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REVIEW

Focus on the road to modelling cardiomyopathy in muscular dystrophy

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Abstract

Alterations in the *DMD* gene, which codes for the protein dystrophin, cause forms of dystrophinopathies such as Duchenne muscular dystrophy, an X-linked disease. Cardiomyopathy linked to *DMD* mutations is becoming the leading cause of death in patients with dystrophinopathy. Since phenotypic pathophysiological mechanisms are not fully understood, the improvement and development of new disease models, considering their relative advantages and disadvantages, is essential. The application of genetic engineering approaches on induced pluripotent stem cells, such as gene-editing technology, enables the development of physiologically relevant human cell models for *in vitro* dystrophinopathy studies. The combination of induced pluripotent stem cells-derived cardiovascular cell types and 3D bioprinting technologies hold great promise for the study of dystrophin-linked cardiomyopathy. This combined approach enables the assessment of responses to physical or chemical stimuli, and the influence of pharmaceutical approaches. The critical objective of *in vitro* microphysiological systems is to more accurately reproduce the microenvironment observed *in vivo*. Ground-breaking methodology involving the connection of multiple microphysiological systems comprised of different tissues would represent a move toward precision body-on-chip disease modelling could lead to a critical expansion in what is known about inter-organ responses to disease and novel therapies that have the potential to replace animal models. In this review, we will focus on the generation, development, and application of current cellular, animal, and potential for bio-printed models, in the study of the pathophysiological mechanisms underlying dystrophin-linked cardiomyopathy in the direction of personalized medicine.

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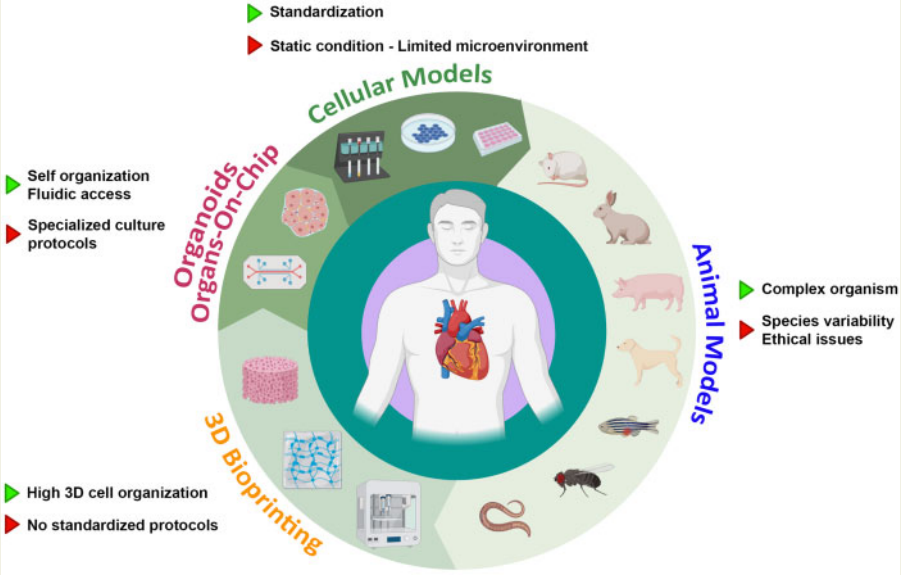
Graphical Abstract



Keywords Duchenne muscular dystrophy • Cardiomyopathy • Disease modelling • Cellular modelling • 3D Bioprinting • Personalized medicine

1. Introduction

The absence, deficit, or alteration of the dystrophin protein leads to dystrophinopathy conditions characterized by degeneration of muscle tissue and therefore progressive loss of strength and reduction of motor skills. Duchenne muscular dystrophy (DMD) is a rare, genetic dystrophinopathy in which the sarcolemma dystrophin protein is completely absent. This absence involves the manifestation of symptoms generally between 2 and 6 years of age.[1–3] DMD mainly affects males with an estimated prevalence of 1/3500–1/9300 live male births, with an early childhood onset signified by delayed ambulation and overall development. Untreated DMD children rarely attain the ability to run or jump.[4] High levels of muscle protein creatine kinase, increased liver enzymes (AST and ALT), but especially genetic testing, contribute to diagnosis and good clinical management.

Alterations of the *DMD* gene, which codes for dystrophin and is located on the X chromosome, cause two forms of dystrophinopathy: DMD and Becker's muscular dystrophy. *DMD*-linked cardiomyopathy (*DMD*-CM) is currently the main cause of morbidity and mortality in people with *DMD* mutations up to the third or fourth decade of life. Some authors[5] have shown that the long-term clinical outcomes of heart transplantation (HTx) in selected patients with dystrophinopathies are similar to those of a matched cohort of transplanted idiopathic dilated cardiomyopathy patients. However, in an era of donor shortage, there is a reluctance to offer HTx to these patients who have a limited life expectancy. The use of left ventricular assist devices has recently been highlighted as an alternative therapeutic option to HTx.[6,7] Early diagnosis of cardiac dysfunction is necessary to allow the therapeutic establishment of various classes of drugs, such as corticosteroids, beta-blockers, ACE inhibitors, and mineralocorticoid diuretics, and new pharmacological and surgical solutions in multimodal and cross-disciplinary care for this patient group.[8–10] Novel functional genomic approaches, such as Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9 (CRISPR-Cas9) gene targeting, are designed with the aim of inducing the expression of a useful gene product by attempting to restore dystrophin protein function and re-establish normal myocyte physiology. Gene editing remains one of the methods with significant potential in regenerative and precision medicine.

The pathogenesis of *DMD*-CM is not fully understood and the limitations of animal models to exactly reproduce human muscle disease and predict relevant clinical and therapeutic effects necessitate further research in modelling *DMD*-CM. The generation of induced pluripotent stem cells (iPSC) from DMD patients and their differentiation into cardiomyocytes (iPSC-CM) are recent strategies of extreme interest for uncovering the pathological mechanisms of *DMD*-CM. In particular, these novel approaches, combined with the field of genome editing, aim to not only correct *DMD* mutations but also offer the possibility to study the mechanisms underlying *DMD*-CM.[11] Additionally, the application of genetic- and bio-engineering approaches to iPSC allows the generation of physiologically relevant human tissue models for *in vitro* DMD studies. For example, the adoption of 3D bioprinting technology grants the control of finer aspects, such as the cell positioning process, cell concentration, and the diameter of the printed cell constructs[12] while also supporting the creation of internal tissue lumen. The potential to obtain 3D models of *DMD*-CM is a promising element in the perspective of unearthing new therapeutic strategies. The combination of iPSC with bioengineering technologies, such as 3D bioprinting, represents a great promise for the study of *DMD*-CM and for the future of personalized

medicine. The *Graphical abstract* shows a schematized overview of state-of-the-art tactics for *DMD*-CM modelling.

In this review, we will focus on the generation, development, and application of current animal, cellular, and 3D bio-printed models to the study of the pathophysiological mechanisms underlying *DMD*-CM.

2. Genetic and pathophysiological underpinnings

The *DMD* gene is the largest known human gene spanning 2.4 Mb, which produces a 14 Kb mRNA transcript from 79 exons. *DMD* encodes several dystrophin isoforms present in striated and smooth muscle, brain, retina, and kidney. Deletions represent the most common type of mutation underlying DMD; other *DMD* mutations are insertions and point mutations usually resulting in premature stop codons and the termination of dystrophin protein synthesis.[13] Due to the large size of introns, splicing enhancers play a central role in dystrophin mRNA maturation. Consequently, effective antisense strategies, inducing exon skipping, use splice junctions and/or exonic splicing enhancer sequences as therapeutic targets.[14,15] Dystrophin, together with the dystrophin-associated glycoprotein complex (DGC), offers structural support and stability to the sarcolemma during muscle contraction by connecting the actin cytoskeleton to the extracellular matrix (ECM). In addition to sarcolemma stabilization, the DGC is involved in gene expression related to muscle activity. Mechanical stress in the heart of dystrophin-deficient mice upregulates genes involved in the intracellular signalling of calcineurin, p38 mitogen kinase activated protein, and c-Jun N-terminal kinase, in addition to the integrin-signalling pathway.[16] The pathophysiological mechanisms underlying *DMD*-CM are frequently represented by sarcolemma instability, calcium dysregulation, reactive oxygen species (ROS) increasing, nitric oxide deregulation, and fibrosis.[17] Multiple factors contribute to the hallmarks of *DMD*-CM, i.e., contractile dysfunction and, death of cardiomyocytes and myocardial fibrosis.

3. DMD modelling strategies

Several *in vitro* and *in vivo* animal models of DMD have been observed or generated using gene editing strategies, such as CRISPR-Cas9, zinc finger nucleases, somatic cell nuclear transfer (SCNT), and transcription activator-like effector nuclease (TALEN).[18–21] These have advanced the understanding of the mechanisms underlying the onset and progression of dystrophinopathies and are essential for testing the impact of new treatment strategies on disease-specific pathophysiology and tissue function loss. We will provide an overview of these models specifically concentrating on their suitability to design cardiac pathology and screen new therapeutic strategies.

3.1 Non-mammalian

Caenorhabditis elegans, Drosophila melanogaster, and *Danio rerio* are the three most commonly used non-mammalian models. They have a short life cycle, generate large progeny size, and are genetically manipulable, which make them excellent for screening several chemical compounds of unknown function. Gieseler *et al.* and, more recently, Hewitt *et al.*, have used the *C. elegans* DMD model to identify new drug candidates and validate drugs known for their ability to improve muscle strength in DMD patients.[22–24] *Drosophila melanogaster* DMD models share muscle weakness and cardiac dysfunction comparable to DCM and morphological changes in the wing vein.[25–27] Taghli-Lamallem *et al.*[25] also showed that the loss of functional dystrophin in Drosophila dys-deficient heart leads to alterations in cardiac performance, such as an increase in heart rate by shortening the diastolic intervals (relaxation phase) of the cardiac cycle. The muscles of *Da. rerio* DMD mutants display serious histological lesions (necrosis, inflammation, and fibrosis) that lead to premature death similarly to DMD patients.[28] These models are useful for further studies as they yield excellent reproducibility because they are unaffected by the inherent complexity and individual variation present in mammalian models.

3.2 Mammalian

3.2.1 Murine

The *mdx* mouse, which is the most widely used model to study DMD, is the outcome of a spontaneous mutation resulting in a stop codon in exon 23 of the *DMD* gene.[29] By 3 months of age *mdx* mice have altered metabolic processing associated with increased oxygen consumption, decreased cardiac efficiency, and increased cell membrane fragility.[30] Heart-to-bodyweight ratios of 6-month-old mice suggest that the *mdx* heart is hypertrophied compared to wild-type hearts. Severe dilated cardiomyopathy and cardiac fibrosis occur in aged *mdx* mice (20–22 months of age).[31,32] However, the dystrophic phenotype of *mdx* mice is mild because dystrophin is replaced by the homologous protein utrophin.[33] The genetic elimination of utrophin in *mdx* mice generated a double-KO model (*mdx*/*utrn*$^{-/-}$) that has a more severe phenotype comparable to the human disease characterized by growth retardation, weight loss, spinal curvature, and premature death.[34] More specifically, the *mdx*/*utrn*$^{-/-}$ model presents skeletal and cardiac muscle degeneration that starts at 2 weeks from birth; however, the cardiac ventricular dilation is not comparable to humans.[35]

