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3D bioprinted human skin tissue model to study scleroderma

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Background

Systemic Sclerosis (**SSc**) is a rare and autoimmune connective tissue disease of unknown pathogenesis, characterized by vasculopathy and fibrosis due to hyperproliferation of fibroblasts and abnormal collagen deposition [1,2]. SSc is a chronic and potentially fatal disease, producing a significant invalidity given by the progressive fibrosis of the skin (*i.e.,* **scleroderma**), in the first place, and other target organs, such as lung and heart [1,3]. In recent years, many animal models resembling clinical and biological phenotypes of SSc have been developed for the study of the disease pathogenesis and related drug discovery; nevertheless, an accurate preclinical model of SSc is not yet available. The consequent inadequacy of these preclinical models makes drug development an obstacle race [4-6]. Moreover, the US government and the European Union, together with many other international organizations, strongly suggest enhancing Replacement, Reduction, and Refinement (**3Rs**) of research on animal models. On the wave of obtaining accurate, reliable and affordable disease models, nowadays different tissue models are being developed for several diseases [7]. The development of **skin tissue models**, especially using **3D bioprinting**, has already had some resonance in atopic dermatitis and other skin diseases [8,9]. Skin tissue bioprinting may allow the generation of a skin surrogate to mirror tissue complexity and recreate the cellular interplay underlying SSc in a multidimensional model, thus representing a milestone in the study of the disease pathogenesis and a valuable **testbed** for **new drug discovery**.

Aims

The **Project Life Cycle** will consist of **4 main phases.**
To achieve the ambitious goal of fabricating a representative skin tissue model:
i) We will develop and characterize **3D printed dermo-epidermal skin tissue equivalent** using primary human dermal fibroblasts (HDFs) and epidermal keratinocytes (HEKs);
ii) The 3D bioprinted construct will be stimulated with pro-inflammatory and pro-fibrotic factors for 6 days (*e.g.,* TGF-β and PDGF-BB both at 10ng/mL, and/or conditioned serum derived from SSc patients) **to induce** the pathological processes of **SSc**;
iii) We will also bioprint **primary cells** derived from the skin of **patients with scleroderma**, thus increasing the model's resemblance to the *in vivo* pathological condition;
iv) We aim to evaluate the effect of the monoclonal antibody **Ontuxizumab** (1μM for 72h) against the human transmembrane glycoprotein **CD248** (endosialin), which is overexpressed in SSc skin and known to play a key role in the fibrotic progression of the disease, thus representing a potential **therapeutic target** [10-12].
The herein described '**Proof of Principle**' *in vitro* human skin tissue model, will provide a valid **drug-screening platform** for investigating novel potential **therapeutic treatments** against SSc, as an alternative to animal testing and in agreement with the principles of the 3Rs.

Materials and Methods

1 3D bioprinting setup

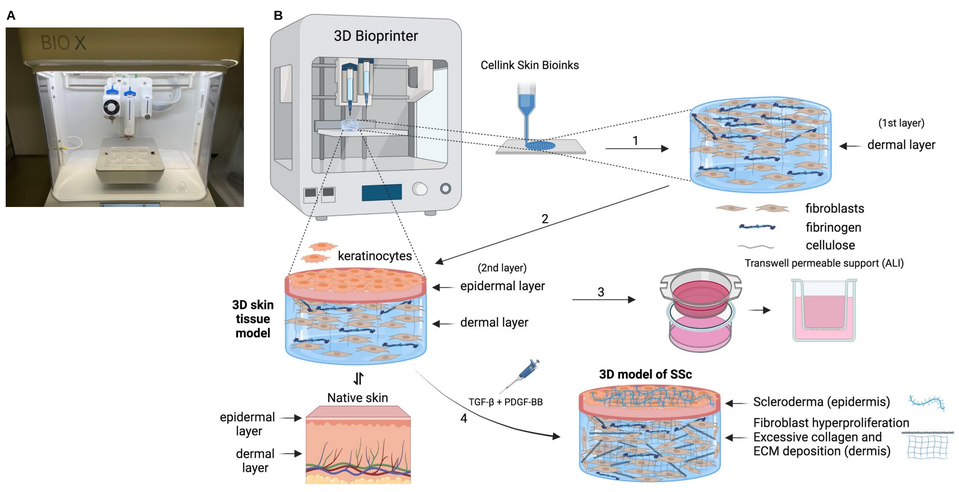


Figure 1. Blueprint of the 3D skin tissue model. A) Photo of the BIO X bioprinter with 3 exchangeable printheads (CELLINK, USA); **B)** 3D printed dermo-epidermal skin tissue equivalent (1-3) and induction of the scleroderma condition (4) using pro-inflammatory and pro-fibrotic cytokines and growth factors (*e.g.,* TGF-β and PDGF-BB) to develop a 3D model of SSc.

