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Bioengineering human skeletal muscle models: Recent advances, current challenges and future perspectives

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ABSTRACT

Engineering models of human skeletal muscle tissue provides unique translational opportunities to investigate and develop therapeutic strategies for acute muscle injuries, and to establish personalised and precision medicine platforms for *in vitro* studies of severe neuromuscular and musculoskeletal disorders. Several myogenic and non-myogenic cell types can be isolated, generated, amplified and combined with scaffolds and biomaterials to achieve this aim. Novel bio-fabrication strategies, which include exogenous stimuli to enhance tissue maturation, promise to achieve an ever-increasing degree of tissue functionalisation both *in vivo* and *in vitro*. Here we review recent advances, current challenges and future perspectives to build human skeletal muscle tissue "in a dish", focusing on the cellular constituents and on applications for *in vitro* disease modelling. We also briefly discuss the impact that emerging technologies such as 3D bioprinting, organ-on-chip and organoids might have to circumvent technical hurdles in future studies.

1. Introduction

Skeletal muscle is the most abundant human tissue, exerting multiple critical functions such as movement generation, posture maintenance, storage of nutrients and metabolites, soft tissue support and body temperature homeostasis. The functional cellular units of skeletal muscle are the muscle fibres (myofibres): long cylindrical multinucleated cells containing the contractile apparatus, densely packed and precisely oriented along the force axis. Layers of connective tissue surround each muscle fibre (endomysium), then fascicles of muscle fibres (perimysium) and ultimately the whole muscle (epimysium), further defining its structure [[48]]. Skeletal muscle contraction and force production are mediated by actin and myosin filaments arranged in units called sarcomeres. Skeletal muscle is innervated by motoneurons that interact with the tissue in specialised structures called neuromuscular junctions (NMJs). In these synapses, the neurotransmitter acetylcholine (ACh) is released and activates signalling pathways ultimately leading to muscle contraction [[143]]. A complex vascular tree runs parallel to myofibres, which grant blood supply [[54]], along with lymphatic vessels which maintain interstitial fluid homeostasis [[4]].

Understanding how muscle is made in the embryo and how it regenerates in the adult is critical to generate faithful muscle models *in vitro*. Most skeletal muscles originate from the segmentation of embryonic paraxial mesoderm on both sides of the neural tube into transient structures called somites (reviewed in [[22]] and further detailed in specific papers of this special issue; please note that there are exceptions to this statement and indeed cranial muscle have a different origin). Somites undergo differentiation into a ventral (sclerotome) and a dorsal (dermomyotome) part: the latter will give rise to dorsal dermis and muscles. Soon after dermomyotome formation, myogenic progenitor cells delaminate from the structure forming the so-called primary myotome. Later, embryonic myoblasts invade the myotome and undergo terminal differentiation into multinucleated fibres, known as primary fibres (E11). As development proceeds, a second wave of myogenesis occurs with foetal myoblasts giving rise to secondary fibres surrounding the primary fibres' scaffold (E14.5–17.5). Arising in the central part of

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the dermomyotome, muscle stem cells (MuSCs, also known as satellite cells) which are critical for postnatal muscle regeneration, can also begin to be identified. MuSCs migrate in the developing muscle, invade the tissue and partially fuse with secondary muscle fibres contributing to the generation of multinucleated fibres. Key transcription factors in the molecular circuitry required for myogenesis are Pax3 and Pax7 [[66,113]]. Downstream of the Pax genes, myogenic factor 5 (Myf5), myogenic regulatory factor 4 (Mrf4) and myogenic differentiation 1 (MyoD), are critical for further tissue specification [[23]].