The identification of more than 7000 mutations in DMD patients compelled the development of sequence-specific therapies, such as exon-skipping and genome editing, but also raised the necessity for more variety in the *DMD* mutations represented in murine models. The *mdx*4cv and *mdx*52 mice are the first dystrophinopathy strains that harbour mutations in the major hotspot region located between exons 45 to 55 or 2 to 10, respectively and are used to test CRISPR-mediated gene repair therapy.[36–38] Additional humanized DMD mouse models were generated, e.g., hDMDdel45/*mdx* and hDMDdel52/*mdx* generated using CRISPR-Cas9 to remove a specific exon of the knock-in humanized dystrophin gene.[39–41] The hDMDdel45/*mdx* model backcrossed to DBA/2J mouse presents histopathological features of DMD, such as poor regeneration, atrophic muscle, elevated plasma creatine kinase levels, and accumulation of fibrosis.[41,42] The deletion of exon 52 in the hDMDdel52/*mdx* model, leads to fibrosis, inflammation, degeneration, and impaired muscle function similar to the *mdx* mouse. However, further studies are needed to characterize skeletal and cardiac muscle performance.[40] Although the C57BL/10ScSn-DMD^{mdx}/J (BL10-*mdx*) mouse is among the most used murine DMD model, the disease phenotype is much more attenuated than that of DMD patients. Therefore, a DMD mouse model generated by crossing BL-10-*mdx* mice on a DBA/2J genetic background, which exhibits a more severe dystrophic phenotype, is becoming increasingly of interest as it has the potential to improve the effectiveness of preclinical studies.[43] In this strain, cardiac pathology is characterized by accumulation of fibrosis and calcification at an earlier age (10 weeks of age) compared to BL-10-*mdx*.[43]

3.2.2 Rat

While murine strains partially mimic the human disease due to their small size and development of minor cardiac dysfunctions, the rat DMD model is 10 times bigger and exhibits more complex and accurate motor coordination compared to *mdx* mice. Dystrophin-deficient rats were generated using TALEN and CRISPRs/Cas9 genetic approaches targeting *DMD* exon 23 and exons 3 and/or 16 respectively.[44–46] TALEN-mutated DMD^{mdx} rats displayed severe skeletal muscle necrosis and regeneration at early life stages and, at 7 months of age presented fibrosis and adipose tissue infiltration that compromised motor activity. Further echocardiographic analysis revealed left ventricular (LV) wall thinning and increased ventricle diameter indicating a progressive dilated cardiomyopathy.[46] DMD rats, generated using CRISPR-Cas9, showed cardiac dysfunctions similar to DMD patients, e.g., by 10 months of age CRISPR-Cas9-mutated rats exhibited decreases in LV fractional shortening (LVFS) and histological accumulation of fibrosis.[47,48]

3.2.3 Rabbit

Recently, Sui *et al.* generated a dystrophic rabbit model using CRISPR-Cas9 target exon 51. *DMD* knockout rabbits exhibited the typical signs of DMD, which included increased serum creatine kinase levels, muscle inflammation, atrophy, necrosis, and fibrosis. Echocardiography also highlighted a significant reduction in left ventricular ejection fraction and LVFS at 4 months of age, which was caused by myocardial inflammation, fibrosis, and fatty cell infiltration.[49] Therefore, this study describes an animal model valid for preclinical *DMD*-CM studies.

3.2.4 Simian

To better understand DMD pathogenesis large animal models of DMD have been generated. Chen *et al.* reported the production of a DMD rhesus monkey model using CRISPR-Cas9 to introduce mutations in exons 4 and 46 of the *DMD* gene. Muscle degeneration was evident in this model at early disease stages compared to other models; however, detailed assessments of cardiac tissue or heart functions were not performed.[50] Nevertheless, this model was recently used to evaluate the effect of tacrolimus on skeletal muscle transduction with Adeno Associated Viral (AAV) vectors containing microdystrophin gene.[51]

3.2.5 Canine

The golden retriever muscular dystrophy (GRMD) dog is characterized by a spontaneous splice site mutation in the dystrophin gene. It displays a clinical course and disease severity much more similar to humans than mice models. The larger body size makes it an attractive means to assess cardiac functions and validate gene therapy approaches.[52,53] However, preclinical studies using canine models are limited due to the high costs and the difficulty of establishing and maintaining the colonies. Despite these drawbacks, several studies, performed on a very limited number of young GRMD dogs, revealed similarities with human dystrophic cardiomyopathy, such as electrocardiographic, echocardiographic, and histopathologic abnormalities including observations from carrier females.[54–58] A notable and most recent clinically relevant application of the GRMD model by Guo *et al.*[59] provided a detailed characterization of the GRMD cardiac phenotype as assessed by a longitudinal 2D echocardiography and cardiac magnetic resonance with late gadolinium enhancement, which allowed semi-quantitative measurement of myocardial fibrosis. The study revealed that EF and FS correlated with age, systolic dysfunction began at 30–45 months of age, circumferential strain was a better readout vs. EF for early disease detection, LV chamber dilation, LV lateral wall lesions, and early occurring septal fibrosis. Most interestingly, they developed a multi-parametric cardiac scoring system, which uncovered the parallel decline of skeletal and cardiac muscle function. This study provides the strongest evidence as to the suitability of the GRMD model for preclinical therapeutic studies. Furthermore, the cardiac scoring system, which is based on standardized myocardial segmentation and nomenclature for tomographic cardiac imaging,[60] will permit the comparison with GRMD dogs in different locations, thus furthering preclinical testing procedures and potential.

3.2.6 Porcine

Porcine models are critically relevant for translational research because they share several similarities with humans in terms of body size and, organ dimensions and functions. Klymiuk *et al.*[61] using gene-targeting technologies have generated a porcine DMD model by deleting exon 52 of the *DMD* gene ($DMD^{\Delta exon52}$) a mutation frequently reported in humans. The animals showed evidence of biochemical and histological hallmarks of DMD, such as mobility impairment and severe myopathy. A significant limitation of this DMD model lies in the fact that affected males do not live to reproductive age, consequently maintaining a breeding colony is impossible. However, a follow-up report from the same group detailed the introduction of the same mutation into female cells followed by the generation, via SCNT, of female pigs that produced male DMD piglets in the first offspring.[18] The $DMD^{\Delta exon52}$ porcine model was also employed for testing a CRISPR-Cas9-based therapeutic approach aimed at restoring an intact *DMD* reading frame.[62]

4. iPSC-CM

Cellular modelling aims to artificially reconstruct pathological condition in a manageable and correctable external environment. Toward this aim, iPSCs represent an unlimited source of cells from each early developmental layer, i.e., endoderm, mesoderm, and ectoderm. Human dermal fibroblasts were commonly used to derive human iPSC; however, there are alternative cell sources that are easier to obtain, e.g., peripheral blood mononuclear cells.[167] iPSCs are generated through a process known as 'cellular reprogramming', consisting of the forced expression of specific transcription factors including Oct3/4, Sox2, Klf4, and c-Myc or other combinations of transcription factors, such as Nanog and Lin28.[63]

The strategies used for iPSC myogenic differentiation are subdivided into two approaches: transgenic (by forced expression of Pax7 or MyoD) or non-transgenic (co-culture, embryoid bodies, small molecules, and extracellular vesicles).[64–69] DMD patients' iPSC-derived cardiomyocytes (iPSC-CM) have shown abnormalities consistent with *DMD*-CM pathophysiology, proving an important means to *in vitro* model *DMD*-CM, which can be utilized for mechanistic studies and drug screening.[70–72] However, the genetic and morphological characterization of iPSC-CM revealed limitations regarding their state of relative functional and structural immaturity. In particular, questions to keep in mind concern the morphological similarity with early foetal cardiomyocytes, the electrophysiological characteristics, and the differences in contraction mechanisms between iPSC-CM and adult cardiomyocytes.[73] To date, nine different mutations in the *DMD* gene have been studied using iPSC-CM models that involved the evaluation of myocardial cell damage markers and Ca^{2+} handling.[74] An example of particularly differential application of iPSC-CM obtained from DMD patients is represented by the work of Gartz *et al.* who demonstrated the cardioprotective effects of exosomes

in the context of *DMD*-CM. Specifically, exosome-induced ROS decrease depended on the activation of MAPK, ERK1/2, and p38 signalling,[75] and microRNA cargo.[76] Electrophysiological studies represent another intriguing application of iPSC-CM for modelling *DMD*-CM. Eisen *et al.*[77] evaluated the cellular mechanisms underlying electrophysiological abnormalities and cardiac arrhythmias in iPSC-CM from a DMD patient and a symptomatic carrier female that displayed lower spontaneous firing rates, increased beat rate variability (female carrier only), arrhythmias, and prolonged action potential duration, decreased pacemaker channel density (male patient only) and increased L-type Ca^{+2} current. More recently, the use of iPSC-CM was employed to test novel mechanisms underlying the development of *DMD*-CM.[78] Indeed, the iPSC-CM obtained by Kamdar *et al.* replicated the phenotype of increased arrhythmias due to irregular calcium transients, which were exacerbated by the addition of isoproterenol, a β-adrenergic agonist. They also demonstrated that *in vitro* β-blocker treatment decreased the incidence of arrhythmogenesis. Importantly, they also undertook a transcriptome study that showed LV tissue samples and iPSC-CM isolated from DMD patients shared similar dysregulated pathways. Although there are many examples of the routine incorporation of iPSC-CM in *DMD*-CM modelling paradigms, the continued application of iPSC-CM in the fields of *bone fide* high throughput drug screening and clinical translation of cell therapy products for replacing damaged cardiomyocytes with corrective iPSC-CM will require resolution of the many challenges within this field.[79–81]

Despite the expanding the repertoire of DMD models, the perfect DMD model has not been developed yet. Nevertheless, each model provides information that is useful both for basic research and preclinical studies concerning DMD pathology. The current clinical and pre-clinical studies of *DMD*-CM are summarized in *Table 1*. It is likely that a successful new therapy will be the result of integrated studies conducted in different animal species and precision iPSC-based models.

5. Application of genetic engineering approaches to *DMD*-CM

It is well known that different models have different purposes, including a wide range of advantages and disadvantages, related to ethics and social perceptions, costs, space, maintenance, phenotypic representation, and suitability for the development of pre-clinical studies.[87,88] We next turn our attention to highlight how research garnered from preclinical DMD models can be extrapolated and combined with ancillary technologies, such as gene editing to aid the growth and further integration of genetic engineering approaches within the DMD context.

5.1 CRISPR-Cas9 gene editing

The expansion and vigorous interest in CRISPR-Cas9 technology, as an innovative genome modification system, lead to an increasing capacity and versatility of gene sequence manipulation and potentially offers outstanding therapeutic possibilities for cardiomyopathy.[89] This technology involves the co-ordinated activity of a Cas9 nuclease and a single guide RNA molecule (sgRNA) that recognizes its genomic target through the pairing of complementary bases between the 5' end of the sgRNA sequence and a predefined DNA sequence (known as the Protospacer and is destined to be the site of exchange of new donor DNA). Cas9 requires a short-recognized sequence called the Protospacer Adjacent Motif for DNA cleavage. This method has the ability to target multiple distinct genomic *loci* by co-expressing a single Cas9 protein with multiple sgRNAs. One of its most interesting applications is the correction of genetic mutations associated with hereditary diseases.[90] Shimo *et al.*[85] removed DMD exons 51–57 in a human rhabdomyosarcoma cell line using the CRISPR-Cas9 system, which allowed, among other things, the evaluation of a splice-switching oligonucleotide with the ability to target most of the rare mutations reported for the *DMD* gene. In a recent study, Jin *et al.*[86] demonstrated that CRISPR-Cas9 directed deletion of mutant exon 23 (ΔEx23) with short palindromic repeats at regular intervals and targeted integration mediated by a homology-directed repair donor vector can correct dystrophin gene expression in iPSC, effectively leading to successful virus-free *DMD* gene therapy. Additionally, compared to packaging CRISPR-Cas9 DNA within viral vectors, delivery of CRISPR-Cas9 as ribo nucleo proteins (RNP) would facilitate strong target cleavage and reduce adverse effects. However, since RNP are quickly degraded, relative to DNA this approach requires a system to efficiently package, protect, and deliver the RNP to target tissues e.g. combining RNP with nanomaterials. Indeed, intramuscular injection of an RNP delivery system based on extracellular nanovesicle-mediated delivery (nanoMEDIC) achieved over 90% exon skipping efficiency in DMD patients' iPSC-derived skeletal myocytes.[91]