2 3D printing layout and CAD model

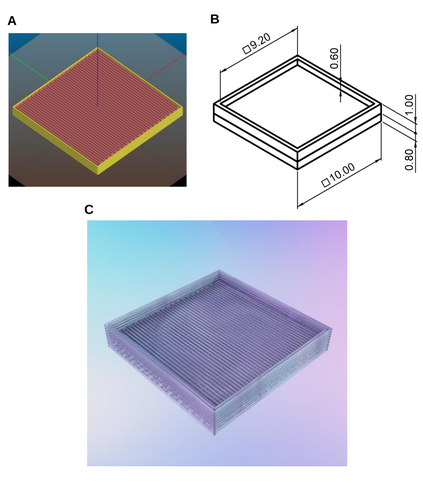
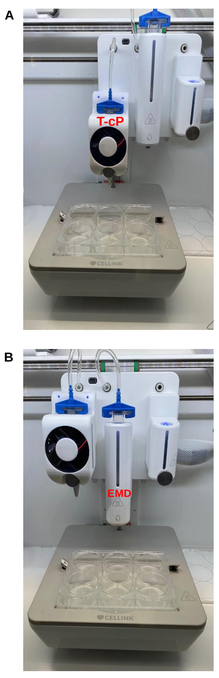


Figure 2. Geometric configuration of the 3D dermis model. A) CAD model of the dermis; **B**) layout and geometry (dimensions are expressed in mm); **C**) 3D rendering of the dermis construct, printed in Fig. 4, with dermal basket (0.6mm high) to accommodate the overlying epidermis.

3 3D bioprinting protocol

Figure 3. Bioprinting process.
Dermis: the HDF-laden GelXA Skin bioink (CELLINK) was printed in gel state through a temperature-controlled printhead (T-cP in Fig. A) set at 27°C, extruded into a dish through a 200μm nozzle, 27kPa pressure, 5mm x s^{-1} printing speed, 300ms pre-flow delay, 15°C printbed temperature. Squared structures (10mm side) with a homogeneous fiber thickness (200μm ca.) were obtained by depositing four layers of fibers at 40% infill density for the **reticular dermis** and two layers at 80% infill density for the **papillary dermis** in a wood-pile structure. After printing, photocuring (405nm) and chemical crosslinking (ionic+thrombin o.n. at 37°C) were performed; HDFs density: 8 x 10^6/mL bioink. Dermis will be cultured for 5-7 days, until proper type I collagen synthesis and deposition will be achieved and assessed by immunostaining, before bioprinting of epidermis.
Epidermis: after 5-7 days in culture, the HEK-laden GelMA FIBRIN bioink (CELLINK) will be extruded through an electro-magnetic droplet printhead (EMD in Fig. B) on top of the dermal basket shown in Fig. 2 at 27kPa, 2 mm x s^{-1} printing speed, 20ms opening time and 100ms cycle time at 37°C; HEKs density: 7-10 x 10^6/mL bioink. After photocuring, epidermis will be grown submerged until confluence, then switched to air-liquid interface (ALI in Fig. 1B) culture on transwell inserts for another 7 to 14 days, before final skin thickness analysis and construct characterization.



Results and Discussion

4 Characterization of the 3D-printed scaffold

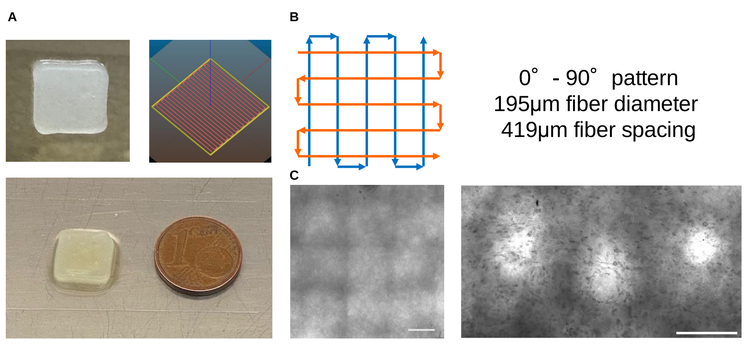


Figure 4. Microscopic characterization of the 3D-printed dermis construct. 3D printing of a six-layer dermis construct with parallel-aligned fibers was performed. **A)** top left: macrograph (10mm x 10mm x 1.2mm, L x W x H), top right: 3D layout, bottom: real size of a construct; dermis scaffolds were extruded through a 200μm nozzle. **B)** Printing scheme of two perpendicular dermis layers in a wood-pile structure (0° - 90° pattern), nozzle diameter = 200μm, mean fiber diameter = 195±6.8μm with a homogeneous fiber spacing of 419.3±5.2μm. **C)** Optical micrographs of a multilayered construct; left: bright-field microscopy image (4X) of the pristine bioink mesh, 24h post-printing, captured from the bottom layer (reticular dermis with a rectilinear pattern of the grid, 40% infill density), showing high-shape fidelity with the CAD model and uniformity of the porous texture; right: bright-field microscopy image (10X) of the HDF-laden bioink taken 48h post-printing; scale bars: 100μm.