Skeletal muscle is characterised by its remarkable regenerative potential which can be divided into four main phases: 1. Tissue injury; 2. Inflammatory response; 3. Activation, proliferation and differentiation of MuSCs; 4. Maturation and remodelling of the newly formed tissue. Tissue injury, (e.g., due to myotoxins, physical means or chronic damage such as in genetic muscle conditions), causes influx of calcium and myofibre necrosis. The necrotic response induces neutrophils invasion and release of an array of pro-inflammatory molecules that recruit tissue-resident macrophages, which act as scavengers and clear tissue debris, to set the foundations for new myofibre formation from MuSCs. MuSCs were first identified thanks to their anatomical position under the basal lamina of adult muscle fibres [[87]]; they are normally quiescent and are marked by the expression of the transcription factor Pax7 [[119,149]], although a subset also expressing Pax3 has been identified in specific muscles such as the diaphragm and the biceps [[26,112]].Upon muscle injury, MuSCs become activated, start proliferating and undergo asymmetric cell division generating committed progenitors called myoblasts while maintaining a self-renewing pool. Myoblasts progressively lose Pax7 and acquire Myf5 and MyoD expression [[35]] and fuse with each other or with existing fibres to repair the tissue. Newly formed myofibres undergo maturation, which entails the re-expression of embryonic and neonatal myosins, in a process that mirrors their developmental pattern [[118]]. It is important to note that most MuSC markers have been identified in mouse and some do not have a direct human counterpart. Besides MuSCs, additional cells capable of myogenic differentiation *in vitro* and *in vivo* have been identified. These cell types have different origins (e.g., muscle, blood vessels, pluripotent cells), proliferation and differentiation capacity: we will provide an overview of the key ones utilised for human skeletal muscle tissue engineering and redirect the reader to a previous review for a more comprehensive discussion [[132]].

Despite their regenerative capacity, the repair machinery of skeletal muscle tissue can be overtaken in conditions such as genetically inherited muscular dystrophies, and irrecoverable loss of muscle tissues after traumatic injury known as volumetric muscle loss (VML) [[58,90]]. In either scenario, patients face persistent impairment of muscle function that causes severe disability.

With the aim of developing advanced therapeutic strategies by repairing or replacing the affected tissues, human skeletal muscle has been a long-standing research target of regenerative medicine and tissue engineering. Ever since the first reported generation of three-dimensional (3D) muscle constructs *in vitro* [[140]], human muscle bioengineering has taken a significant leap forward in the past years with novel technologies and strategies that enable the generation of multi-scale 3D constructs and further expand its application in *in vitro* disease modelling and drug screening [e.g., Ref. [83]]. Here we review advances in human cell-based *in vitro* and *in vivo* skeletal muscle engineering approaches developed in the last five years (Fig. 1). We also discuss strategies to improve tissue maturation as well as current hurdles and perspectives on emerging technologies. A particular emphasis is given to the cellular components required to bioengineer human muscle, and to applications for *in vitro* disease modelling. We will not be discussing studies based upon rodent or other cell types which have not been validated with human cells. Although we provide here a general overview on biomaterials, scaffolds and *in vivo* applications, we invite the reader to consult recent review articles on those specific topics [e.g., Refs. [96,111]].

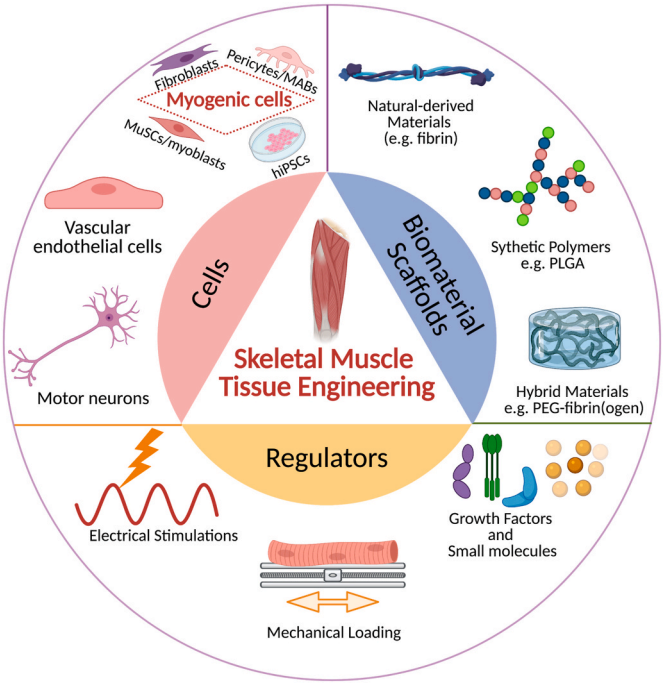

Fig. 1. Tissue engineering triad: multilineage cellular components, biomaterial scaffolds and exogenous stimuli (regulators) to recreate functional, biomimetic human skeletal muscle tissues. Adapted from Ref. [24]. MuSCs: muscle stem cells; MABs: mesoangioblast; hiPSCs: human induced pluripotent stem cells; PEG: Polyethylene glycol; PLGA: poly(lactide-*co*-glycolide. Figure created with BioRender.com.

