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Current Opinion in Cell Biology

# Advanced models of human skeletal muscle differentiation, development and disease: Three-dimensional cultures, organoids and beyond

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**Abstract**

Advanced *in vitro* models of human skeletal muscle tissue are increasingly needed to model complex developmental dynamics and disease mechanisms not recapitulated in animal models or in conventional monolayer cell cultures. There has been impressive progress towards creating such models by using tissue engineering approaches to recapitulate a range of physical and biochemical components of native human skeletal muscle tissue. In this review, we discuss recent studies focussed on developing complex *in vitro* models of human skeletal muscle beyond monolayer cell cultures, involving skeletal myogenic differentiation from human primary myoblasts or pluripotent stem cells, often in the presence of structural scaffolding support. We conclude with our outlook on the future of advanced skeletal muscle three-dimensional cultures (e.g. organoids and biofabrication) to produce physiologically and clinically relevant platforms for disease modelling and therapy development in musculoskeletal and neuromuscular disorders.

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**Current Opinion in Cell Biology** 2021, **73**:92–104

This review comes from a themed issue on **Differentiation and development**

Edited by **Karen Echeverri** and **Michael Kyba**

For a complete overview see the Issue and the Editorial

**https://doi.org/10.1016/j.ceb.2021.06.004**



**Keywords**

Skeletal muscle, Stem cells, iPS cells, 3D cultures, Organoids, Tissue engineering, Disease modelling.

## Introduction

The skeletal muscle, an architecturally complex tissue that accounts for the largest tissue mass in the human body, is responsible for supporting posture, voluntary movement, guarding soft tissues and body openings, as well as regulating several metabolic and homoeostatic functions. Functional skeletal muscle not only contains myofibres and their progenitor cells but also requires their constant interaction with other cell types and tissues including, but not limited to, connective tissue, vasculature and motor neurons [1]. The hierarchical organisation of skeletal muscle (Figure 1a) consists of organised bundles of fascicles which in turn are composed of bundles of myofibres embedded within three layers of extracellular matrix (the endomysium, perimysium and epimysium) [2]. The importance of the interplay between different compartments of the skeletal muscle niche (Figure 1b) is exemplified on acute injury, when multiple mechanisms are initiated within the different compartments that eventually converge to activate tissue-resident muscle stem cells (MuSCs, also known as satellite cells). For instance, damaged blood vessels can release cytokines [3] or inflammatory cells [4] to support regeneration at an injury site.

Normal tissue function and repair/regeneration can be overcome in large acute muscle injuries as well as in chronic severe musculoskeletal disorders such as muscular dystrophy [5], where different components of the skeletal muscle tissue functional units and niche are compromised. Given ethical considerations and limited tissue availability, it is often difficult to study skeletal muscle developmental dynamics, regeneration and disease pathogenesis in human subjects or their biopsies. Although traditional cell culture and animal models have been used to elucidate some molecular aspects behind these processes, limitations in using different species [6] and systems lacking physiologically relevant extracellular cues [7] make it difficult to translate such

findings to the human context. Bioengineering human models with higher fidelity to native skeletal muscle tissues can overcome these limitations and enable researchers to advance our fundamental understanding of the mechanistic processes behind muscle development and regeneration. Such insights can be further applied to disease modelling, biomarker detection, drug screening and regenerative medicine.

In this review, we will start with a brief overview of skeletal myogenic cell generation and differentiation followed by a discussion on recently developed three-dimensional (3D) platforms, developed with human biopsy-derived myoblasts (primary or immortalised) or pluripotent stem cells. We then conclude with our perspectives on the future of artificial skeletal muscle models by discussing methods to develop physiologically complex models able to deliver clinically relevant phenotypic readouts that can be used as outcome measures for therapy development. We will not highlight studies based on platforms using rodent myogenic cells, nor those involving top-down approaches such as tissue decellularization, for which we redirect the reader to recent comprehensive reviews [8,9].