5 Post-printing analysis of dermis viability

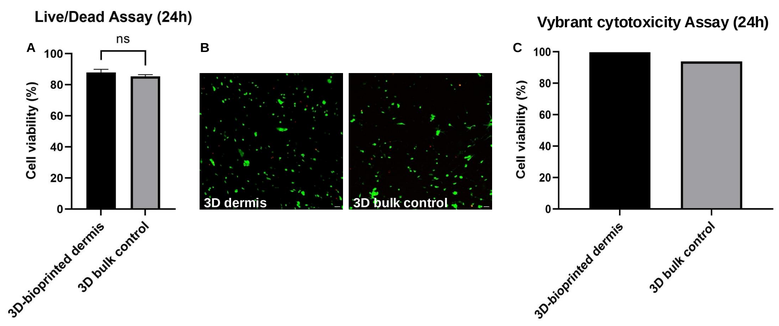


Figure 5. Analysis of cell viability 24h after bioprinting. Biocompatibility of both the bioprinting process and the GelXA Skin bioink was assessed 24h after printing (t_0) *via* a Live/Dead assay (**A**) on HDFs cultured into the 3D-bioprinted dermis *vs.* the 3D bulk control with the same volume of bioink and cell density used in the bioprinted hydrogel: comparable, with no significant difference (ns) p=0.24, and very high levels of cell viability, close to 90%, were detected between 3D dermis and bulk control; representative confocal images (10X, Olympus, Japan) are shown in (**B**), with live cells in green and dead cells in red, scale bars: 50μm. Excellent biocompatibility and higher levels of cell viability (close to 99%) in the 3D-bioprinted dermis were also confirmed by the Vybrant cell viability/cytotoxicity assay (**C**) compared with the 3D bulk control (94%).

6 Analysis of dermis proliferation and viability during a week in culture

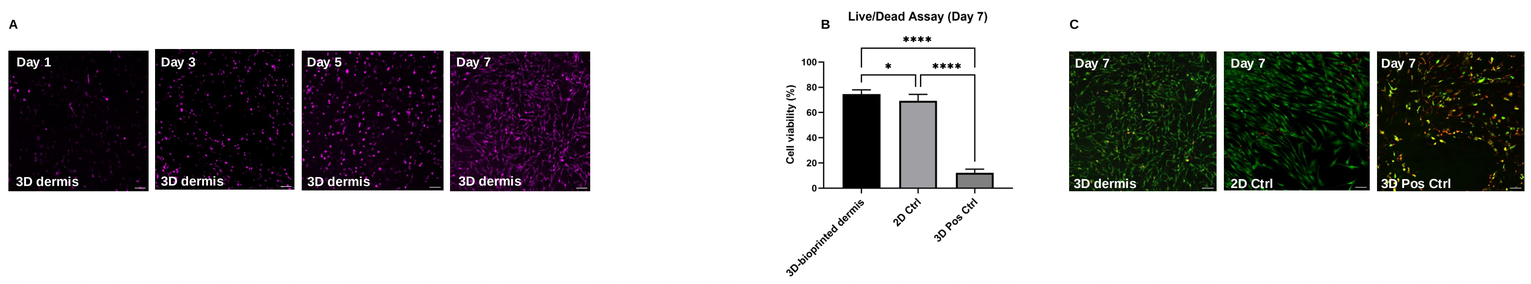


Figure 6. Analysis of dermis cell growth and viability. A) Confocal fluorescence imaging of HDFs grown in culture from day 1 thru day 7 after 3D bioprinting; cells were stained with the CellTracker Deep Red fluorescent dye (violet), showing progressive growth and increasing number, scale bars: 100μm. **B**) Live/Dead Assay after 7 days in culture showed higher cell viability (green cells in **C**) in the 3D dermis *vs.* 2D adhesion control (2D Ctrl) and 3D positive control (3D Pos Ctrl) of cell death (red cells in **C**, *p<0.05, ****p<0.0001); **C**) representative confocal images (10X, Olympus, Japan), scale bars: 100μm.

Conclusions and Future Perspectives

- Our *Proof of Principle* **3D human skin tissue model** may represent a reliable platform to mimic the condition of **SSc**.
- The herein proposed **3D scleroderma model** may represent a human-relevant setting to corroborate the efficacy of the CD248 inhibition in skin manifestations of SSc.

References

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Acknowledgments

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