2. Cell sources for human skeletal muscle tissue engineering

The selection of an appropriate cell source with intrinsic myogenic potential and optimal complementarity with the chosen biomaterial is vital for all cell-based muscle engineering strategies. Several studies employ non-human myogenic cell lines, such as immortalised murine C2C12 myoblasts, which represent robust and effective cell sources in muscle bioengineering [[16]]. Yet, the degree of similarities between the resulting skeletal muscle models and the human native counterpart remain to be further clarified due to limited comparative studies.

2.1. Satellite cells and myoblasts

Due to their physiological role in muscle regeneration and self-renewal ability, MuSCs and their myoblast progeny (reviewed in [[132]]) are the obvious candidate to generate skeletal muscle *in vitro*. However, obtaining an adequate yield of homogenous MuSCs remains challenging, not only due to technical difficulties using most isolation and purification methods (reviewed in [[104]]), but also because of the transcriptional and functional heterogeneity within the human MuSC pool [[33]]. Identifying specific transcriptional profiles for distinguishing quiescent and activated subsets of human MuSCs [[32]] and surface markers such as CD82 and caveolin-1 which associate with quiescent subsets [[5,13]] could expand the use of human MuSCs for skeletal muscle engineering in the upcoming years. Emerging high-yield purification methods for human MuSCs also open a promising avenue for further applications of this cell type [[52,129]]. Furthermore, recapitulating the native niche environment is vital to maintain MuSC self-renewal and proliferative potential upon *in vitro* expansion. For instance, *in vitro* culturing of human MuSCs on soft biopolymeric films that mimicked physiological matrix stiffness can extend human MuSCs quiescence up to 10 days [[94]]. Recreating the endogenous MuSC niche in the context of both cellular constituents and scaffold substrate can generate MuSC-encapsulated 3D bioconstructs that sustain

proliferative and myogenic abilities of MuSCs upon transplantation into mouse models of VML [[109]], highlighting the importance of scaffold engineering when employing MuSCs as the myogenic cell source.

Human MuSC-derived (primary) myoblasts encapsulated in natural or synthetic hydrogels have been shown to generate skeletal muscle tissue constructs *in vitro* which possess aligned, multi-nucleated myotubes, some also with contractile ability [[1,2,38,40,47,68,72,82,83,130,138]]. Notably, some of the aforementioned studies demonstrated the presence of $Pax7^{+}$ nuclei within the produced constructs, suggesting potential regenerative capacity of these engineered muscles [e.g., Refs. [47,82]]. A drawback associated with using primary myoblasts for muscle bioengineering is their limited proliferative capacity due to cellular senescence [[114,127]].

The combined expression of human telomerase reverse transcriptase (hTERT) with cell cycle regulators such as cyclin-dependent kinase (CDK)-4 (with or without cyclin D1) or Bmi-1 has been shown to successfully bypass the restricted proliferative lifespan of primary human myoblasts derived from both normal and dystrophic muscle biopsies [[15,84,124]]. Although there are concerns regarding genomic alterations in immortalized myoblasts, this has not been observed comparing the transcriptomic profiles of hTERT/CDK4 immortalized myoblasts with their parental $CD56^{+}$ primary myoblast population [[135]]. Immortalized human myoblasts have been integrated into several tissue-engineered 3D muscle platforms [[108]]. Examples relevant for disease modelling include models to study the function and diseases of NMJs [[1]] and multilineage 3D constructs containing endothelium and endomysium *in vitro* to study fibrosis in Duchenne muscular dystrophy (DMD) [[17]]. More recently, DMD immortalized myoblast-derived 3D microtissues have also been used to model formation of revertant fibers in DMD [[43]]. A recent proof-of-principle study further validated the feasibility of employing immortalized myoblasts to generate skeletal muscle tissues that shared comparable level of reduction in contractile force to primary human myotubes *in vitro* upon treatment with myotoxic drugs [[97]], highlighting the potential of these models in preclinical drug screening. However, spontaneous fusion between immortalised myoblasts could generate genomic instability in hybrid cells which warrants careful monitoring for possible pre-clinical studies [[91]].