## Cellular constituents of advanced human muscle models: beyond primary myoblasts

### Immortalising biopsy-derived skeletal myogenic cells

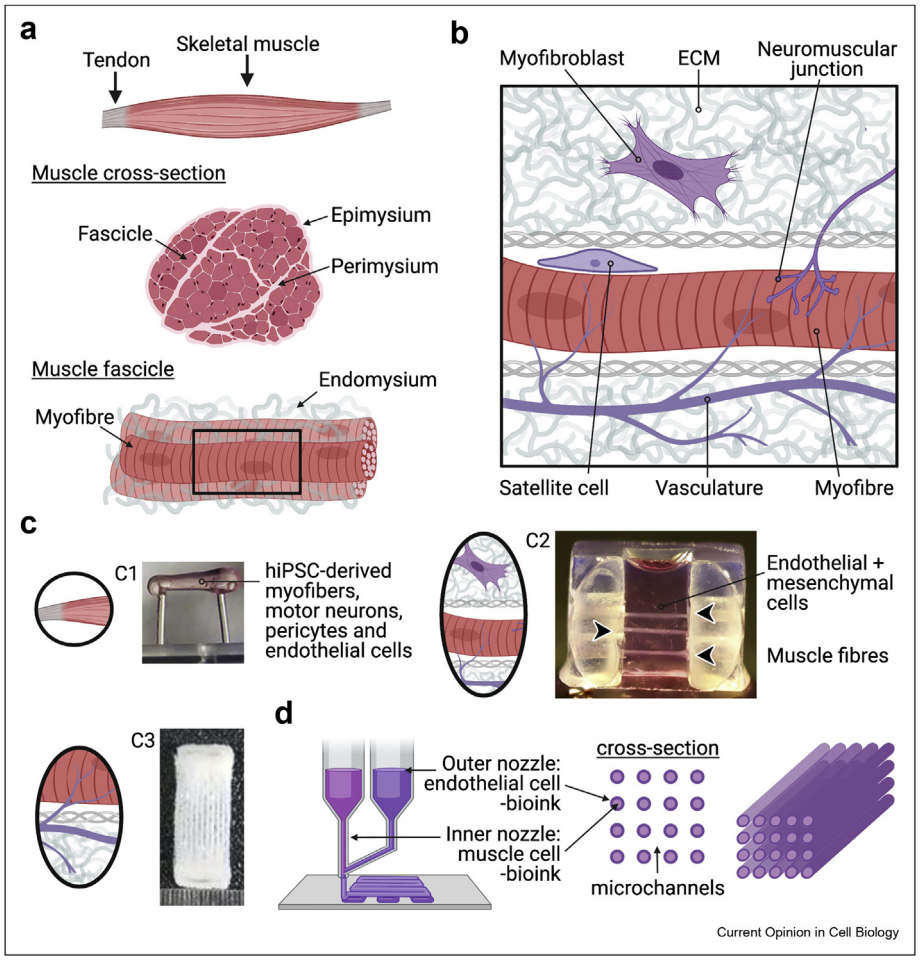
The ability to culture primary myogenic cells from human skeletal muscle biopsies *ex vivo* is crucial for modelling skeletal muscle function and disease [10,11]. However, the limited availability of patient tissue biopsies and restricted proliferative capacity of the extracted myoblasts make it difficult to use these cells extensively [12]. As a result, several immortalisation strategies have been applied to overcome Hayflick's limit while maintaining the myogenic differentiation potential of isolated primary myoblasts *in vitro*. The most used strategies rely on the dual expression of cell cycle regulators (e.g. CDK4 and Bmi-1) and the catalytic subunit of human telomerase [13–15]. Other strategies include expression of Simian Virus 40 (SV40) large T-antigen [16] and cyclin D1 genes [17] to produce clonal human myogenic cell lines with robust differentiation potential [18,19] that are amenable to genetic manipulation, transplantation, disease modelling and tissue engineering [20,21,23]. However, primary cell immortalisation relies on the supply of biopsy-derived myogenic cells which are not always available for disease-specific (e.g. tissue fibrosis or exhaustion of MuSCs in degeneration–regeneration cycles), diagnostic (e.g. fewer muscle biopsies are performed because more diagnoses are being made with genetic testing) and ethical issues (*ad hoc* biopsies for research purposes are not feasible in children with severe muscle disorders). A further concern with primary and immortalised cell lines is their limitation in modelling processes requiring extended time-resolution such as developmental myogenesis of early-onset muscle disorders, given the adult/mature state of the cells [24]. In these cases, pluripotent stem cells (PSCs; including induced PSCs (iPSCs) and embryonic stem cells) provide a particularly useful solution to these hurdles.