5.2 Exon skipping

Exon skipping allows the restoration of the disrupted *DMD* reading frame and therefore leads to the successful production of a shortened but functional dystrophin protein.[92] Skipping exon-53, using anti-sense oligonucleotides (ASO), is a promising therapy to correct the disruption of the reading frame that the underlying *DMD* mutation disturbs and, in turn, leads to the absence of the functional protein. Komaki *et al.*[93] have recently completed a phase I study based on the systemic administration of a phosphorodiamidate morpholino oligomer (PMO) to induce the exon-53 skipping in DMD. Also, of remarkable interest is exon-51 skipping, which could interest a large portion of the DMD patient population. Eteplirsen is a PMO designed to re-establish out-of-frame *DMD* gene mutations and expression of a truncated dystrophin protein in patients responsive to exon-51 skipping.[94] Antoury *et al.*[95] successfully identified exon deletions in the *DMD* gene using extracellular mRNA (exRNA) isolated from the urine of DMD patients. This approach is a distinct advantage as traditionally the detection of the activity of ASO therapy in DMD patients involves carrying out multiple muscle biopsies to check the removal of the target exon from mRNA transcribed from *DMD* and quantification of dystrophin protein production. Studying exRNA in the urine of six DMD therapy-naïve patients, they found gene-specific deletion transcripts. Furthermore, exon-51 skipping activity after treatment with Eteplirsen was also confirmed by this elegant non-invasive 'liquid biopsy' which holds great promise for evaluating the target engagement efficacy of novel ASOs.[95] Restoring dystrophin expression by inhibiting *DMD* translation termination, induced by nonsense mutations, is the principle on which read-through therapy rests. A Phase 1 study in patients with documented stop codon mutations receiving gentamicin for 6 months, one of the first identified compounds with such properties, showed increased dystrophin expression in some patients.[96]

5.3 Gene therapy

Gene therapy should lead to safe and long-term therapeutic effects. However, limitations associated with delivery vectors are well known, e.g. the need for high tropism to skeletal, diaphragmatic, and cardiac muscles, which necessitates systemic delivery and high doses increasing the potential for immune system activation.[97] Therefore, it is still necessary to undertake innovative multidisciplinary research to drive

Table 1 Clinical, pre-clinical gene therapy studies and non-mammalian and mammalian models

Gene therapies	CI	Therapy	Start date	No. patients	Mechanism	Phase/State
Exon skipping	NCT02255552	Exondys 51 (Eteplirsen)	2014	109	Skips exon 51	III/Completed
	NCT02500381	SRP-4053 SRP-4045	2016	222	Skips exon 53; exon 45	III/Ongoing
	NCT02667483	DS-5141b	2015	7	Skips exon 45	I-II/Ongoing
	NCT02740972	NS-065/NCNP-01	2016	16	Skips exon 53	II/Completed
Stop codon read through	NCT02369731	Translarna	2015	270	Reverses the effects of non-sense mutations	Ongoing
AAV-mediated therapies	NCT03333590	GALGT2	2017	6	Increases muscle protein production	I-II/Completed
	NCT03769116	SRP-9001	2018	41	Introduces a gene coding for mi-cro-dystrophin	II/Ongoing
	NCT03368742	SGT-001	2017	16	Introduces a gene coding for mi-cro-dystrophin	I-II/Ongoing
	NCT04281485	PF-06939926	2017	99	Produces a shorter version of the dystro-phin protein.	III/Ongoing

Gene therapy	Authors	Mechanism	Pre-clinical study
CRISPR/Cas9	Kim *et al.*[82]	Cytidine deaminase fused to catalytically inactive Cas9 introducing a point mutation leading to a premature stop codon in the exon 20 of the *DMD* gene	
	Amoasii *et al.*[83,84]	Short palindromic/CRISPR/Cas9 repeats eliminating exon 50 or using a single guide RNA that created reframing mutations and allowed skipping of exon 51	
	Young *et al.*[41]	CRISPR deletion of exons 45–55 for the generation of humanized DMD mouse models	
	Sui *et al.*[49]	Dystrophic rabbit model generation by co-injection of Cas9 mRNA and sgRNA targeting exon 51 into rabbit zygotes.	
	Min *et al.*[39]	Correction of exon 44 deletion mutations by CRISPR-Cas9 gene editing in cardiomyocytes obtained from patient-derived iPSC	
	Shimo *et al.*[85]	CRISPR editing of exons 51–57 allowing the evaluation of a splice-switching oligonucleotide	
	Jin *et al.*[86]	CRISPR editing of exon 23 with short palindromic repeats at regular intervals	
	Moretti *et al.*[62]	AAV6-Cas9-g51-mediated excision of exon 51	

Current animal models	Phenotype
Non-mammalian models	
Caenorhabditis elegans	Short life cycle, readily genetically malleable
Drosophila melanogaster	Muscle weakness, cardiac dysfunction
Zebrafish	Histological lesions (necrosis, inflammation and fibrosis), premature death
Mammalian-models	
mdx mouse	Dilated cardiomyopathy, cardiac fibrosis in aged/stressed mice
mdx/utrn$^{-/-}$ mouse	Skeletal and cardiac muscle degeneration, severe phenotype
hDMDdel45/mdx mouse	Dystrophic phenotype, poor regeneration, atrophic muscle
hDMDdel52/mdx mouse	Fibrosis, inflammation, degeneration and regeneration and impaired muscle function
DBA/2J mouse	Severe dystrophic phenotype, cardiac fibrosis and calcification
Dmd^{mdx} rat	Adipose tissue infiltration, reduction of motor activity, dilated cardiomyopathy
DMD KO rabbit	Myocardial inflammation, fibrosis and fatty cell infiltration
DMD monkey	Muscle degeneration at the early stage of disease
GRMD dog	Clinical course and disease severity similar to DMD patients
DMD$^{\Delta exon52}$ porcine	Mobility impairment and severe myopathy

Illustration of some genetic engineering applications in modelling DMD-related cardiomyopathy and cardiac phenotypes characterizing mammalian and non-mammalian models. AAV, adeno-associated virus; CI, ClinicalTraials.gov Identifier; CRISPR, clustered regularly interspaced short palindromic repeats; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy.

continued advances in gene therapy aimed at ameliorating the life expectancy of patients affected by DMD. Indeed, a recent study reported by Sarcar *et al.*[98] used a genome-wide *in silico* data mining approach to detect novel robust and evolutionarily conserved muscle-specific transcriptional cis-regulatory modules (CRMs) which outperformed previously reported promoters of micro-dystrophin expression in therapeutic validation experiments in SCID/mdx mice.

6. Potential of advanced bio-engineered techniques to model DMD

Traditional approaches to modelling muscular dystrophies[99–102] remain very limited compared to the physiological and multicellular complexity of biological human tissues[103–106] where multiple dynamic forces operate to produce critical factors that regulate cell differentiation and, tissue development and function.[107] For example, the plastic or glass substrates used in standard 2D cell-culture practice hinder the maturation of complex cellular structures, such as thick muscle syncytia, displaying coordinated electrical activity and contractile forces approaching ~10 mN. Nevertheless, *in vitro* model systems still represent the fundamental scientific research tool. Therefore, optimizing the *in vitro* recapitulation of complex micro-environmental processes of native tissues is the most important challenge to expand the limited capacity to model multifaceted diseases and screen innovative drugs.[108,109]

6.1 Multidimensional modelling

Using multidimensional recapitulation of the native tissues (e.g. by incorporating cellular heterogeneity, ECM components and, electrical and mechanical forces) supports extended culture times,[110–112] increased protein content,[113] cell/tissue maturation levels[111,114] and disease recapitulation ability.[115] Technology from the industrial manufacturing fields (e.g. printing, materials chemistry, fluidics, and microfabrication) has been transferred to basic biological research to overcome some important limitations of conventional 2D modelling. Indeed, several tissue-like constructs have already been generated through this combination of approaches, such as cartilage,[116] cornea,[117] bone,[118] heart,[113] brain,[119] vascular networks,[120,121] and muscles.[122,123]

6.2 Printing dimensionality

The most popular technical variations in the bioprinting context are based on the principles of inkjet, laser-assisted, extrusion, stereolithography, acoustic, and magnetic technologies (*Figure 1*).[119,123–128] A thorough description of these technologies is beyond the scope of this review and so the reader is referred to ancillary literature regarding their cardiac applications.[129,130] In relation to modelling *DMD*-CM, Macadangdang *et al.*[131] were able to manufacture anisotropically nanofabricated substrata (ANFS) with a nanotopographic surface, which mimicked ECM organization, for the culture of healthy donors' and DMD patients' iPSC-CM. Culture of iPSC-CM on the biomimetic nanotopographic ANFS aided the stratification of disease phenotype in DMD patients' iPSC-CM which was attributed to a blunted cellular response to the topographic cues provided by the ANFS. In follow-up, experiments involving the long-term culture of DMD iPSC-CM on the same ANFS biomimetic nanotopographic surfaces revealed greater hypertrophic responses in DMD iPSC-CM compared with healthy iPSC-CM.[21] The authors suggested that the absence of full-length dystrophin was less able to self-organize without strong external topographical cues and was consequently more susceptible to disorganization under stress. Intriguingly this deficit could be linked to decreased signalling of yes-associated protein (YAP), which is already known to be downregulated in DMD patients' skeletal muscle,[132] indeed Yasutake *et al.*[133] recently determined that altered YAP activity, caused by impaired actin dynamics, reduced the proliferation of DMD iPSC-CM.

As previously mentioned, the derivation of iPSC represented the biggest step towards precision medicine, giving the possibility to develop specific models that took the patient, disease, and genetic background into account. Therefore, the combination of bioprinting with iPSC is the most futuristic and promising prospect in the field of biomedical research.[134,135]

6.3 Other potential 3D platforms

6.3.1 Engineered heart tissues

The generation of iPSC in 3 D *in vitro* systems will enable a better study of *DMD*-CM, allowing the evaluation of responses to mechanical, electrical, chemical stimuli, and the development of new potential pharmaceutical approaches. The limited ability to characterize parameters, such as contractile force due to random orientation of iPSC-CM in 2 D cultures, represents a defect in single-cell assays of iPSC-CM analyses. Engineered heart tissues (EHT) are formed by combining cardiomyocytes (and sometimes other cardiovascular cell types) with aqueous extracellular matrices (e.g. collagen, fibrin, and other heterogeneous mixtures) which are transferred to casting moulds.[136] Following a short period of culture (usually 1 week), the cells spontaneously undergo differentiation maturation, fusion, and assemble within and remodel the 3D matrix. Subsequent mechanical stimulation further enhances the morphological, functional, and mechanical properties of EHT[137] that makes them vastly superior cardiac disease models and even tissue replacement therapy.[138–140] Therefore, the human EHT format might be employed in conjunction with numerous *in vitro* analytical instruments or gene therapy techniques, such as calcium measurements, electrophysiology, and adeno-associated virus transduction.[141]

Indeed, Long *et al.*[100] used the EHT paradigm with healthy, DMD, and genome-edited corrected-DMD iPSC-CM co-cultures with non-matched healthy foreskin fibroblasts. Contractile dysfunction was readily detected in DMD EHTs compared to healthy and edited EHTs, which showed normal force of contraction and maximal inotropic capacity. Intriguingly, the group also elegantly tackled the 'therapeutic efficiency' issue (i.e. what is the optimum percentage of corrected iPSC-CM needed to ameliorate the cardiac phenotype), by carefully titrating the percentages of corrected-DMD iPSC-CM that determined between 30 and 50% mosaic dystrophin expression was needed to partially or maximally restore normal contractile phenotype. Critically this is comparable to results observed *in vivo* experiments.[142] Therefore, it is conceivable that the pathological features of *DMD*-CM might be shaped with high fidelity using this 3D platform.[143] *Figure 2* shows a schematic representation of the application of 3D bioprinting using iPSC-CM, as a tool for personalized medicine and cardiac disease modelling of DMD patients' pathophysiology. However, the potential of these technologies has not yet been fully realized. Consequently, the future perspective must aim to include the implementation of biochemical and molecular biology studies performed using these advanced 3D models.