2.2. Skeletal muscle derived-pericytes and mesoangioblasts

Non-canonical stem/progenitor cells residing in skeletal muscle interstitium also contribute to myogenesis and regeneration, albeit less efficiently than MuSCs [reviewed in Refs. [45,134]]. Among them, an attractive cell sources for human muscle bioengineering include perivascular progenitor cells surrounding microvessels of skeletal muscles whose *in vitro* progeny has been termed mesoangioblasts. Notably, human mesoangioblasts have also undergone clinical investigation as an advanced medicinal product in a first in human cell therapy study for DMD [[36]], providing important clinical information potentially translatable in future studies based upon their application in tissue replacement strategies. Human and mouse mesoangioblasts survival and differentiation improved when cultured and delivered in polyethylene glycol-fibrinogen (PF) hydrogels [[50]]. The same PF hydrogels were used to bioengineer human mesoangioblast-derived skeletal muscle tissues which enabled replacement of ablated tibialis anterior muscles in mouse models upon transplantation [[49]]. More recently, the same team used a variety of murine and human myogenic cells (including mesoangioblasts) to biofabricate myo-substitutes for volumetric muscle injuries [[38]].

2.3. Human induced pluripotent stem cell-derived myogenic cells

Human induced pluripotent stem cells (hiPSCs) are generated by reprogramming somatic cells using specific factors and can supply virtually unlimited progenitors to derive cell lineages of the three embryonic germ layers [[131]]. Current methods to obtain hiPSC-derived myogenic progenitor cells (iMPCs) can be broadly classified into transgene-based and transgene-free protocols [reviewed in Refs. [76,80]].

Transgene-based approaches allow robust production of human iMPCs via expression of myogenic regulators such as Pax3/7 or MyoD [e.g., Refs. [39,57,133]]. To decrease potential risks linked to genomic manipulation (e.g., insertional mutagenesis) and to recapitulate skeletal muscle specification during development, several transgene-free protocols based on small molecules to derive myogenic cells from hiPSCs have been developed in the last decade [e.g., Refs. [19,28,30,63]]. However, these protocols often require prolonged differentiation time (>30 days) with a relative lower yield and purity of the derived myogenic population compared to the iMPCs derived using transgene-based approach [reviewed in Ref. [65]].

Although variability in differentiation capacity and limited maturation of the resulting cultures provide some bottlenecks to widespread use iMPC-derived myotubes [[145]], the potential of iMPC applications in skeletal muscle tissue engineering with respect to human disease modelling, drug screening and tissue replacement could be remarkable. Monolayer cultures of human iMPC-derived myotubes have been studied to model various genetic muscle disorders *in vitro*, displaying disease-specific phenotypes with relevance for drug development [e.g., Refs. [27,28,71,77,93,128,136,139]]. Notably, human iMPCs have also recently been bioengineered in 3D functional skeletal muscle tissues [[46,83,110,120]]. Specifically, Rao *et al.* generated functional iMPC-derived myobundles capable of generating contractile force, whereas our group pioneered the bioengineering of multi-lineage 3D skeletal muscle tissues containing hiPSC-derived myofibers, vascular endothelial cells, pericytes, and motor neurons [[83]]. Using this platform, we also reported the first human iMPC-derived 3D model of a muscle disease [[128]], further highlighting the potential of this emerging technology for precision medicine.

