of cells prior to matrix injection. The gelation rate and degree of covalent cross-linking of Gtn-HPA *in vivo* can be independently tuned by the horseradish peroxidase and peroxide, respectively. The heterogeneous population of periodontal ligament (PDL) cells isolated from goat teeth, comprising epithelial as well as connective tissue cell types, was found to survive the covalent cross-linking process with 95% viability. In our proliferation model, PDGF-BB released

from Gtn-HPA increased the number of ligament cells by many fold. A migration model demonstrated the permissiveness of Gtn-HPA of migration of PDL cells, and the presence of PDGF-BB significantly increased the number of migrating cells and the distance migrated in a dose-dependent manner. At one dose, ESWs in conjunction with PDGF-BB enhanced proliferation by 30%. These data suggest the potential advantages of Gtn-HPA matrix incorporating PDGF-BB for the treatment of the early loss of periodontal tissue.

### Sustained Regeneration of High-volume Adipose Tissue for Breast Reconstruction using Computer Aided Design and Biomaterials

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**Introduction:** Breast reconstruction is undertaken for cosmetic and psychological reasons following tumour resection. Reconstruction using silicone-based implants leads to formation of a rigid fibrous tissue surrounding the implant giving an unnatural appearance to the breast. Reconstruction using autologous tissue is also associated with tissue resorption and necrosis. We hypothesise that these problems may be overcome with a tissue engineering approach whereby fully vascularised adipose tissue can be regenerated *in vivo* using scaffolds fabricated by additive manufacturing.

**Methods:** A 3D model of the breast was extracted from a mastectomy patient using a laser digitiser [1]. The model was scaled down to 3 mL and 95% porous designs were fabricated using a 3D printer (Replicator, Makerbot Industries). Scaffolds were seeded with 20×10<sup>6</sup> human umbilical cord-derived perivascular cells (HUCPVC), cultured statically for 4 weeks, subsequently in a Tis-Xell bioreactor (QuinXell technologies, Singapore) for 2 weeks, and subcutaneously implanted into nude rats for 24 weeks. At the time of implantation, 1.3×10<sup>6</sup> Green Fluorescence Protein labelled Human Umbilical Vein Endothelial Cells (HUVEC) suspended in Matrigel were also injected into the scaffold.

**Results:** The HUCPVC proliferated within the scaffolds and were seen to spread across pores prior to implantation. The percentage of adipose tissue compared to overall tissue area increased from 37.2% to 81.2% at week 24. At 24 weeks, HUVECs were found to have self-assembled into a functional capillary network within the pores of the scaffold.

**Conclusions:** A clinically viable route to design and fabricate patient-specific scaffolds from 3D imaging data has been demonstrated.

### Long-term Pulsatile Parathyroid Hormone (PTH) Delivery from a Biodegradable and Implantable Device Promoting Bone Growth *In Vivo*

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PTH, the only FDA-approved anabolic agent to treat osteoporosis, is sensitive to the delivery method to achieve its ideal therapeutic effect. Current PTH treatment requires pulsatile administration by daily injection, which is inconvenient. An implantable and biodegradable device capable of long-term pulsatile PTH delivery would be favorable.

Pulsatile and continuous devices were fabricated to determine the optimal PTH delivery mode by using surface-eroding and biocompatible polyanhydride. The pulsatile device consisted of alternating drug layers and polyanhydride layers and the continuous device was fabricated by compressing drug-encapsulated polyanhydride microspheres into disks. *In vitro*, model drug and bioactive PTH releases were determined using MicroBCA protein and cAMP assays,

respectively. Pulsatile, continuous PTH devices and BSA control devices were subcutaneously implanted in 10d old mice for 3 weeks. The mice were then euthanized and vertebrae harvested for histological analyses and blood serum collected for ELISA analyses.

The pulsatile device delivered 21 pulses of bioactive PTH and the continuous device delivered bioactive PTH in a linear manner over 3 weeks. Pulsatile PTH significantly increased bone area and the serum bone formation marker (P1NP) levels, while continuous PTH significantly decreased bone area and increased the serum bone resorption marker (TRAP 5b) levels vs. controls. TRAP staining showed that PTH delivery (both pulsatile and continuous) increased osteoclast numbers per bone perimeter.

The implantable pulsatile device holds promise for treating conditions of bone loss without the burden of daily injections.

#### **Effects of Tubular Perfusion Pulsatile Bioreactor on Endothelial Progenitor Cells Seeded on Biodegradable Vascular Grafts**

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Small diameter vascular grafts (<6 mm) continue to have issues with long term patency stemming from thrombosis and incompatible mechanical properties. One strategy to producing antithrombotic potential is by creating an active endothelium layer by seeding cells onto the vascular graft prior to implantation. We investigated a system to accomplish this. Previous studies show that poly(glycolic acid) felt solvent cast with poly(DL-caprolactone-co-L-lactic acid) solution is mechanically compatible with native tissue [1]. For cell culture, we are using a Tubular Perfusion System (TPS) Bioreactor [3]. The cellular component was chosen to be endothelial progenitor cells (EPCs). These cells have been shown to differentiate into endothelial cells when exposed to shear stress [2]. The objective of this study was to determine if this off-the-shelf TEVG preparation system would demonstrate successful neotissue formation and endothelial-like differentiation of EPCs within the biodegradable scaffolds in comparison with grafts seeded and cultured in a static environment. Grafts were seeded then cultured for 14 days in either the TPS bioreactor or tissue-culture dishes. Proliferation was measured using cell counting and DNA quantification by pico green. Assays showed higher proliferation rates and an overall higher population for the dynamic cultured group. This was confirmed using H&E staining. PCR tested for differentiation and endothelial-like function. Von Willebrand Factor, NOS3, and VEGF were more highly expressed in the dynamic group showing active endothelial function. Selecting this method for both rapid endothelium formation and ease of use by only needing off-the-shelf components presents a promising option for the seeding and culture of vascular grafts.