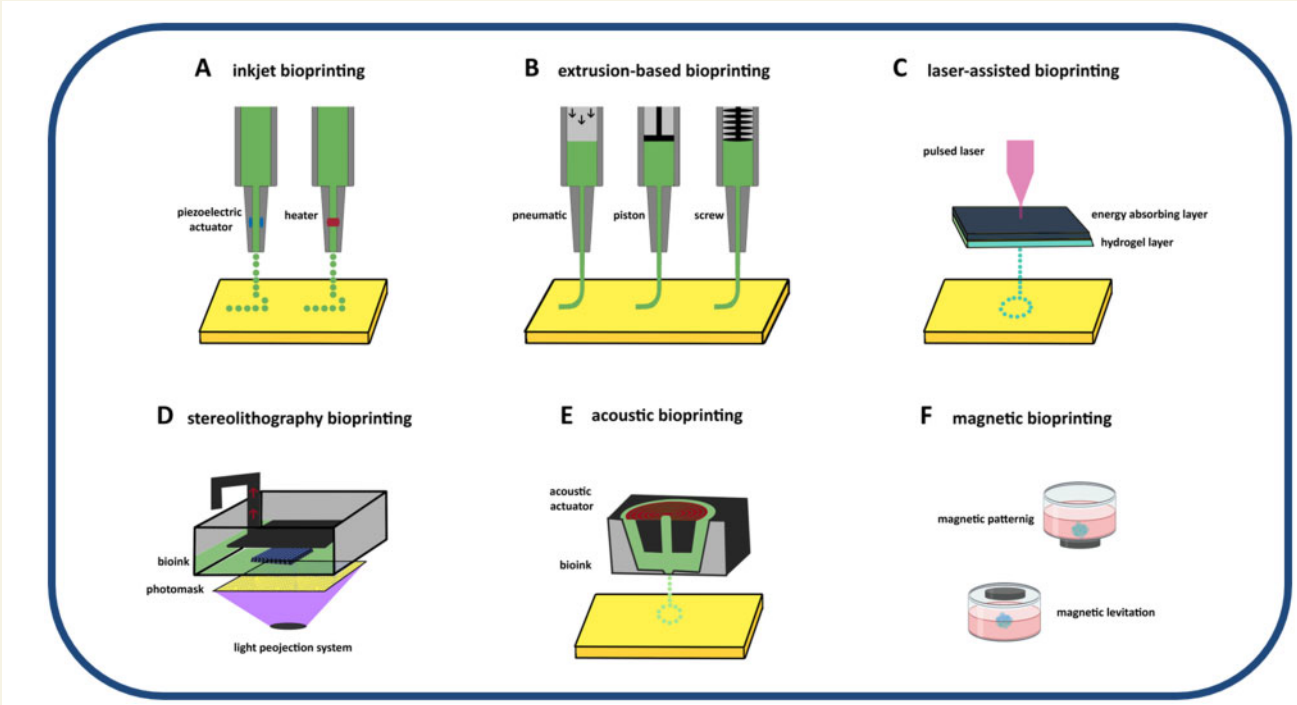


Figure 1 3D bioprinting approaches. (*A*) Inkjet bioprinting generates drops of low viscous bioink containing viable cells through thermal or piezoelectric actuators. (*B*) Extrusion bioprinting consists of a pneumatic, screw, or pistons-based method that allows the deposition of continuous filaments of high viscous hydrogel. (*C*) Laser-assisted bioprinting uses an exciting, pulsed laser source, which vaporizes the energy-absorbing layer in contact with the liquid or gelatinous bioink pre-solution, inducing the expulsion of cell-laden droplets layer. (*D*) Stereolithography is a projection bioprinting method, which employs a light projector to crosslink, plane-by-plane, photosensitive bioinks. (*E*) Acoustic bioprinting generates acoustic waves at the air-bioink interface inducing the bioink drops formation. (*F*) Magnetic bioprinting employs magnetic or paramagnetic bioadditives that facilitate the aggregation of the cells and their spatial organization following predefined patterns.

6.4 Aiming toward high throughput scale

6.4.1 Organoids

The development of novel drugs for the treatment of human diseases is one of the most expensive and time-consuming research processes. Therefore, the development of widely reproducible platforms of advanced cellular technology that recapitulate the complexity of human tissues *in vitro* currently represents one of the most difficult challenges. In the last decade, the organoid model has assumed an increasingly central role in efficiently recreating physiological and pathophysiological conditions *in vitro*.[144] Organoids are tissue-like cellular aggregates with heterogeneous composition, which can self-organize by recapitulating the microenvironment and cell-matrix interactions, thus exhibiting complex physiological as well as pathological functions. To date, the methodologies developed for the generation of organoids are numerous and continuously updated to obtain organ-specific systems that can mimic the liver,[145] brain,[146] prostate,[147] small intestine,[148] and extend to recreating the tumour microenvironment[149] and of direct relevance to this review, the myocardium[150] including the recapitulation of genetic cardiomyopathy.[151] The huge methodological facilitation represented by the use of the patient-specific iPSC differentiated into all the different cell lineages involved in *DMD*-CM (e.g. cardiomyocytes, endothelial cells, cardiac fibroblasts, and immune cells) and combined with selected genetic- or bio-engineered strategies, could be used to provide a coherent and efficient method to study the contribution of any cardiac cell type, matrix material or additional factors in pathological DMD cardiac dynamics and to test new drugs.[152]

6.4.2 3D microfabrication

Despite their success and potential, single organoid models fail to effectively reproduce the complex inter-organ interactions that occur physiologically. Microfabrication is an expanding technology used to manipulate miniature organs housed in a chip-like device that is advantageous to build truly holistic *in vitro* models of human physiology and disease. Indeed, modularity, 3D capability and construction adaptability of different complex organ modelling systems make these small organ-on-chip models excellent candidates for drug and toxicity screening.

The production of microphysiological organs-on-chip began with photolithography for the creation of the desired substrate, where an elastomeric biocompatible, non-toxic, and low-cost material, called polydimethylsiloxane (PDMS),[153] was poured, thus creating a positive copy that was sealed on a slide, forming closed-loop channels.[154–158] Perfusion of the platform with culture medium allowed the simulation of blood flow-induced shear stress on myocardial tissue making this model potentially useful for studying biomimetic signals on cardiac function. In addition, electrical stimulation and cell anisotropy remain two important factors for the fabrication of heart-on-a-chip devices. Indeed, Grosberg *et al.* printed microcontact patterns of fibronectin on a deformable PDMS film, creating the muscle thin film (MTF) technique. Rat primary

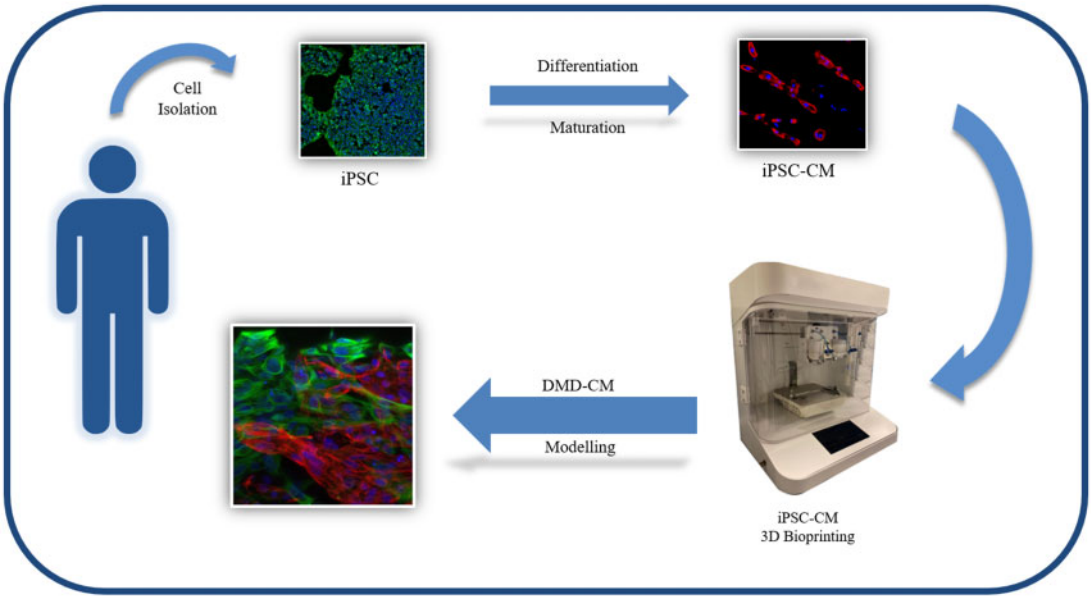


Figure 2 3D bioprinting application using iPSC-CM in cell therapy and cardiac disease modelling. Representative scheme of cell isolation, characterization, and differentiation of iPSC-CM; the use of 3D bioprinting techniques aimed at modelling cardiomyopathies and cell therapy in DMD is schematized. This figure was created with an adapted image from BioRender.com (2021) , and using the images developed by the group of the Unit of Vascular Biology and Regenerative Medicine, Centro Cardiologico Monzino-IRCCS and the Microfluidics and Biomimetic Microsystems Laboratory, Politecnico di Milano, Milan, Italy. DMD, Duchenne muscular dystrophy; iPSC, induced pluripotent stem cells; iPSC-CM, iPSC-derived cardio myocytes.

neonatal ventricular cardiomyocytes were seeded into the MTF platforms and electrically stimulated by platinum electrodes.[159] This study showed that stimulation increased cellular alignment, differentiation, and function of EHT making this model ideal for evaluating pharmacological intervention on the contractile function of multiple cardiac micro-tissues.[160,161] Afterward, Xiao *et al.*[162] designed a perfusable cardiac microtissue with a poly (tetrafluoroethylene) microtube to induce cardiomyocyte alignment and stretching along with the shape of the tube that also developed spontaneous beating and, sarcomeric troponin-T and connexin-43 expression. In the context of DMD, skeletal muscle myoblasts from DMD patients were used to create a DMD tongue-on-chip microphysiological device that illustrated the failure of DMD skeletal myoblasts to develop an equivalent level of contractile strength as seen for healthy myoblasts, a deficit that the authors attributed to the inability of DMD skeletal myoblasts to respond to extracellular cues for adaptive growth and remodelling.[99] Although these microphysiological models are useful for representing tissue-specific phenotypic disease features, they also require the incorporation of other factors in order to illustrate the cooperative nature of human organ systems. Recently, new technology was developed by integrating hepatic and cardiac tissue in a precise and reproducible manner by bioprinting, which are then housed in modular perfusable devices connected to a lung module via an immobilized semi-porous cell-laden membrane, thus effectively creating an air–liquid interface. Each organ model was created with native human tissue-derived-cell types, with similar relative proportions and ECM-based supporting bio-inks or -materials. This three-organ microphysiological system was used to reveal inter-organ responses to drugs (epinephrine and propranolol, used to assess the metabolic capabilities of the liver module and the downstream reduced impact on the cardiac module) and toxic agents (bleomycin, an anti-cancer drug known to cause lung fibrosis and inflammation).[163] Despite the revolutionary effectiveness of multi-organ microphysiological systems in the closest approximation to real human organ-system activity, in low-cost drug discovery and drug trials, as well as improved toxicity screenings, there are still some limitations to address. In particular, the most urgent need is to miniaturize the system to produce a cost-effective and operator-friendly pre-clinical system suitable for high-throughput applications, e.g., complex pharmacokinetic studies.

Furthermore, such tandem organ-on-chip systems generated with cells from diverse healthy donors or patients with a particular genetic profile, such as a *DMD* mutation, would provide test platforms that better represent heterogeneity in the human population, thereby improving overall drug development process.