2.4. Trans-differentiation

Trans-differentiation (also known as direct reprogramming or myogenic conversion) is an intriguing option explored to obtain myogenic cells without transitioning though the pluripotent stage, but via direct conversion of a fully committed somatic cell type (usually fibroblasts) to the skeletal myogenic lineage [[78]]. Similarly to the aforementioned transgene-based protocols for deriving iMPCs, the majority of the transdifferentiation strategies induce myogenesis upon constitutive or inducible (over) expression of MyoD [e.g., Refs. [[12],20,70,78,117]]. Functional 3D skeletal muscle models with contractile ability upon electrical stimulation can be generated using MyoD-transduced primary human fibroblasts [[123,147]]. Nevertheless, contractile force generated by transdifferentiated cells appear to be lower than those produced by primary human skeletal myoblasts [[147]]. In addition, skeletal muscle tissue constructs fabricated with different lines of healthy donor fibroblasts displayed variations in contractile phenotype [[123]]. These observations can be correlated with possible incomplete genome reprogramming upon MyoD expression compared to primary human skeletal myoblasts [[85]]. Nonetheless, given the easy accessibility of the parental cell types and the relatively rapid differentiation time, this approach remains an attractive alternative option for pilot experiments of disease modelling and drug screening.

3. Biomaterials for human skeletal muscle tissue engineering

Most skeletal muscle engineering platforms combine myogenic cells with biomaterials in specific scaffolds. These scaffolds are designed to provide the structural framework which facilitates cell proliferation, migration, fusion and myogenic differentiation. Scaffold-free approaches involving self-assembly of myogenic cultures and construction of multi-layered cell sheets or micropatterned on-chip culture device

have also been used as an alternative [e.g., Refs. [29,41,115]]. The purpose of these engineered scaffolds is ultimately to produce extracellular matrix (ECM)-mimicking structures with low immunogenicity, high biodegradability and biocompatibility along with a uniform substrate elasticity and optimized conductivity that matches with the mechanical features of native skeletal muscles. Generally, these cell-laden scaffolds are assembled *in vitro* with myogenic cells (see above) and can be used for either *in vitro* modelling studies or for *in vivo* regenerative experiments in preclinical or clinical settings (Fig. 2).

Regardless of the scaffold type, a variety of studies have used different biomaterials to construct bioengineered muscle tissue, with natural, synthetic and composite polymers (which will be discussed in detail in dedicated sections). Decellularised scaffolds have been extensively used for skeletal muscle engineering. This approach entails removing the cellular component of donor tissues/organs, whilst maintaining the ECM architecture using perfused detergents. An advantage of this method is the generation of a biocompatible scaffold with native topography, mechanical properties and vascular network of the tissue of interest; however, it also presents several issues of sourcing and batch-to-batch variation that pose challenges to clinical translation. These decellularied scaffolds will not be discussed further in this review, but we wish to redirect the interested reader to a recent comprehensive article on the topic [[79]].

3.1. Natural polymers

In line with the goal of resembling the 3D microenvironment of ECM, natural polymers originated from bona fide ECM proteins such as collagen type I, fibrin, gelatin, keratin and chitosan represent optimal choices with respect to their intrinsic biodegradability, mediation of cell-matrix interactions and inclusion of native functional groups that allow conjugation with growth factors and small molecules (reviewed in [[100]]).