### PSC-derived skeletal myogenic cells: lessons from developmental myogenesis

Myogenic differentiation protocols of PSCs take inspiration from biochemical signalling processes that occur during developmental and/or regenerative myogenesis. During embryogenesis, precursor cells for trunk and limb muscles originate from structures of condensed paraxial mesoderm into bilaterally segmented compartments known as somites. Key signalling pathways governing this complex process include those triggered by Sonic hedgehog, Wnt and bone morphogenetic protein produced by the notochord, dorsal neural tube and surface ectoderm and lateral plate mesoderm, respectively [25]. The dermomyotome, an epithelial cell layer at the dorsal end of the somites underneath the ectoderm, is a signalling hotspot for myogenic specification and determination and gives rise to the dermis, skeletal muscle precursor cells, endothelial and vascular smooth muscle cells [26,27]. Crucially, the dorsomedial lip of the dermomyotome is also the site for expression of skeletal myogenic regulatory transcription factors such as myogenic differentiation 1 (MyoD) and myogenic factor 5 (Myf5), that initiate specification of skeletal muscle progenitors [28]. These embryonic myoblasts then migrate under the dermomyotome to form the myotome and then fuse to form embryonic muscle fibres during primary myogenesis. Notably, around this time, Paired box genes 3 and 7 (Pax3/7) positive cells from the dermomyotome migrate into the underlying myotome to sustain muscle growth and establish the future MuSC pool.

Primary myogenesis is followed by foetal or secondary myogenesis (~E14.5-E17.5), characterised by the formation of secondary muscle fibres surrounding the existing primary myofibres and by the onset of innervation. At this stage, MuSCs expressing C-Met, M-Cadherin and Pax7 become identifiable in their characteristic niche between the basal lamina and myofibres (Figure 1b) [26,29]. The MuSCs contribute to the formation of multinucleated fibres by partially fusing with secondary muscle fibres during development. In adult skeletal muscles, MuSCs are normally quiescent and are only activated on injury to first proliferate and then asymmetrically divide into a pool of progenitors that return to quiescence for maintenance and a pool of committed myoblasts that will progressively lose *Pax7* expression while upregulating Myf5 and MyoD [30]. Subsequently, these myoblasts proliferate and fuse with each other and/or other muscle fibres to recover the

**Figure 1**




**Recreating human skeletal muscle architecture *in vitro*.** Created with BioRender.com. **(a)** Physiological structure of human skeletal muscle tissue. **(b)** Schematic of the skeletal muscle tissue niche including myofibres, vasculature, satellite cells, myofibroblasts, motor neuron endings (in the neuromuscular junction) and extracellular matrix (ECM). **(c)** Selected examples of culture platforms that integrate different components of the skeletal muscle tissue niche. C1) humaninduced pluripotent stem cell (hiPSC) derived cells (including myofibres, motor neurons, pericytes and endothelial cells) are differentiated in a fibrin hydrogel held under tension between two silicone posts; copyright 2018 Elsevier [40]. C2) myoblasts were first formed into myotubes in a fibrin gel that were subsequently surrounded by a fibrin gel solution containing endothelial cells and fibroblasts; copyright 2018 Elsevier [55]. C3) 3D bioprinted cylindrical tubes of myogenic progenitors with or without encasement by an endothelial cell layer in parallel with hollow microchannels**;** copyright 2019 Elsevier [59]. **(d)** Illustration of a coaxial 3D bioprinting setup. 3D, three-dimensional.

injured tissue mass in a process that resembles embryonic myogenesis [31].

In the past decade, several methods have been established to differentiate myogenic cells from PSCs (summarised in Table 1). The two main strategies to induce myogenic differentiation of human PSCs are 1) transgene-based, involving the exogenous expression of key myogenic regulators (e.g. Pax3/7 or MyoD) [32,33] sometimes together with epigenetic modulators (e.g. BRG1/BRM-associated factor 60 (BAF60) or Jumonji domain-containing protein D3 (JMJD3)) [34,35] and 2) transgene-free methods which use a cocktail of signalling molecules, growth factors and inhibitors to recapitulate developmental myogenesis [36–39].

Although skeletal muscle models based on monolayer [bidimensional and two-dimensional (2D)] cell cultures are well-established and widely used to study muscle differentiation and disease because they are simple, inexpensive, and user-friendly, their physiological relevance may be limited [40]. Indeed, these 2D models often do not replicate the complexity of the native muscle tissue functional units and niche, where cells of different lineages constantly interact via a range of biochemical and physical factors in different 3D compartments (Figure 1) [41–44]. To overcome these