7. Concluding considerations and future perspectives

The approaches presented in this review might provide the basis for implementing upcoming therapeutic strategies for DMD patients. The advent of iPSC technology largely solved problems regarding ethical issues, high production costs, and immune rejection related to other cell-therapy approaches; however, challenges remain for the clinical development of iPSC-based treatments. Promisingly, recent studies based on iPSC-CM have contributed to the identification of specific myocardial disease mechanisms relevant to the pathogenesis of *DMD*-CM, representing new potential therapeutic targets and a powerful means to understand the consequences of various *DMD* mutations, potentially encompassing multiple organ systems.[164] Understanding the multi-organ mechanisms underlying *DMD*-CM is essential for improving the

prognosis, management, and treatment of cardiac implications not only in the male population, which is more represented, but also in the female carrier population who also can present with HF or be predisposed to risks during pregnancy.[165]

Using iPSC in tissue bioengineering and modelling of multifactorial diseases is constantly evolving. A crucial long-term goal of micro-physiological systems (organ-on-chips) will be to reproduce the inter-communication between organ systems, which is steadily becoming more essential to understand human disease, including cardiovascular diseases, and treatment responses in a whole-body context, i.e., 4D multi-organ systems or body-on-chip comprised of heart, lung, and liver.[163,166] If fully realized a future could be imagined where it is achievable to completely replace animal models with precision theranostic patients-on-chip systems. This will require further pioneering proof-of-concept studies to holistically recreate, with sufficient resolution for both disease-modelling and clinical impact, the *in vivo* complexities that render *DMD*-CM truly manifest.

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References

1. Birnkrant DJ, Bushby K, Bann CM, Apkon SD, Blackwell A, Brumbaugh D, Case LE, Clemens PR, Hadjiyannakis S, Pandya S, Street N, Tomezsko J, Wagner KR, Ward LM, Weber DR; DMD Care Considerations Working Group. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, andgastrointestinal and nutritional management. *Lancet Neurol* 2018;**17**:251–267.
2. Crisafulli S, Sultana J, Fontana A, Salvo F, Messina S, Trifirò G. Global epidemiology of Duchenne muscular dystrophy: an updated systematic review and meta-analysis. *Orphanet J Rare Dis* 2020;**15**:141.
3. Ciafaloni E, Fox DJ, Pandya S, Westfield CP, Puzhankara S, Romitti PA, Mathews KD, Miller TM, Matthews DJ, Miller LA, Cunniff C, Druschel CM, Moxley RT. Delayed diagnosis in Duchenne muscular dystrophy: data from the Muscular Dystrophy Surveillance, Tracking, and Research Network (MD STARnet). *J Pediatr* 2009;**155**:380–385.
4. Flanigan KM. Duchenne and Becker muscular dystrophies. *Neurol Clin* 2014;**32**: 671–688, viii.
5. Wu RS, Gupta S, Brown RN, Yancy CW, Wald JW, Kaiser P, Kirklin NM, Patel PC, Markham DW, Drazner MH, Garry DJ, Mammen PP. Clinical outcomes after cardiac transplantation in muscular dystrophy patients. *J Heart Lung Transplant* 2010;**29**: 432–438.
6. Perri G, Filippelli S, Adorisio R, Iacobelli R, Iodice F, Testa G, Paglietti MG, D'Amario D, Massetti M, Amodeo A. Left ventricular assist device as destination therapy in cardiac end-stage dystrophinopathies: midterm results. *J Thorac Cardiovasc Surg* 2017;**153**:669–674.
7. Adorisio R, D'Amario D, Perri G, Amodeo A. Comment on: 'Implantation of a left ventricular assist device to provide long term support for end-stage Duchenne muscular dystrophy-associated cardiomyopathy' by Stoller et al. *ESC Heart Fail* 2018;**5**: 651–652.
8. D'Amario D, Amodeo A, Adorisio R, Tiziano FD, Leone AM, Perri G, Bruno P, Massetti M, Ferlini A, Pane M, Niccoli G, Porto I, D'Angelo GA, Borovac JA, Mercuri E, Crea F. A current approach to heart failure in Duchenne muscular dystrophy. *Heart* 2017;**103**:1770–1779.
9. Matsumura T, Tamura T, Kuru S, Kikuchi Y, Kawai M. Carvedilol can prevent cardiac events in Duchenne muscular dystrophy. *Intern Med* 2010;**49**:1357–1363.
10. Gloss D, Moxley RT 3rd, Ashwal S, Oskoui M. Practice guideline update summary: corticosteroid treatment of Duchenne muscular dystrophy: report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology* 2016;**86**:465–472.
11. Piga D, Salani S, Magri F, Brusa R, Mauri E, Comi GP, Bresolin N, Corti S. Human induced pluripotent stem cell models for the study and treatment of Duchenne and Becker muscular dystrophies. *Ther Adv Neurol Disord* 2019;**12**:1756286419833478.
12. Atta-Ur-Rahman, , Anjum S. Frontiers in stem cell and regenerative medicine research. *Bentham eBooks*. 2017;**4**: 3–35.
13. Meyers TA, Townsend D. Cardiac pathophysiology and the future of cardiac therapies in duchenne muscular dystrophy. *Int J Mol Sci* 2019;**20**:4098.
14. Martone J, Lisi M, Castagnetti F, Rosa A, Di Carlo V, Blanco E, Setti A, Mariani D, Colantoni A, Santini T, Perone L, Di Croce L, Bozzoni I. Trans-generational epigenetic regulation associated with the amelioration of Duchenne Muscular Dystrophy. *EMBO Mol Med* 2020;**12**:e12063.
15. Incitti T, De Angelis FG, Cazzella V, Sthandier O, Pinnarò C, Legnini I, Bozzoni I. Exon skipping and Duchenne muscular dystrophy therapy: selection of the most active U1 snRNA antisense able to induce dystrophin exon 51 skipping. *Mol Ther* 2010;**18**:1675–1682.
16. Deconinck N, Dan B. Pathophysiology of Duchenne muscular dystrophy: current hypotheses. *Pediatric Neurology* 2007;**36**:1–7.
17. Nanni S, Re A, Ripoli C, Gowran A, Nigro P, D'Amario D, Amodeo A, Crea F, Grassi C, Pontecorvi A, Farsetti A, Colussi C. The nuclear pore protein Nup153 associates with chromatin and regulates cardiac gene expression in dystrophic mdx hearts. *Cardiovasc Res* 2016;**112**:555–567.
18. Klymiuk N, Seeliger F, Bohlooly-Y M, Blutke A, Rudmann DG, Wolf E. Tailored pig models for preclinical efficacy and safety testing of targeted therapies. *Toxicol Pathol* 2016;**44**:346–357.
19. Montag J, Petersen B, Flögel AK, Becker E, Lucas-Hahn A, Cost GJ, Mühlfeld C, Kraft T, Niemann H, Brenner B. Successful knock-in of hypertrophic cardiomyopathy-mutation R723G into the MYH7 gene mimics HCM pathology in pigs. *Sci Rep* 2018;**8**:4786.
20. Yang D, Yang H, Li W, Zhao B, Ouyang Z, Liu Z, Zhao Y, Fan N, Song J, Tian J, Li F, Zhang J, Chang L, Pei D, Chen YE, Lai L. Generation of PPARγ mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. *Cell Res* 2011;**21**: 979–982.
21. Pioner JM, Guan X, Klaiman JM, Racca AW, Pabon L, Muskheli V, Macadangdang J, Ferrantini C, Hoopmann MR, Moritz RL, Kim DH, Tesi C, Poggesi C, Murry CE, Childers MK, Mack DL, Regnier M. Absence of full-length dystrophin impairs normal maturation and contraction of cardiomyocytes derived from human-induced pluripotent stem cells. *Cardiovasc Res* 2020;**116**:368–382.
22. Gieseler K, Grisoni K, Ségalat L. Genetipionc suppression of phenotypes arising from mutations in dystrophin-related genes in *Caenorhabditis elegans*. *Curr Biol* 2000; **10**:1092–1097.
23. Hewitt JE, Pollard AK, Lesanpezeshki L, Deane CS, Gaffney CJ, Etheridge T, Szewczyk NJ, Vanapalli SA. Muscle strength deficiency and mitochondrial dysfunction in a muscular dystrophy model of *Caenorhabditis elegans* and its functional response to drugs. *Dis Models Mech* 2018;4;11(12):dmm036137.
24. Gaud A, Simon JM, Witzel T, Carre-Pierrat M, Wermuth CG, Ségalat L. Prednisone reduces muscle degeneration in dystrophin-deficient Caenorhabditis elegans. *Neuromuscul Disord* 2004;**14**:365–370.
25. Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, Ocorr K, Bodmer R. Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. *Aging Cell* 2008;**7**:237–249.
26. Kucherenko MM, Marrone AK, Rishko VM, Magliarelli H. D F, Shcherbata HR. Stress and muscular dystrophy: a genetic screen for dystroglycan and dystrophin interactors in drosophila identifies cellular stress response components. *Dev Biol* 2011;**352**:228–242.
27. Kucherenko MM, Pantoja M, Yatsenko AS, Shcherbata HR, Fischer KA, Maksymiv DV, Chernyk YI, Ruohola-Baker H. Genetic modifier screens reveal new components that interact with the Drosophila dystroglycan-dystrophin complex. *PLoS One* 2008;**3**:e2418.
28. Widrick JJ, Alexander MS, Sanchez B, Gibbs DE, Kawahara G, Beggs AH, Kunkel LM. Muscle dysfunction in a zebrafish model of Duchenne muscular dystrophy. *Physiol Genomics* 2016;**48**:850–860..
29. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science (New York, N.Y.)* 1989;**244**:1578–1580.
30. Khairallah M, Khairallah RJ, Young ME, Allen BG, Gillis MA, Danialou G, Deschepper CF, Petrof BJ, Des Rosiers C. Sildenafil and cardiomyocyte-specific cGMP signaling prevent cardiomyopathic changes associated with dystrophin deficiency. *Proc Natl Acad Sci USA* 2008;**105**:7028–7033.
31. Bostick B, Yue Y, Duan D. Gender influences cardiac function in the mdx model of Duchenne cardiomyopathy. *Muscle Nerve* 2010;**42**:600–603.
32. Wehling-Henricks M, Jordan MC, Roos KP, Deng B, Tidball JG. Cardiomyopathy in dystrophin-deficient hearts is prevented by expression of a neuronal nitric oxide synthase transgene in the myocardium. *Hum Mol Genet* 2005;**14**:1921–1933.