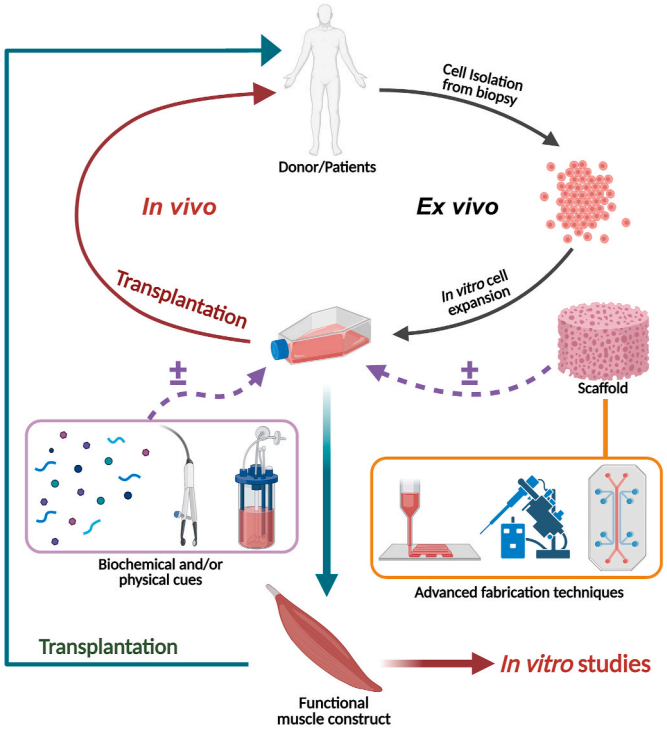

Fig. 2. *In vitro* or *in vivo* human cell-based skeletal muscle tissue engineering workflow. *In vivo* tissue engineering approaches involve direct implantation of the produced cell-laden construct, while *in vitro* approaches involve functionalization of the generated construct *in vitro* for downstream applications not only restricted to transplantation. Figure created with BioRender.com.

To date, fibrin- or collagen-based hydrogels are amongst the most extensively used natural scaffolds in human muscle tissue engineering (Table 1). In a comparative study, fibrin-based hydrogels supported optimal MuSCs differentiation *in vitro*, followed by collagen-based hydrogels [[107]]. Nonetheless, in a 3D bioconstruct seeded with human MuSCs and supportive muscle resident cells, collagen I hydrogels were effective in maintaining MuSCs myogenic potential upon transplantation [[109]].

Fibrin contains biologically relevant domains, such as arginine-glycine-aspartic acid tripeptides (RGD) that facilitate cell adhesion, as well as provide binding sites for growth factors, including vascular endothelial growth factor (VEGF) which is key to promote angiogenesis [[95]]. One more substantial advantage for selecting fibrin is its dual function in recruiting and promoting anti-inflammatory activity of macrophages, which is critical for tissue replacement [[64]]. An established strategy to generate fibrin hydrogels for muscle bioengineering is via the polymerization of fibrinogen by thrombin [[1,2,40,68,82,83,97,110,120,123,130,147]]. Such method enables fine-tuning in the composition ratio of fibrinogen and thrombin, which in turn introduces flexibility in modulating the stiffness of the resulting 3D matrix [[42]].

Another commonly used natural hydrogel constituent is alginate, which can be chemically modified by coupling with integrin-binding RGD peptides, similar to fibrin, to promote cell adhesion [[9]]. Other non-skeletal myogenic cells such as human adipose tissue-derived cells have also been tested for tissue replacement in VML using electrospun fibrin microfiber bundles, albeit with limited results in terms of myogenic differentiation both *in vivo* and *in vitro* [[55]]. For these and other cell sources, supplementation with growth factors and/or other ECM proteins such as Laminin-111 may help generating more mature and functional tissues [[86]].

Other natural biomaterials including gelatin, which is the denatured form of collagen [[18,53]], polysaccharide biopolymers such as chitosan [[59,60]], and the intermediate filament protein keratin [[11,105]], have also been employed in hydrogel fabrication for muscle bioengineering. Notably, several of these studies are based upon rodent cells, thus requiring further validation for application in human muscle modelling and potential clinical applications. Of note, scaffolds manufactured using natural polymers may present limitations due to difficulties in sourcing and purification, as well as in batch-to-batch variability. Moreover, some natural ECM hydrogels tend to have a comparatively shorter degradation period.

3.2. Synthetic polymers

Compared to natural ECM components, synthetic polymers possess higher tunability in chemistry, electroactivity and substrate stiffness but might lack biocompatibility. The most widely investigated biodegradable polymers are mainly polyurethane (PU), poly(ethyleneglycol) (PEG), poly(ε-caprolactone) (PCL), Poly(L-lactide) (PLLA) and poly (lactide-*co*-glycolide) (PLGA).