**Table 1**

**Overview of key transgene- and small molecule-based skeletal myogenic differentiation studies of human iPSCs/ESCs.**

**A. Transgene-mediated**

| Transgene | Cell source | Culture method, disease models and remarks | References |
|---|---|---|---|
| MyoD | hiPSC | 2D culture, DMD, inducible SMARCD3 gene expression | [35] |
| | hESC, hiPSC | 2D culture, MyoD mRNA transfection and siRNA mediated knockdown of POU5F1 | [75] |
| | hiPSC | Facioscapulohumeral muscular dystrophy, transposon-mediated delivery of tetracycline inducible MyoD | [76] |
| | hiPSC | 2D culture, DMD | [77] |
| | hiPSC | 2D culture, Pompe disease, transposon-mediated delivery of MyoD | [78] |
| | hiPSC | 2D culture, amyotrophic lateral sclerosis, transposon-mediated delivery of MyoD | [79] |
| | hiPSC | 2D culture, epigenetic modulator JMJD3 | [34] |
| | hESC | 2D culture, GAG-binding motif for cell penetration peptide | [80] |
| | hiPSC | 2D culture | [81] |
| | hiPSC | 2D culture, exon skipping for DMD | [82] |
| | hESC, hiPSC | 2D culture, limb girdle muscular dystrophy iPSCs, DMD iPSCs. Inducible MyoD expression | [83] |
| | hiPSC | 2D culture, DMD patient hiPSCs for gene correction by TALEN and CRISPR-Cas9 | [84] |
| | hiPSC | EB culture, Carnitine palmitoyltransferase II deficiency patient iPSCs | [85] |
| | hiPSC | 2D culture, DMD patient-derived | [86] |
| | hESC | Myosphere culture, overexpression of MyoD and BAF60C | [88] |
| | hiPSC | 2D culture, Miyoshi myopathy patient-derived | [89] |
| | hESC | EB culture, adenoviral delivery | [90] |
| | hESC | 2D culture, Tet-ON system in the lentiviral vector | [91] |
| | hiPSC | 2D culture, limb girdle muscular dystrophy iPSCs, DMD iPSCs, inducible MyoD expression | [33] |
| | hiPSC | mRNA-mediated | [92] |
| Pax7 | hESC, hiPSC | EB culture | [32] |
| Pax7 | hESC, hiPSC | GSK3-β inhibitor-induced commitment, PAX7-induced differentiation, maturation cocktail | [66] |
| Myf5 | mESC, hESC | EB culture, Lenti-mediated Tet-ON system | [93] |

**B. Small molecule-induced**

| Small molecules and/or culture platform | Cell source | Culture method and remarks | References |
|---|---|---|---|
| ITS-A, LDN, Wnt activators, BMP inhibitors, CHIR, GSK3 inhibitor, IGF1, HGF, DAPT (notch pathway inhibitor) | hiPSC | 2D culture, dual codifferentiation into skeletal muscle cells and motor neurons | [94] |
| CHIR, LDN, SB431542, HGF, IGF-1 | ESC, hiPSC | 2D culture | [95] |
| Wnt activators, TGF-β inhibitors, CHIR, LDN, BMP receptor inhibitors | hiPSC | Sphere-based culture | [96] |
| FGF2, LY294002, BMP4, CHIR | hESC | 2D culture | [97] |
| GSK3-β inhibitor, ascorbic acid, Alk5 inhibitor, EGF, dexamethasone, insulin | hESC | 2D culture | [98] |
| GSK3-β inhibitor, BMP inhibitor, HGF, IGF, bFGF | hESC, hiPSC | 2D culture | [36,99] |
| GSK3-β inhibitor, BMP, VEGF inhibitor, bFGF | hESC, hiPSC | EB culture | [100] |
| bFGF, EGF | hESC, hiPSC | Free-floating spherical culture | [101] |
| GSK3-β inhibitor, CHIR, FGF2 | hiPSC | 2D culture, FACS sorting | [102,103] |
| Chitosam-polycaprolactone nanofibres, Wnt3a | hESC | 2D culture, C-MET$^{+}$ sorting | [104] |
| GSK3-β inhibitor, bFGF, forskolin | hiPSC | EB culture | [39] |
| LiCl, BMP4, activin A | hESC, mESC | 2D culture | [105] |
| – | hESC, hiPSC | EB culture | [106] |

(*continued on next page*)