33. Rybakova IN, Patel JR, Davies KE, Yurchenco PD, Ervasti JM. Utrophin binds laterally along actin filaments and can couple costameric actin with sarcolemma when overexpressed in dystrophin-deficient muscle. *Mol Biol Cell* 2002;**13**:1512–1521.
34. Deconinck A, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson JG, Tinsley JM, Davies KE. Utrophin-dystrophin-deficient mice as a model for duchenne muscular dystrophy. *Cell* 1997;**90**:717–727.
35. Janssen PM, Hiranandani N, Mays TA, Rafael-Fortney JA. Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice. *Am J Physiol Heart Circ Physiol* 2005;**289**:H2373–8.
36. Araki E, Nakamura K, Nakao K, Kameya S, Kobayashi O, Nonaka I, Kobayashi T, Katsuki M. Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy. *Biochem Biophys Res Commun* 1997;**238**:492–497.
37. Chapman VM, Miller DR, Armstrong D, Caskey CT. Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice. *Proc Natl Acad Sci USA* 1989; **86**:1292–1296.
38. Echigoya Y, Lim KRQ, Nakamura A, Yokota T. Multiple exon skipping in the duchenne muscular dystrophy hot spots: prospects and challenges. *J Pers Med* 2018;**8**: 41.
39. Min YL, Li H, Rodriguez-Caycedo C, Mireault AA, Huang J, Shelton JM, McAnally JR, Amoasii L, Mammen PPA, Bassel-Duby R, Olson EN. CRISPR-Cas9 corrects Duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells. *Sci Adv* 2019;**5**:eaav4324.
40. Veltrop M, van Vliet L, Hulsker M, Claassens J, Brouwers C, Breukel C, van der Kaa J, Linssen MM, den Dunnen JT, Verbeek S, Aartsma-Rus A, van Putten M. A dystrophic duchenne mouse model for testing human antisense oligonucleotides. *PLoS One* 2018;**13**:e0193289.
41. Young CS, Mokhonova E, Quinonez M, Pyle AD, Spencer MJ. Creation of a novel humanized dystrophic mouse model of Duchenne muscular dystrophy and application of a CRISPR/Cas9 gene editing therapy. *J Neuromuscul Dis* 2017;**4**:139–145.
42. 't Hoen PA, de Meijer EJ, Boer JM, Vossen RH, Turk R, Maatman RG, Davies KE, van Ommen GJ, van Deutekom JC, den Dunnen JT. Generation and characterization of transgenic mice with the full-length human DMD gene. *J Biol Chem* 2008; **283**:5899–5907.
43. van Putten M, Putker K, Overzier M, Adamzek WA, Pasteuning-Vuhman S, Plomp JJ, Aartsma-Rus A. Natural disease history of the D2-mdx mouse model for Duchenne muscular dystrophy. *FASEB J* 2019;**33**:8110–8124.
44. Aoki Y, Yokota T, Nagata T, Nakamura A, Tanihata J, Saito T, Duguez SM, Nagaraju K, Hoffman EP, Partridge T, Takeda S. Bodywide skipping of exons 45-55 in dystrophic mdx52 mice by systemic antisense delivery. *Proc Natl Acad Sci USA* 2012;**109**: 13763–13768.
45. Ménoret S, Fontanière S, Jantz D, Tesson L, Thinard R, Rémy S, Usal C, Ouisse LH, Fraichard A, Anegon I. Generation of Rag1-knockout immunodeficient rats and mice using engineered meganucleases. *FASEB J* 2013;**27**:703–711.
46. Larcher T, Lafoux A, Tesson L, Remy S, Thepenier V, François V, Le Guiner C, Goubin H, Dutilleul M, Guigand L, Toumaniantz G, De Cian A, Boix C, Renaud JB, Cherel Y, Giovannangeli C, Concordet JP, Anegon I, Huchet C. Characterization of dystrophin deficient rats: a new model for Duchenne muscular dystrophy. *PLoS One* 2014;**9**:e110371.
47. Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X, Zhao Y, Liu M. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat Biotechnol* 2013;**31**:681–683.
48. Sugihara H, Kimura K, Yamanouchi K, Teramoto N, Okano T, Daimon M, Morita H, Takenaka K, Shiga T, Tanihata J, Aoki Y, Inoue-Nagamura T, Yotsuyanagi H, Komuro I. Age-dependent echocardiographic and pathologic findings in a rat model with duchenne muscular dystrophy generated by CRISPR/Cas9 genome editing. *Int Heart J* 2020;**61**:1279–1284.
49. Sui T, Lau YS, Liu D, Liu T, Xu L, Gao Y, Lai L, Li Z, Han R. A novel rabbit model of Duchenne muscular dystrophy generated by CRISPR/Cas9. *Dis Model Mech* 2018; **11**: doi: 10.1242/dmm.032201.
50. Chen Y, Zheng Y, Kang Y, Yang W, Niu Y, Guo X, Tu Z, Si C, Wang H, Xing R, Pu X, Yang SH, Li S, Ji W, Li XJ. Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. *Hum Mol Genet* 2015;**24**:3764–3774.
51. Ishii A, Okada H, Hayashita-Kinoh H, Shin JH, Tamaoka A, Okada T, Takeda S. rAAV8 and rAAV9-mediated long-term muscle transduction with tacrolimus (FK506) in non-human primates. *Mol Ther Methods Clin Dev* 2020;**18**:44–49.
52. Kornegay JN. The golden retriever model of Duchenne muscular dystrophy. *Skelet Muscle* 2017;**7**:9.
53. Cooper BJ, Winand NJ, Stedman H, Valentine BA, Hoffman EP, Kunkel LM, Scott MO, Fischbeck KH, Kornegay JN, Avery RJ. The homologue of the duchenne locus is defective in X-linked muscular dystrophy of dogs. *Nature* 1988;**334**:154–156.
54. Valentine BA, Cummings JF, Cooper BJ. Development of Duchenne-type cardiomyopathy. Morphologic studies in a canine model. *Am J Pathol* 1989;**135**:671–678.
55. Moise NS, Valentine BA, Brown CA, Erb HN, Beck KA, Cooper BJ, Gilmour RF. Duchenne's cardiomyopathy in a canine model: electrocardiographic and echocardiographic studies. *J Am Coll Cardiol* 1991;**17**:812–820.
56. Chetboul V, Escriou C, Tessier D, Richard V, Pouchelon JL, Thibault H, Lallemand F, Thuillez C, Blot S, Derumeaux G. Tissue Doppler imaging detects early asymptomatic myocardial abnormalities in a dog model of Duchenne's cardiomyopathy. *Eur Heart J* 2004;**25**:1934–1939.
57. Fine DM, Shin JH, Yue Y, Volkmann D, Leach SB, Smith BF, McIntosh M, Duan D. Age-matched comparison reveals early electrocardiography and echocardiography changes in dystrophin-deficient dogs. *Neuromuscul Disord* 2011;**21**:453–461.
58. Kane AM, DeFrancesco TC, Boyle MC, Malarkey DE, Ritchey JW, Atkins CE, Cullen JM, Kornegay JN, Keene BW. Cardiac structure and function in female carriers of a canine model of Duchenne muscular dystrophy. *Res Vet Sci* 2013;**94**:610–617.
59. Guo LJ, Soslow JH, Bettis AK, Nghiem PP, Cummings KJ, Lenox MW, Miller MW, Kornegay JN, Spurney CF. Natural history of cardiomyopathy in adult dogs with golden retriever muscular dystrophy. *J Am Heart Assoc* 2019;**8**:e012443.
60. Cerqueira MD, Weissman NJ, Dilsizian V, Jacobs AK, Kaul S, Laskey WK, Pennell DJ, Rumberger JA, Ryan T, Verani MS; American Heart Association Writing Group on Myocardial Segmentation and Registration for Cardiac Imaging. Standardized myocardial segmentation and nomenclature for tomographic imaging of the heart. A statement for healthcare professionals from the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association. *Circulation* 2002;**105**: 539–542.
61. Klymiuk N, Blutke A, Graf A, Krause S, Burkhardt K, Wuensch A, Krebs S, Kessler B, Zakhartchenko V, Kurome M, Kemter E, Nagashima H, Schoser B, Herbach N, Blum H, Wanke R, Aartsma-Rus A, Thirion C, Lochmüller H, Walter MC, Wolf E. Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Hum Mol Genet* 2013;**22**:4368–4382.
62. Moretti A, Fonteyne L, Giesert F, Hoppmann P, Meier AB, Bozoglu T, Baehr A, Schneider CM, Sinnecker D, Klett K, Fröhlich T, Rahman FA, Haufe T, Sun S, Jurisch V, Kessler B, Hinkel R, Dirschinger R, Martens E, Jilek C, Graf A, Krebs S, Santamaria G, Kurome M, Zakhartchenko V, Campbell B, Voelse K, Wolf A, Ziegler T, Reichert S, Lee S, Flenkenthaler F, Dorn T, Jeremias I, Blum H, Dendorfer A, Schnieke A, Krause S, Walter MC, Klymiuk N, Laugwitz KL, Wolf E, Wurst W, Kupatt C. Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy. *Nat Med* 2020;**26**:207–214.
63. Parrotta EI, Lucchino V, Scaramuzzino L, Scalise S, Cuda G. Modeling cardiac disease mechanisms using induced pluripotent stem cell-derived cardiomyocytes: progress, promises and challenges. *Int J Mol Sci* 2020;**21**:4354.
64. Goudenege S, Lebel C, Huot NB, Dufour C, Fujii I, Gekas J, Rousseau J, Tremblay JP. Myoblasts derived from normal hESCs and dystrophic hiPSCs efficiently fuse with existing muscle fibers following transplantation. *Mol Ther J Am Soc Gene Ther* 2012;**20**:2153–2167.
65. Shoji E, Woltjen K, Sakurai H. Directed myogenic differentiation of human induced pluripotent stem cells. *Methods Mol Biol* 2016;**1353**:89–99.
66. Savarese M, Johari M, Johnson K, Arumilli M, Torella A, Töpf A, Rubegni A, Kuhn M, Giugliano T, Gläser D, Fattori F, Thompson R, Penttilä S, Lehtinen S, Gibertini S, Ruggieri A, Mora M, Maver A, Peterlin B, Mankodi A, Lochmüller H, Santorelli FM, Schoser B, Fajkusová L, Straub V, Nigro V, Hackman P, Udd B. Improved criteria for the classification of titin variants in inherited skeletal myopathies. *J Neuromuscul Dis* 2020;**7**:153–166.
67. Zhu S, Wurdak H, Wang J, Lyssiotis CA, Peters EC, Cho CY, Wu X, Schultz PG. A small molecule primes embryonic stem cells for differentiation. *Cell Stem Cell* 2009; **4**:416–426.
68. Chal J, Oginuma M, Al Tanoury Z, Gobert B, Sumara O, Hick A, Bousson F, Zidouni Y, Mursch C, Moncuquet P, Tassy O, Vincent S, Miyanari A, Bera A, Garnier JM, Guevara G, Hestin M, Kennedy L, Hayashi S, Drayton B, Cherrier T, Gayraud-Morel B, Gussoni E, Relaix F, Tajbakhsh S, Pourquié O. Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nat Biotechnol* 2015;**33**:962–969.
69. Baci D, Chirivì M, Pace V, Maiullari F, Milan M, Rampin A, Somma P, Presutti D, Garavelli S, Bruno A, Cannata S, Lanzuolo C, Gargioli C, Rizzi R, Bearzi C. Extracellular vesicles from skeletal muscle cells efficiently promote myogenesis in induced pluripotent stem cells. *Cells* 2020;**9**:1527.
70. Gowran A, Spaltro G, Casalnuovo F, Vigorelli V, Spinelli P, Castiglioni E, Rovina D, Paganini S, Di Segni M, Gervasini C, Nigro P, Pompilio G. Generation of induced pluripotent stem cells from a Becker muscular dystrophy patient carrying a deletion of exons 45-55 of the dystrophin gene (CCMi002BMD-A-9 Δ45-55). *Stem Cell Res* 2018;**28**:21–24.
71. Lin B, Li Y, Han L, Kaplan AD, Ao Y, Kalra S, Bett GC, Rasmusson RL, Denning C, Yang L. Modeling and study of the mechanism of dilated cardiomyopathy using induced pluripotent stem cells derived from individuals with Duchenne muscular dystrophy. *Dis Model Mech* 2015;**8**:457–466.
72. Gowran A, Rasponi M, Visone R, Nigro P, Perrucci GL, Righetti S, Zanobini M, Pompilio G. Young at heart: pioneering approaches to model nonischaemic cardiomyopathy with induced pluripotent stem cells. *Stem Cells Int* 2016;**2016**:4287158.
73. Rovina D, Castiglioni E, Niro F, Mallia S, Pompilio G, Gowran A. "Betwixt Mine Eye and Heart a League Is Took": the progress of induced pluripotent stem-cell-based models of dystrophin-associated cardiomyopathy. *Int J Mol Sci* 2020;**21**:6997.
74. van Mil A, Balk GM, Neef K, Buikema JW, Asselbergs FW, Wu SM, Doevendans PA, Sluijter JPG. Modelling inherited cardiac disease using human induced pluripotent stem cell-derived cardiomyocytes: progress, pitfalls, and potential. *Cardiovasc Res* 2018;**114**:1828–1842.