Scaffolds constituted solely by synthetic polymers are less common (Table 1) than those based upon natural polymers for human skeletal muscle tissue engineering. Nonetheless, recent advance in engineering novel synthetic protein hydrogels with customized mechanical properties by modulating crosslinkers and load-bearing modules can broaden applications of synthetic materials for muscle bioengineering. Designed synthetic proteins can be used to generate self-healable hydrogels with passive elasticity similar to native skeletal muscles [[144]]. Wang and colleagues designed an electrospun synthetic nanofibre yarn produced by blending PANi, which is a type of electroactive polymer, PCL and silk, can provide structural cues in guiding the alignment of seeded myoblasts into myotubes [[142]]. Other 3D nanofibres constructed by combining PCL with PANi demonstrated that optimization of macrostructures by fine-tuning PANi concentration was critical for myoblast differentiation

Table 1
Myogenic cells and biomaterials for human skeletal muscle tissue engineering. ECM: Extracellular matrix; HA: Hyaluronic acid; NMJ: Neuromuscular junction; PAM: Polyacrylamide; hiPSC: Human induced pluripotent stem cells; hESC: Human embryonic pluripotent stem cells; PEG: Polyethylene glycol; VML: Volumetric muscle loss; DMD: Duchenne muscular dystrophy.

Cell source	Biomaterial	Scaffold	Application	Outcome and notes	Reference
Primary human myoblasts	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D model	Aligned myobundles with $Pax7^+$ cells showing electrical-induced contraction and response to drug treatment	[82]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D models	Formation of contractile myobundles, recapitulation of drug-induced mitochondrial toxicity	[40]
	Fibrinogen/ gelatin/HA/ glycerol	Hydrogel bioink	*In vitro* 3D construction followed by *in vivo* implantation	Production of multi-layered myobundles, functional muscle restoration upon implantation in rodents	[72]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D model	3D skeletal muscle tissues with aligned and multinucleated myotubes	[83]
	Fibrinogen and thrombin	Hydrogel-seeded micropatterned surface	*In vitro* 3D model	Generation of aligned sheets of myotubes; contraction upon electrical stimulation; response to drug treatment	[130]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D models	Contractile 3D muscle tissues, formation of functional NMJ by co-culturing with hESC-derived motor neurons (primary and immortalized myoblasts)	[1]
	Collagen type I and matrigel	Hydrogel	*In vitro* 3D models	Contractile 3D micromuscle tissues showing improved maturation upon optogenetic stimulations	[92]
	PAM	Hydrogel	*In vitro* 3D model	Generation of mature myobundles resembling native skeletal muscle at single fibre scale with spontaneous contractility	[138]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D model	Development of a 96-well micro-muscle tissue array device with contractile myofibers	[2]
	Collagen type I	Hydrogel	*In vitro* 3D model	Formation of multinucleated myofibers with $Pax7^+$ nuclei, capable of functional regeneration following chemical injury	[47]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D model	Contractile myobundles reproducing myotoxic effects in releasing injury-related biomarkers	[68]
	Alginate and PEG-fibrinogen	Hydrogel	*In vitro* 3D model, *in vivo* applications	Myoblast-laden hydrogel microfibres with multinucleated myotube bundles for VML restoration	[38]
Immortalized human myoblasts	Fibrin	Hydrogel	*In vitro* 3D model	Multilineage skeletal muscle model containing vascularized fibres and fibroblast-rich endomysium to model DMD fibrosis	[17]
	Collagen type I, Fibrin and PEG-Fibrinogen	Hydrogel	*In vitro* 3D model	3D culture system allowing formation of multinucleated and aligned myotubes	[108]
	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D model	Engineered muscle tissues with contractibility 12-times greater than primary human myoblasts, induced atrophy led to decreased contractile force in the muscle model	[97]
	Fibrinogen and geltrex	Hydrogel	*In vitro* 3D model	3D minitissues capable of recapitulating DMD phenotypes; showing revertant dystrophin-positive myotubes	[43]
Human mesoangioblasts	PEG-fibrinogen	Hydrogel	*In vitro* 3D construction followed by *in vivo* implantation	Transplantation of injectable mesoangioblasts-laden hydrogels promoted local vascularization and muscle regeneration	[49,50]
hiPSC-derived myogenic cells	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D disease model, *in vivo* implantation	Aligned multinucleated and functional myotubes using both healthy and dystrophic hiPSC-derivatives; isogenic and multilineage with vascular cells and motoneurons; implantable *in vivo*; model muscular dystrophy	[83]
			In vitro 3D model	High fidelity, mutation-specific recapitulation of phenotypes of skeletal muscle laminopathies in 3D cultures	[128]
			In vitro 3D model with *in vivo* implantation	Engineered skeletal muscle tissues with contractile multinucleated myotubes, capable of engraftment in mice	[110]
			In vitro 3D models	Cotreatment with maturation-inducing cocktail increased contractile force generation	[120]
Transdifferentiated human fibroblasts	Fibrinogen and thrombin	Hydrogel	*In vitro* 3D models	Skeletal muscle tissue constructs showing multinucleated myotubes with electrical-induced contractibility	[123, 147];

and myotube maturation, further emphasizing the importance of optimizing the ratio of each constituent in the selected biomaterial [[126]].