**Table 1** (*continued*)

| B. Small molecule-induced | | | |
|---|---|---|---|
| **Small molecules and/or culture platform** | **Cell source** | **Culture method and remarks** | **References** |
| TGF-β inhibitor | hESC | GNE–/– EB culture | [107] |
| OP9 coculture, insulin | hESC | $CD73^+$ MSC sorting, $NCAM^+$ sorting | [108] |
| OP9 and C2C12 coculture | hESC | $CD73^+$ MSC sorting | [109] |

hiPSC, human-induced pluripotent stem cell; hESC, human embryonic stem cell; mESC, mouse embryonic stem cell; DMD, Duchenne muscular dystrophy; EB, embryoid body; 2D, two-dimensional; MSC, mesenchymal stem cell; IGF, insulin growth factor; HGF, hepatocyte growth factor; LDN,LDN193189; CHIR, CHIR99021; ITS, insulin transferrin-selenium; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; bFGF, basic FGF; GAG, glycosaminoglycan; JMJD3, jumonji domain-containing protein D3; TGF, transforming growth factor; FACS, fluorescence-activated cell sorting; MSC, mesenchymal stem cell; NCAM, neural cell adhesion molecule; EGF, epidermal growth factor.

limitations, muscle biologists have started to exploit the potential of bioengineering to develop 3D human skeletal muscle platforms with a higher degree of complexity and maturation, better resembling native tissues.

## Recapitulating 3D tissue complexity

Strategies to engineer 3D human skeletal muscles can be broadly classified into either 1) self-organised, organoid-like 3D cultures or 2) scaffold-based platforms. Recent notable studies using 3D culture platforms containing human myogenic cells are summarised in Table 2 and discussed in the following sections.

### Self-organised 3D skeletal muscle organoids

The principles behind organoid generation could be traced back to Steinberg's differential adhesion hypothesis [45], as per which different cell types tend to segregate themselves based on their adhesive properties. Two recent studies have elegantly shown the generation of human organoids with functional neuromuscular junctions (NMJs) able to stimulate skeletal myofibres via the activation of neuronal circuits [46,47]. Anderson et al. [46] first generated spinal and muscle spheroids before assembling the spheroids together to obtain 3D cortico-motor assembloids, which are complex multicellular models with functional neural circuits. More recently, hiPSC skeletal muscle organoids containing paraxial mesoderm and neuromesodermal progenitors have been induced to foetal hypaxial myogenesis, generating PAX7-positive myogenic and PDGFRa-positive fibroadipogenic progenitor populations which could offer useful inights into human developmental somitogenesis and muscle histogenesis [111]. Although these models provide us with insights into the complexity of muscle tissue and its interface with the neural network (necessary for better modelling of neuromuscular disorders), they do not replicate key architectural features of skeletal muscles such as myofibre alignment, owing to the absence of tension normally provided by tendinous attachments to the bone.

### Scaffold-based platforms to model skeletal muscle tissue architecture

Skeletal muscle is a highly mechanically active tissue undergoing frequent contraction cycles that expose cells, organelles and the surrounding extracellular matrix to physical forces which could in turn impact myogenesis and differentiation. For instance, culturing cells on substrates with physiological rigidity enhances muscle stem cell renewal [43], myogenic differentiation [41] and optimise myotube maturation [48]. Moreover, spatially aligning differentiating myoblasts—either by patterning lines of adhesive protein or by fabricating alternating lines of physiologically stiff and soft hydrogels—further enhances myotube formation and maturation [48,49]. Thus, providing mechanical cues via structural support from a scaffold to cultured myogenic cells is necessary to enhance the physiological relevance of the resulting advanced skeletal muscle models.

Several research groups have successfully created 3D muscle models by embedding differentiating human myoblasts in hydrogels (including fibrin, collagen and Matrigel) anchored between two attachment points [50–54,63]. These experimental setups recreate mechanical cues present in the native skeletal muscle niche that are absent in most organoid systems by providing embedded cells with a surrounding matrix that they can attach to, while also presenting an axis of tension in the hydrogel held between the two attachment points that guides myotube alignment. Such tension and alignment of myotubes promote sarcomere maturation and reveal disease-specific phenotypes normally seen with less prevalence in 2D cultures. This was indeed demonstrated by our group for skeletal muscle disorders caused by defective nuclear envelope proteins using patient-specific iPSCs [7,40] with the resulting engineered muscles showing characteristic disease-associated nuclear shape abnormalities secondary to *LMNA* mutations. This finding has been recently validated in an independent study using a miniaturised 3D platform [112]. Other groups have used similar platforms to differentiate