75. Gartz M, Darlington A, Afzal MZ, Strande JL. Exosomes exert cardioprotection in dystrophin-deficient cardiomyocytes via ERK1/2-p38/MAPK signaling. *Sci Rep* 2018; **8**:16519.
76. Gartz M, Lin CW, Sussman MA, Lawlor MW, Strande JL. Duchenne muscular dystrophy (DMD) cardiomyocyte-secreted exosomes promote the pathogenesis of DMD-associated cardiomyopathy. *Dis Model Mech* 2020;**13**:dmm045559.doi: 10.1242/dmm.045559.
77. Eisen B, Ben Jehuda R, Cuttitta AJ, Mekies LN, Shemer Y, Baskin P, Reiter I, Willi L, Freimark D, Gherghiceanu M, Monserrat L, Scherr M, Hilfiker-Kleiner D, Arad M, Michele DE, Binah O. Electrophysiological abnormalities in induced pluripotent stem cell-derived cardiomyocytes generated from Duchenne muscular dystrophy patients. *J Cell Mol Med* 2019;**23**:2125–2135.
78. Kamdar F, Das S, Gong W, Klaassen Kamdar A, Meyers TA, Shah P, Ervasti JM, Townsend D, Kamp TJ, Wu JC, Garry MG, Zhang J, Garry DJ. Stem cell-derived cardiomyocytes and beta-adrenergic receptor blockade in Duchenne muscular dystrophy cardiomyopathy. *J Am Coll Cardiol* 2020;**75**:1159–1174.
79. Musunuru K, Sheikh F, Gupta RM, Houser SR, Maher KO, Milan DJ, Terzic A, Wu JC; American Heart Association Council on Functional Genomics and Translational Biology; Council on Cardiovascular Disease in the Young; and Council on Cardiovascular and Stroke Nursing. Induced pluripotent stem cells for cardiovascular disease modeling and precision medicine: a scientific statement from the American Heart Association. *Circ Genom Precis Med* 2018;**11**:e000043.
80. Oikonomopoulos A, Kitani T, Wu JC. Pluripotent stem cell-derived cardiomyocytes as a platform for cell therapy applications: progress and hurdles for clinical translation. *Mol Ther* 2018;**26**:1624–1634.
81. Liu G, David BT, Trawczynski M, Fessler RG. Advances in pluripotent stem cells: history, mechanisms, technologies, and applications. *Stem Cell Rev Rep* 2020;**16**: 3–32.
82. Kim K, Ryu SM, Kim ST, Baek G, Kim D, Lim K, Chung E, Kim S, Kim JS. Highly efficient RNA-guided base editing in mouse embryos. *Nat Biotechnol* 2017;**35**:435–437.
83. Amoasii L, Long C, Li H, Mireault AA, Shelton JM, Sanchez-Ortiz E, McAnally JR, Bhattacharyya S, Schmidt F, Grimm D, Hauschka SD, Bassel-Duby R, Olson EN. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci Transl Med* 2017;**9**:eaan8081 doi: 10.1126/scitranslmed.aan8081. Erratum in: *Sci Transl Med*. **2018**;10 (425).
84. Amoasii L, Li H, Zhang Y, Min YL, Sanchez-Ortiz E, Shelton JM, Long C, Mireault AA, Bhattacharyya S, McAnally JR, Bassel-Duby R, Olson EN. In vivo non-invasive monitoring of dystrophin correction in a new Duchenne muscular dystrophy reporter mouse. *Nat Commun* 2019;**10**:4537.
85. Shimo T, Hosoki K, Nakatsuji Y, Yokota T, Obika S. A novel human muscle cell model of Duchenne muscular dystrophy created by CRISPR/Cas9 and evaluation of antisense-mediated exon skipping. *J Hum Genet* 2018;**63**:365–375.
86. Jin Y, Shen Y, Su X, Weintraub NL, Tang Y. Effective restoration of dystrophin expression in iPSC Mdx-derived muscle progenitor cells using the CRISPR/Cas9 system and homology-directed repair technology. *Comput Struct Biotechnol J* 2020;**18**: 765–773.
87. Lim KRQ, Nguyen Q, Dzierlega K, Huang Y, Yokota T. CRISPR-generated animal models of duchenne muscular dystrophy. *Genes (Basel)* 2020;**11**:342.
88. Wells DJ. Tracking progress: an update on animal models for Duchenne muscular dystrophy. *Dis Model Mech* 2018;**11**:dmm035774. doi: 10.1242/dmm.035774.
89. Musunuru K. The hope and hype of CRISPR-Cas9 genome editing: a review. *JAMA Cardiol* 2017;**2**:914–919.
90. Ousterout DG, Kabadi AM, Thakore PI, Majoros WH, Reddy TE, Gersbach CA. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nat Commun* 2015;**6**:6244.
91. Gee P, Lung MSY, Okuzaki Y, Sasakawa N, Iguchi T, Makita Y, Hozumi H, Miura Y, Yang LF, Iwasaki M, Wang XH, Waller MA, Shirai N, Abe YO, Fujita Y, Watanabe K, Kagita A, Iwabuchi KA, Yasuda M, Xu H, Noda T, Komano J, Sakurai H, Inukai N, Hotta A. Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping. *Nat Commun* 2020;**11**:1334.
92. Dzierlega K, Yokota T. Optimization of antisense-mediated exon skipping for Duchenne muscular dystrophy. *Gene Ther* 2020;**27**:407–416.
93. Komaki H, Nagata T, Saito T, Masuda S, Takeshita E, Sasaki M, Tachimori H, Nakamura H, Aoki Y, Takeda S. Systemic administration of the antisense oligonucleotide NS-065/NCNP-01 for skipping of exon 53 in patients with Duchenne muscular dystrophy. *Sci Transl Med* 2018;**10**:eaan0713.
94. Khan N, Eliopoulos H, Han L, Kinane TB, Lowes LP, Mendell JR, Gordish-Dressman H, Henricson EK, McDonald CM; Eteplirsen Investigators and the CINRG DNHS Investigators. Eteplirsen treatment attenuates respiratory decline in ambulatory and non-ambulatory patients with duchenne muscular dystrophy. *J Neuromuscul Dis* 2019;**6**:213–225.
95. Antoury L, Hu N, Balaj L, Das S, Georghiou S, Darras B, Clark T, Breakefield XO, Wheeler TM. Analysis of extracellular mRNA in human urine reveals splice variant biomarkers of muscular dystrophies. *Nat Commun* 2018;**9**:3906.
96. Łoboda A, Dulak J. Muscle and cardiac therapeutic strategies for Duchenne muscular dystrophy: past, present, and future. *Pharmacol Rep* 2020;**72**:1227–1263.
97. Asher DR, Thapa K, Dharia SD, Khan N, Potter RA, Rodino-Klapac LR, Mendell JR. Clinical development on the frontier: gene therapy for duchenne muscular dystrophy. *Expert Opin Biol Ther* 2020;**20**:263–274.
98. Sarcar S, Tulalamba W, Rincon MY, Tipanee J, Pham HQ, Evens H, Boon D, Samara-Kuko E, Keyaerts M, Loperfido M, Berardi E, Jarmin S, In't Veld P, Dickson G, Lahoutte T, Sampaolesi M, De Bleser P, VandenDriessche T, Chuah MK. Next-generation muscle-directed gene therapy by in silico vector design. *Nat Commun* 2019;**10**:492.
99. Nesmith AP, Wagner MA, Pasqualini FS, O'Connor BB, Pincus MJ, August PR, Parker KK. A human in vitro model of Duchenne muscular dystrophy muscle formation and contractility. *J Cell Biol* 2016;**215**:47–56.
100. Long C, Li H, Tiburcy M, Rodriguez-Caycedo C, Kyrychenko V, Zhou H, Zhang Y, Min YL, Shelton JM, Mammen PPA, Liaw NY, Zimmermann WH, Bassel-Duby R, Schneider JW, Olson EN. Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. *Sci Adv* 2018;**4**: eaap9004. doi: 10.1126/sciadv.aap9004.
101. Khodabukus A, Prabhu N, Wang J, Bursac N. In vitro tissue-engineered skeletal muscle models for studying muscle physiology and disease. *Adv Healthc Mater* 2018; **7**:e1701498.
102. Decary S, Mouly V, Hamida CB, Sautet A, Barbet JP, Butler-Browne GS. Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. *Hum Gene Ther* 1997;**8**:1429–1438.
103. Anderson PA. The heart and development. *Semin Perinatol* 1996;**20**:482–509.
104. Cheng CS, Davis BN, Madden L, Bursac N, Truskey GA. Physiology and metabolism of tissue-engineered skeletal muscle. *Exp Biol Med (Maywood)* 2014;**239**:1203–1214.
105. Deng Y, Bao F, Dai Q, Wu LF, Altschuler SJ. Scalable analysis of cell-type composition from single-cell transcriptomics using deep recurrent learning. *Nat Methods* 2019;**16**:311–314.
106. Menden K, Marouf M, Oller S, Dalmia A, Magruder DS, Kloiber K, Heutink P, Bonn S. Deep learning-based cell composition analysis from tissue expression profiles. *Sci Adv* 2020;**6**:eaba2619.
107. D'Amario D, Gowran A, Canonico F, Castiglioni E, Rovina D, Santoro R, Spinelli P, Adorisio R, Amodeo A, Perrucci GL, Borovac JA, Pompilio G, Crea F. Dystrophin cardiomyopathies: clinical management, molecular pathogenesis and evolution towards precision medicine. *J Clin Med* 2018;**7**:291.
108. Vandenburgh H, Shansky J, Benesch-Lee F, Barbata V, Reid J, Thorrez L, Valentini R, Crawford G. Drug-screening platform based on the contractility of tissue-engineered muscle. *Muscle Nerve* 2008;**37**:438–447.
109. Vandenburgh H, Shansky J, Benesch-Lee F, Skelly K, Spinazzola JM, Saponjian Y, Tseng BS. Automated drug screening with contractile muscle tissue engineered from dystrophic myoblasts. *FASEB J* 2009;**23**:3325–3334.
110. Madden L, Juhas M, Kraus WE, Truskey GA, Bursac N. Bioengineered human myobundles mimic clinical responses of skeletal muscle to drugs. *Elife* 2015;**4**:e04885.
111. Rao L, Qian Y, Khodabukus A, Ribar T, Bursac N. Engineering human pluripotent stem cells into a functional skeletal muscle tissue. *Nat Commun* 2018;**9**:126.
112. Khodabukus A, Baar K. Regulating fibrinolysis to engineer skeletal muscle from the C2C12 cell line. *Tissue Eng Part C Methods* 2009;**15**:501–511.
113. Liu N, Ye X, Yao B, Zhao M, Wu P, Liu G, Zhuang D, Jiang H, Chen X, He Y, Huang S, Zhu P. Advances in 3D bioprinting technology for cardiac tissue engineering and regeneration. *Bioact Mater* 2020;**6**:1388–1401.
114. Maiullari F, Costantini M, Milan M, Pace V, Chirivì M, Maiullari S, Rainer A, Baci D, Marei HE, Seliktar D, Gargioli C, Bearzi C, Rizzi R. A multi-cellular 3D bioprinting approach for vascularized heart tissue engineering based on HUVECs and iPSC-derived cardiomyocytes. *Sci Rep* 2018;**8**:13532.
115. Giacomelli E, Meraviglia V, Campostrini G, Cochrane A, Cao X, van Helden RWJ, Krotenberg Garcia A, Mircea M, Kostidis S, Davis RP, van Meer BJ, Jost CR, Koster AJ, Mei H, Míguez DG, Mulder AA, Ledesma-Terrón M, Pompilio G, Sala L, Salvatori DCF, Slieker RC, Sommariva E, de Vries AAF, Giera M, Semrau S, Tertoolen LGJ, Orlova VV, Bellin M, Mummery CL. Human-iPSC-derived cardiac stromal cells enhance maturation in 3D cardiac microtissues and reveal non-cardiomyocyte contributions to heart disease. *Cell Stem Cell* 2020;**26**:862–879.e11.
116. Zhu W, Cui H, Boualam B, Masood F, Flynn E, Rao RD, Zhang ZY, Zhang LG. 3D bioprinting mesenchymal stem cell-laden construct with core-shell nanospheres for cartilage tissue engineering. *Nanotechnology* 2018;**29**:185101.
117. Sorkio A, Koch L, Koivusalo L, Deiwick A, Miettinen S, Chichkov B, Skottman H. Human stem cell based corneal tissue mimicking structures using laser-assisted 3D bioprinting and functional bioinks. *Biomaterials* 2018;**171**: 57–71.
118. Datta P, Ozbolat V, Ayan B, Dhawan A, Ozbolat IT. Bone tissue bioprinting for craniofacial reconstruction. *Biotechnol Bioeng* 2017;**114**:2424–2431.
119. Knowlton S, Anand S, Shah T, Tasoglu S. Bioprinting for neural tissue engineering. *Trends Neurosci* 2018;**41**:31–46..
120. Miri AK, Khalilpour A, Cecen B, Maharjan S, Shin SR, Khademhosseini A. Multiscale bioprinting of vascularized models. *Biomaterials* 2019;**198**:204–216.
121. Grigoryan B, Paulsen SJ, Corbett DC, Sazer DW, Fortin CL, Zaita AJ, Greenfield PT, Calafat NJ, Gounley JP, Ta AH, Johansson F, Randles A, Rosenkrantz JE, Louis-Rosenberg JD, Galie PA, Stevens KR, Miller JS. Multivascular networks and functional intravascular topologies within biocompatible hydrogels. *Science* 2019;**364**: 458–464.
122. Ostrovidov S, Salehi S, Costantini M, Suthiwanich K, Ebrahimi M, Sadeghian RB, Fujie T, Shi X, Cannata S, Gargioli C, Tamayol A, Dokmeci MR, Orive G, Swieszkowski W, Khademhosseini A. 3D bioprinting in skeletal muscle tissue engineering. *Small* 2019;**15**:e1805530.