#### **Enhanced Anticancer Potency using an Acid-responsive ZnO-incorporated Liposomal Drug-delivery System**

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The development of stimuli-responsive nanocarriers is becoming important in chemotherapy. Liposomes, with an appropriate triggering mechanism, can efficiently deliver their encapsulated cargo in a controlled manner. We explored the use of acid-sensitive zinc oxide nanoparticles (ZNPs) as modulators of the responsive properties of liposomes. Nanocomplexes formed by the incorporation of ZNPs in liposomes (ZNP-liposomes) were designed to demonstrate the pH-responsive release of a drug (daunorubicin) without premature drug leakage and with the maintenance of the relevant therapeutic concentrations. The nanocomplexes were spherical in shape with a narrow size distribution and showed a high drug encapsulating efficiency. Under

acidic conditions, the ZNP-liposome nanocomplexes released the loaded drug more rapidly than bare liposomes. Using flow cytometry, confocal microscopy and an MTT assay, we demonstrated that these nanocomplexes were readily taken up by cancer cells, resulting in significantly enhanced cytotoxicity. On exposure to the acidic conditions inside cancer cells, the ZNPs rapidly decomposed, releasing the entrapped drug molecules from the ZNP-liposome nanocomplexes, producing widespread cytotoxic effects. The incorporated ZNPs were multimodal in that they not only resulted in a pH-responsive drug-delivery system, but they also had a synergistic chemo-photodynamic anticancer action. This design provides a significant step towards the development of multimodal liposome structures.

#### **Infection Sensitive Drug Loaded Electrospun Nanofiber Membranes**

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Infection is the major reason for guided tissue regeneration membrane failure in clinical application. Localized and sensitive drug release from membrane is an efficient way to prevent infection from happening. A biomimetic surface modification of electrospun PCL nanofiber membrane was demonstrated. Drug was covalently grafted on the surface of the nanofiber membrane by a series of reaction to fabricate enzyme sensitive drug release profile from membrane. We used dopamine self-polymerization to form thin poly(dopamine) film on the surface of PCL nanofibers in a dopamine solution. Hydroxyl groups were introduced on the surface of the nanofibers. Silane (KH550) grafting was carried out on the surface of the poly(dopamine)-coated PCL nanofibers to introduce amine groups. An ester has been synthesized from acryloyl chloride and drug metronidazole. Then esterified drug was grafted on the surface of the nanofiber from C=C on the ester and the amine groups on the nanofibers by means of Michael addition reaction. The modification process of the nanofibers was characterized by SEM, IR, EDS and XPS. Drug released from the surface of the nanofibers under the action of enzyme. The membrane showed enzyme sensitive drug release kinetics with no cytotoxicity.

#### **Nanogels for Dual Delivery of Bone Morphogenetic Proteins and Vascular Endothelial Growth Factor in Bone Regeneration**

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The presence of recombinant human Bone Morphogenetic Protein (rhBMP-2) and Vascular Endothelial Growth Factor (VEGF) in the extra-cellular matrix show synergistic effect on the osteogenic and vasculogenic differentiation of stem cells. However, the co-expression of those proteins should be in a controlled manner to decrease side effects and increase growth factors efficacy. In this work, we have designed biodegradable nanogels (NGs) based on polyethylene glycol (PEG) chain extended with short segments of lactide (L) and glycolide (G) monomers (GL-PEG-LG) to control the release rate of rhBMP-2 and VEGF growth factors. NGs were synthesized for one and three weeks release of VEGF and BMP-2 proteins, respectively. Endothelial progenitor cells (EPCs) encapsulated in gels loaded with NGs with sustained release of VEGF in the first week of incubation had the highest VE-cadherin expression. On the other hand, mesenchymal stem cells (MSCs) encapsulated in gels loaded with NGs with sustained release of BMP-2 in the first 4 weeks of incubation had the highest ALP expression. The synthesized NGs can be used for concurrent release of BMP-2 and VEGF in biphasic matrices to induce vascularized osteogenesis.

#### **Dynamic Culture of a Full-Scale Human Femur**

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The repair of large segmental bone defects due to trauma, inflammation, and tumor surgery remains a major clinical problem. While small ( $<11\text{ cm}^3$ ) tissue engineered bone constructs have been developed *in vitro*, the need for viable constructs to regenerate tissues after massive loss remains unmet<sup>1</sup>. The objective of this work was to develop a strategy for the culture of a full-scale human femur. 3D printing technology was used to fabricate a macro-porous mold in the shape of the superior half of a human femur. We then filled the mold with over 7000 3 mm-diameter alginate beads, each containing  $1 \times 10^5$  human mesenchymal stem cells (hMSCs), and subsequently cultured the construct within the tubular perfusion system (TPS) bioreactor. While dynamically cultured, fluid flow from the TPS bioreactor was able to enter the mold through the

pores and apply shear stress to the surface of the alginate scaffolds. In addition, as previously demonstrated, oxygen diffusion and nutrient transport enhanced differentiation of hMSCs compared to static conditions<sup>2</sup>. After 8 days of culture, we aggregated the scaffolds into a single-piece construct and demonstrated viability of the cells throughout the  $200\text{ cm}^3$  composite. Additionally, gene expression analysis of the cells indicated progression towards early osteogenesis by day 8 compared to statically cultured cells. With this system, we believe we have created the largest, tissue-engineered bone construct to date, therefore accelerating the clinical application of bioengineered bone grafts.

<sup>1</sup> *Processes* 2014, 2(2), 494–525.

<sup>2</sup> *Bone* 2011, 48(2), 171–181.