**Table 2**

**Summary of significant 3D artificial human skeletal muscle studies since 2018.**

| Platform | Cell types | Source | Physical cues | Electrical cues | Vascularisation | Functional readout | Disease modelling | References |
|---|---|---|---|---|---|---|---|---|
| Organoids on low adhesion plates | Cortical neurons<br>Spinal MNs<br>Skeletal yogenic cells | hiPSCs | - | Optogenetic stimulation | - | $Ca^{2+}$ transients<br>Contraction | - | [46] |
| | Neuromesodermal progenitors | hPSCs | - | - | - | $Ca^{2+}$ transients<br>Contraction | MG patient antibodies reduce NMJ function | [47] |
| Cells in hydrogel held between two attachment points | Myoblasts | Human biopsy | Tension along attachment sites | Electrical stimulation | - | Contraction | - | [50] |
| | Myoblasts | Human biopsy | Tension | EPS | - | Contraction | Reduced α-glucosidase enzyme activity and elevated glycogen content | [51] |
| | Myoblasts | Human biopsy | Tension | - | - | - | Creatine kinase release | [52] |
| | Myoblasts | Human biopsy | Tension | EPS | - | $Ca^{2+}$ transients<br>Contraction | Atrophy and lower contractility in senescent muscles | [53] |
| | Immortalised myoblasts | Human biopsy | Tension | EPS | - | $Ca^{2+}$ imaging<br>Contraction | - | [54] |
| | Myoblasts | Human biopsy | Tension | Electrical field stimulation | - | Contraction | Regeneration observed after barium chloride injury | [61] |
| | Myoblasts | Human biopsy | Tension | EPS | - | Contraction | Chemotherapeutic agent reduced contractile force | [74] |
| | Skeletal myogenic cells<br>MNs<br>Pericytes<br>ECs | hiPSCs | Tension | - | ECs form vessel-like networks in vitro<br>Functional vascularisation upon implantation in mice | $Ca^{2+}$ transients | Engineered muscles from laminopathy patients nuclear abnormalities | [7,40] |
| | Myoblasts<br>Tenocytes | Human biopsy, rat tail | Tension,<br>Spatial bio-printing of tenocytes around post | EPS | - | $Ca^{2+}$ transients | - | [56] |
| | Myoblasts<br>Fibroblasts<br>MNs | Human biopsy, ESCs | Tension<br>MN spheroids over muscle bundle | Optogenetic stimulation | - | $Ca^{2+}$ transients<br>Contraction | MG antibodies reduced excitability of muscle | [22] |
| Two compartments of fibrin hydrogel: muscle fibres | Immortalised myoblasts<br>Fibroblasts<br>ECs | Human biopsy | Tension<br>Myofibres spatially segregated from fibroblasts + ECs | - | ECs form network of microvessels | - | Fibrosis markers upregulated in Duchenne muscular dystrophic muscles | [55] |

(*continued on next page*)

**Table 2. (*continued*)**

| Platform | Cell types | Source | Physical cues | Electrical cues | Vascularisation | Functional readout | Disease modelling | References |
|---|---|---|---|---|---|---|---|---|
| embedded in endomysium | | | | | | | | |
| Strips of cell-hydrogel bio printed with microchannels | Myoblasts<br>Immortalised neural progenitors | Human biopsy, cell line | Tension (between pillar structures) | Electrical stimulation of peroneal nerve after rodent implantation | Upon implantation | Force measurement of tibialis anterior after implantation | - | [58] |
| | Myoblasts<br>HUVECs | Human biopsy, cell line | Tension<br>Spatial coaxial bioprinting of myotubes encapsulated by ECs | Electrical stimulation of peroneal nerve after rodent implantation | EC layer<br>Post implantation | Force measurement of tibialis anterior after implantation | - | [59] |
| Three compartment microfluidic device: myobundle, MN spheroid, EC monolayer | Skeletal myogenic cells<br>Neural stem cells<br>ECs | hiPSCs | Tension between pillars<br>Spatial segregation | Electrical stimulation | EC barrier | Contraction | ALS constructs contracted less and had more MN degradation | [64] |
| Two compartment BioMEMS device: myoblasts, MNs | Myoblasts<br>MNs | Human biopsy, hiPSCs | Compartments spatially segregated by microtunnels | Electrical stimulation | - | Contraction | - | [62] |
| Cells in hydrogel bundles anchored by frame structure | Myoblasts | Human biopsy | Tension along attachment sites | Exercise by electrical stimulation | - | $Ca^{2+}$ transients<br>Contraction<br>Acylcarnitine and amino acid levels | - | [68] |
| | Myoblasts<br>Dermal fibroblasts | Human biopsy | Tension | Exercise by electrical stimulation | - | Contraction | - | [69] |
| | Myoblasts | Human biopsy | Tension | Exercise-mimetic electrical stimulation | - | $Ca^{2+}$ transients<br>Contraction | Muscle atrophy and proinflammatory cytokine secretion | [70] |

hiPSC, human induced pluripotent stem cell; 1°, primary; MG, myasthenia gravis; NMJ, neuromuscular junction; EPS, electrical pulse stimulation; IOPD, infantile-onset Pompe disease; EC, endothelial cell; MN, motor neuron; ESC, embryonic stem cell; ALS, amyotrophic lateral sclerosis; 3D, three-dimensional; HUVEC, human umbilical vein endothelial cells.