123. Kim JH, Seol YJ, Ko IK, Kang HW, Lee YK, Yoo JJ, Atala A, Lee SJ. 3D bioprinted human skeletal muscle constructs for muscle function restoration. *Sci Rep* 2018;**8**: 12307.
124. Billiet T, Vandenhaute M, Schelfhout J, Van Vlierberghe S, Dubruel P. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* 2012;**33**:6020–6041.
125. Wüst S, Müller R, Hofmann S. Controlled positioning of cells in biomaterials-approaches towards 3D tissue printing. *J Funct Biomater* 2011;**2**:119–154.
126. Cui X, Li J, Hartanto Y, Durham M, Tang J, Zhang H, Hooper G, Lim K, Woodfield T. Advances in extrusion 3D bioprinting: a focus on multicomponent hydrogel-based bioinks. *Adv Healthc Mater* 2020;**9**:e1901648.
127. Peng W, Datta P, Ayan B, Ozbolat V, Sosnoski D, Ozbolat IT. 3D bioprinting for drug discovery and development in pharmaceutics. *Acta Biomater* 2017;**57**:26–46.
128. Leberfinger AN, Ravnic DJ, Dhawan A, Ozbolat IT. Concise review: bioprinting of stem cells for transplantable tissue fabrication. *Stem Cells Transl Med* 2017;**6**: 1940–1948.
129. Wang Z, Lee SJ, Cheng HJ, Yoo JJ, Atala A. 3D bioprinted functional and contractile cardiac tissue constructs. *Acta Biomater* 2018;**70**:48–56.
130. Redaelli A, Cooper-White J. Bioengineering of the heart. *APL Bioeng* 2020;**4**:010402.
131. Macadangdang J, Lee HJ, Carson D, Jiao A, Fugate J, Pabon L, Regnier M, Murry C, Kim DH. Capillary force lithography for cardiac tissue engineering. *J Vis Exp* 2014; 50039.
132. Vita GL, Polito F, Oteri R, Arrigo R, Ciranni AM, Musumeci O, Messina S, Rodolico C, Di Giorgio RM, Vita G, Aguennouz M. Hippo signaling pathway is altered in Duchenne muscular dystrophy. *PLoS One* 2018;**13**:e0205514.
133. Yasutake H, Lee JK, Hashimoto A, Masuyama K, Li J, Kuramoto Y, Higo S, Hikoso S, Hidaka K, Naito AT, Miyagawa S, Sawa Y, Komuro I, Sakata Y. Decreased YAP activity reduces proliferative ability in human induced pluripotent stem cell of duchenne muscular dystrophy derived cardiomyocytes. *Sci Rep* 2021;**11**:10351.
134. Soman SS, Vijayavenkataraman S. Applications of 3D bioprinted-induced pluripotent stem cells in healthcare. *Int J Bioprint* 2020;**6**:280.
135. Gu Q, Tomaskovic-Crook E, Wallace GG, Crook JM. 3D bioprinting human induced pluripotent stem cell constructs for in situ cell proliferation and successive multilineage differentiation. *Adv Healthc Mater* 2017;**6**: doi: 10.1002/adhm.201700175.
136. Eschenhagen T, Fink C, Remmers U, Scholz H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. *FASEB J* 1997;**11**:683–694..
137. Fink C, Ergün S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J* 2000;**14**:669–679.
138. Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med* 2006;**12**:452–458.
139. Yildirim Y, Naito H, Didié M, Karikkineth BC, Biermann D, Eschenhagen T, Zimmermann WH. Development of a biological ventricular assist device: preliminary data from a small animal model. *Circulation* 2007;**116**:I16–23.
140. Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T. Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation* 2002;**106**:I151–7.
141. Breckwoldt K, Letuffe-Brenière D, Mannhardt I, Schulze T, Ulmer B, Werner T, Benzin A, Klampe B, Reinsch MC, Laufer S, Shibamiya A, Prondzynski M, Mearini G, Schade D, Fuchs S, Neuber C, Krämer E, Saleem U, Schulze ML, Rodriguez ML, Eschenhagen T, Hansen A. Differentiation of cardiomyocytes and generation of human engineered heart tissue. *Nat Protoc* 2017;**12**:1177–1197. Erratum in: *Nat Protoc.* **2019**;**14**(9):2748.
142. Bostick B, Yue Y, Long C, Duan D. Prevention of dystrophin-deficient cardiomyopathy in twenty-one-month-old carrier mice by mosaic dystrophin expression or complementary dystrophin/utrophin expression. *Circ Res* 2008;**102**:121–130.
143. Maffioletti SM, Sarcar S, Henderson ABH, Mannhardt I, Pinton L, Moyle LA, Steele-Stallard H, Cappellari O, Wells KE, Ferrari G, Mitchell JS, Tyzack GE, Kotiadis VN, Khedr M, Ragazzi M, Wang W, Duchen MR, Patani R, Zammit PS, Wells DJ, Eschenhagen T, Tedesco FS. Three-dimensional human iPSC-derived artificial skeletal muscles model muscular dystrophies and enable multilineage tissue engineering. *Cell Rep* 2018;**23**:899–908.
144. Huch M, Knoblich JA, Lutolf MP, Martinez-Arias A. The hope and the hype of organoid research. *Development* 2017;**144**:938–941.
145. Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RG, Grompe M, Clevers H. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* 2013; **494**:247–250.
146. Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA. Cerebral organoids model human brain development and microcephaly. *Nature* 2013;**501**:373–379.
147. Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, Dowling C, Wanjala JN, Undvall EA, Arora VK, Wongvipat J, Kossai M, Ramazanoglu S, Barboza LP, Di W, Cao Z, Zhang QF, Sirota I, Ran L, MacDonald TY, Beltran H, Mosquera JM, Touijer KA, Scardino PT, Laudone VP, Curtis KR, Rathkopf DE, Morris MJ, Danila DC, Slovin SF, Solomon SB, Eastham JA, Chi P, Carver B, Rubin MA, Scher HI, Clevers H, Sawyers CL, Chen Y. Organoid cultures derived from patients with advanced prostate cancer. *Cell* 2014;**159**:176–187.
148. Jung P, Sato T, Merlos-Suárez A, Barriga FM, Iglesias M, Rossell D, Auer H, Gallardo M, Blasco MA, Sancho E, Clevers H, Batlle E. Isolation and in vitro expansion of human colonic stem cells. *Nat Med* 2011;**17**:1225–1227.
149. van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, McLaren-Douglas A, Blokker J, Jaksani S, Bartfeld S, Volckman R, van Sluis P, Li VS, Seepo S, Sekhar Pedamallu C, Cibulskis K, Carter SL, McKenna A, Lawrence MS, Lichtenstein L, Stewart C, Koster J, Versteeg R, van Oudenaarden A, Saez-Rodriguez J, Vries RG, Getz G, Wessels L, Stratton MR, McDermott U, Meyerson M, Garnett MJ, Clevers H. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 2015;**161**: 933–945.
150. Hofbauer P, Jahnel SM, Papai N, Giesshammer M, Deyett A, Schmidt C, Penc M, Tavernini K, Grdseloff N, Meledeth C, Ginistrelli LC, Ctortecka C, Šalic Š, Novatchkova M, Mendjan S. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 2021;**184**:3299–3317.e22.
151. Mitragotri S, Lahann J. Physical approaches to biomaterial design. *Nat Mater* 2009;**8**: 15–23.
152. Richards DJ, Coyle RC, Tan Y, Jia J, Wong K, Toomer K, Menick DR, Mei Y. Inspiration from heart development: biomimetic development of functional human cardiac organoids. *Biomaterials* 2017;**142**:112–123.
153. Huh D, Kim HJ, Fraser JP, Shea DE, Khan M, Bahinski A, Hamilton GA, Ingber DE. Microfabrication of human organs-on-chips. *Nat Protoc* 2013;**8**:2135–2157.
154. Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014;**32**:760–772.
155. Duffy DC, McDonald JC, Schueller OJ, Whitesides GM. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Anal Chem* 1998;**70**:4974–4984.
156. Halldorsson S, Lucumi E, Gómez-Sjöberg R, Fleming RMT. Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosens Bioelectron* 2015;**63**:218–231.
157. Annabi N, Selimović Š, Acevedo Cox JP, Ribas J, Afshar Bakooshli M, Heintze D, Weiss AS, Cropek D, Khademhosseini A. Hydrogel-coated microfluidic channels for cardiomyocyte culture. *Lab Chip* 2013;**13**:3569–3577.
158. Annabi N, Shin SR, Tamayol A, Miscuglio M, Bakooshli MA, Assmann A, Mostafalu P, Sun JY, Mithieux S, Cheung L, Tang XS, Weiss AS, Khademhosseini A. Highly elastic and conductive human-based protein hybrid hydrogels. *Adv Mater* 2016;**28**:40–49.
159. Grosberg A, Alford PW, McCain ML, Parker KK. Ensembles of engineered cardiac tissues for physiological and pharmacological study: heart on a chip. *Lab Chip* 2011; **11**:4165–4173.
160. Ribas J, Sadeghi H, Manbachi A, Leijten J, Brinegar K, Zhang YS, Ferreira L, Khademhosseini A. Cardiovascular organ-on-a-chip platforms for drug discovery and development. *Appl In Vitro Toxicol* 2016;**2**:82–96
161. Agarwal A, Goss JA, Cho A, McCain ML, Parker KK. Microfluidic heart on a chip for higher throughput pharmacological studies. *Lab Chip* 2013;**13**:3599–3608.
162. Xiao Y, Zhang B, Liu H, Miklas JW, Gagliardi M, Pahnke A, Thavandiran N, Sun Y, Simmons C, Keller G, Radisic M. Microfabricated perfusable cardiac biowire: a platform that mimics native cardiac bundle. *Lab Chip* 2014;**14**:869–882.
163. Skardal A, Murphy SV, Devarasetty M, Mead I, Kang HW, Seol YJ, Shrike Zhang Y, Shin SR, Zhao L, Aleman J, Hall AR, Shupe TD, Kleensang A, Dokmeci MR, Jin Lee S, Jackson JD, Yoo JJ, Hartung T, Khademhosseini A, Soker S, Bishop CE, Atala A. Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Sci Rep* 2017;**7**:8837.
164. Pioner JM, Fornaro A, Coppini R, Ceschia N, Sacconi L, Donati MA, Favilli S, Poggesi C, Olivotto I, Ferrantini C. Advances in stem cell modeling of dystrophin-associated disease: implications for the wider world of dilated cardiomyopathy. *Front Physiol* 2020;**11**:368.
165. Lim KRQ, Sheri N, Nguyen Q, Yokota T. Cardiac involvement in dystrophin-deficient females: current understanding and implications for the treatment of dystrophinopathies. *Genes (Basel)* 2020;**11**:765.
166. Sung JH, Wang YI, Narasimhan Sriram N, Jackson M, Long C, Hickman JJ, Shuler ML. Recent advances in body-on-a-chip systems. *Anal Chem* 2019;**91**:330–351.
167. Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, Nakata H, Tohyama S, Hashimoto H, Kodaira M, Okada Y, Seimiya H, Fusaki N, Hasegawa M, Fukuda K. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* 2010;**7**:11–14.