primary or iPSC-derived myogenic progenitors to model acute and chronic muscle injuries, disorders and ageing [51–53,63] (additional examples are discussed in subsequent sections). Although these culture models recreate the tensional cues from the attachment of muscles to tendons, the majority lacks the multicellular complexity typical of native skeletal muscle tissues as they have been made purely with cells of a single lineage (often using biopsy-derived myoblasts).

### Introducing lineage complexity together with spatial compartmentalisation

Previous work in our group suggests that increasing lineage complexity by including iPSC-derived endothelial cells and pericytes together with myogenic cells (Figure 1c, panel C1) in human 3D skeletal muscle constructs is associated with improved force recovery after injury upon implantation in mice subjected to volumetric muscle injuries [40]. Although cells were not spatially patterned in these constructs, the intrinsic self-organising properties of myotubes and vascular networks resulted in artificial muscles containing vessel-like networks in the matrix surrounding myofibres. Alternatively, cells can be spatially patterned as performed by Bersini et al. [55] (Figure 1c, panel C2), by differentiating myogenic progenitors in fibrin hydrogels and subsequently embedding the muscle fibres in a hydrogel containing endothelial cells and myofibroblasts. The physiological conditions of the skeletal muscle tissue niche reproduced by these constructs made it possible to observe an increased deposition of collagen I and fibronectin in a 3D model of Duchenne muscular dystrophy that could not be seen in 2D. Other notable spatial patterning methods used to create multilineage artificial muscles anchored at two attachment points include the seeding of motor neuron spheroids on top of muscle bundles [22,40] and the bioprinting of tenocytes around post attachment sites with myoblasts in the hydrogel region between posts [56].

Precise spatial patterning of cells and extracellular matrix to create compartmentalised 3D constructs is currently best achieved by bioprinting techniques. Kim et al. [57] used a 3D bioprinting strategy to create aligned strips of myogenic progenitor-laden bioink with hollow microchannels supported by poly(ε-caprolactone) pillars. The organised structure of these constructs enhanced functional recovery, vascularisation and neural integration in the tibialis anterior muscle after implantation into rats. Integrating neural progenitors into the cell bioink layer further improved neuromuscular junction formation and muscle function with reduced signs of fibrosis after implantation [58]. 'Prevascularised' muscle constructs printed by a coaxial technique (Figure 1c, panel C3 and 1d), where the strips of the cell-laden hydrogel are spatially segregated with an inner strip of myogenic progenitor bioink encased in a layer of endothelial cell-loaded bioink, further enhanced functional vascularisation and recovery upon implantation [59]. Apart from the benefit to *in vivo* vascularisation, the ability to perfuse muscle constructs through hollow microchannels could have further advantages *in vitro*, such as testing antibody-mediated immune responses (e.g. in myasthenia gravis) or the effects of small-molecule treatments on disease-specific muscle constructs.

### Increasing muscle function with simulated innervation

The complex process of skeletal muscle innervation is simplified *in vitro* by electrical stimulation [60–62], chemical treatment [22,40] or optogenetic manipulation [22,46]. Several studies have measured functional parameters of the resulting muscle contraction from such treatments (e.g. $Ca^{2+}$ dynamics and force of contraction). Osaki et al. [64] used this measure to find that artificial muscle microfluidic devices innervated by amyotrophic lateral sclerosis (ALS) iPSC-derived motor neuron spheroids spatially separated from muscle bundles had impaired contraction force compared with control muscles and that the impairment could be partially recovered by treatment with ALS drug candidates.

For diseases such as Duchenne muscular dystrophy where muscles are primarily affected, it is important to generate myotubes that are mature enough to reveal phenotypic readouts for relevant disease modelling. To enhance human skeletal myotube maturation *in vitro* (in terms of gene expression, architecture and contractile ability), cells are usually treated with specific growth factors and small molecules during differentiation [65,66]. Xu et al. [65] showed that exposing myogenic differentiation cultures to endothelial cell growth medium-2 supplements for short time periods enhanced the contractile force generated by myotubes. In another study, Selvaraj et al. [66] used a cocktail of small molecules to enhance myofibril sarcomeric organisation in iPSC-derived myotubes, namely, the transforming growth factor-β (TGF-β) signalling inhibitor SB431542, the γ-secretase and Notch pathway inhibitor DAPT, the glucocorticoid dexamethasone, the MAPK/ERK Kinase (MEK) inhibitor PD0325901 and the adenylyl cyclase activator forskolin. Both studies also demonstrated upregulation of genes (*MYOG* and *MYH3*) and microRNAs (*MIR206* and *MIR113B*) associated with mature muscles.

A way to mimic physiological muscle overuse is to apply long-term electrical field stimulation training. Using this approach, a recent study revealed contractile performance decline in dystrophic iPSC-derived myotubes compared with healthy controls [67]. Electrical stimulation has also been applied to 'exercise' artificial 3D

muscles with prolonged intermittent electrical stimulation regimes that induce hypertrophy and improve metabolic flux [68]. Takahasi et al. [69] showed that by applying electrical pulse stimulation exercise to myofibre sheets cocultured with dermal fibroblasts, more exercise-related cytokines were released. More recently, advanced muscle models have been used to study the anti-inflammatory effects of muscle exercises using exercise-mimetic electrical stimulation on myobundles made from primary human myoblasts [70]. Applying a similar approach to exercise PSC-derived 3D muscle constructs might further advance the maturation of patient-specific artificial muscles to broaden the spectrum of phenotypic readouts for advanced disease modelling.

## Future perspectives

The aphorism from the statistician George E. P. Box, '*all models are wrong, but some are useful*', concisely summarises the current landscape of cellular modelling of skeletal muscle tissue development, differentiation and disease. Although none of the existing models discussed in this review fully recapitulate all aspects of the physiological skeletal muscle tissue niche, the ability to recreate at least some features has been invaluable to improve our understanding of skeletal muscle growth, disease and regeneration. Excitingly, recent studies are also focussing on closely studying and modelling developmental myogenesis and early (i.e. foetal) muscle disease pathogenesis taking advantage of emerging technologies [37,71,72]. Looking forward, we see the need for better integration of the two main methodologies used to differentiate human iPSCs into functional skeletal myofibres (i.e. transgene- and small molecule-based protocols) alongside the two key strategies to produce artificial skeletal muscle tissues, namely, the organoid systems with scaffold-based 3D culture platforms. Scaffold-based culture platforms (bioprinting in particular [59]) are likely to provide superior structural support and spatial cues more than simpler, self-assembling organoid systems. Regardless of the underlying platform/scaffold, the use of iPSCs makes it possible to obtain a virtually unlimited number of cells from a minimally invasive source to create isogenic (and often isochronic) multilineage tissues for disease modelling, drug development, cell therapy or tissue replacement. Nonetheless, at variance with models based upon non-human cells [110], additional work is required to enhance the maturation of human iPSC-derived platforms: this is particularly relevant to model late-onset diseases, for which the relatively immature myofibres currently generated by the majority of available protocols might not recapitulate phenotypic readouts of adult skeletal muscles with high fidelity. We foresee this problem being rapidly addressed by the field, with promising results already obtained by stimulating cultures *in vitro* chemically [66] or electrically [68]. At the same time, more clinically relevant phenotypic readouts of muscle function need to be consistently measured in these artificial tissues (e.g. creatine kinase release and contraction defects [73]). However, also in this case, suboptimal maturation might pose a challenge. Furthermore, scaling down models without compromising tissue architecture and composition to dimensions amenable to medium-/high-throughput screening platforms will become increasingly important in the next decade, and progress is also being made on that front [74,112]. Close multidisciplinary collaborations between muscle biologists, tissue engineers and clinicians are likely to provide solutions to address all the aforementioned challenges in the near future.

## Conflict of interest statement

FST provides consulting services to Aleph Farms via UCL Consultants. The other authors do not declare conflict of interest.

## Acknowledgements

This work was supported by the European Research Council (759108 – HISTOID) and the Francis Crick Institute, which receives its core funding from the Cancer Research UK, the UK Medical Research Council and the Welcome Trust (FC001002); Muscular Dystrophy UK (19GRO-PS48-0188; 17GRO-PS48-0093-1), the BBSRC and the NIHR (the views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR or the Department of Health). Work on 3D human skeletal muscle models in the Tedesco laboratory was also supported by the AFM-Telethon and Duchenne Parent Project. For the purpose of Open Access, the author has applied a CC BY public copyright license to any author accepted manuscript version arising from this submission.

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