In addition, recent improvements in deriving chemically modified polymers could enhance the cytocompatibility of synthetic materials, such as PEG macromer linked with maleimide groups (PEG-4MAL), which has been used to improve MuSCs viability, proliferation and differentiation upon transplantation into injured tibialis anterior muscles of dystrophic and aged mice [[61]]. Furthermore, Narayanan and colleagues designed aligned PLGA fibres at nanometer scale which enhanced the resemblance in scaffold architecture to native ECM [[99]]. One thing to be noted is that besides the aforementioned scaffolds for human skeletal muscle tissue engineering, there is a variety of promising synthetic materials arising from chemistry and engineering labs that are just starting to be trialled with rodent cell lines like C2C12 myoblasts, which will undoubtedly progress to human muscle in the future.

3.3. Composite scaffolds

Semi-synthetic/hybrid materials are generated by cross-linking both classes of biomaterials to combine the tuneable mechanical properties and engineering flexibility of synthetic polymers with the bioactivity and complex interaction capabilities of natural ECM components. These composite materials have recently been exploited in a number of combinations for skeletal muscle tissue engineering. These include PEG and fibrinogen (PF) [[38,49,50]]; Gelatin methacryloyl (GelMA) [[37]], PU

and fibrinogen [[44]], PCL and collagen or gelatin [[25,106]], PVA and chitosan [[10,67]]. Among these configurations, PF is a promising candidate as it can be mechanically tuned by modulating the proportion of PEG while maintaining innate protease-degradation domains and cell-adhesion motifs provided by fibrinogen [[6]]. PF hydrogels have also been shown to be compatible with human myogenic cells for generating 3D muscle constructs *in vivo* and *in vitro* [[38,49,50]]. Overall, combining synthetic and natural polymers has significantly broadened the possibilities for manufacturing next-generation muscle engineering scaffolds.

4. Combining cells, polymers, scaffolds and exogenous stimuli

In addition to scaffold engineering, application of stimuli *in vitro* (e.g., physical, electrical or biochemical cues) and recapitulation of the multicellular milieu have been exploited to mimic microenvironmental signals required for differentiation, alignment and maturation of skeletal muscle [[17,69,83,92,109,120]]. Delivery of bioactive molecules such as growth factors and cytokines within bio-scaffolds could facilitate the induction of a more mature artificial muscle construct resembling the native counterpart. For instance, activation, proliferation and differentiation of myogenic cells can be modulated via secretion of hepatocyte growth factor (HGF), insulin-like growth factor (IGF), VEGF, fibroblast growth factor (FGF), and stromal cell-derived factor-1 (SDF-1) triggered by skeletal muscle injury to activate quiescent MuSCs and promote tissue regeneration and ECM synthesis [[14]].

Previous studies (mostly with non-human cells) indicated that incorporation of growth factors in designed scaffolds can increase cell viability, migration, and maturation of myoblasts and increase secretion of ECM components [e.g., Refs. [116,141]]. Furthermore, different pro-angiogenic factors (e.g., VEGF) can accelerate the recovery rate of injured skeletal muscle tissues and facilitate implantation of the engineered tissues into the injury site. However, integration of growth factors in scaffolds is often challenged by their low biostability and degradation time [[8,125]].

Another aspect to consider when designing biomimetic scaffolds is the crosstalk between different cell lineages residing in native skeletal muscle. To address this issue, some groups have co-cultured/-differentiated myogenic cells and motor neurons (with or without vascular cells) simultaneously to mimic the multilineage cellular environment of native muscle and foster maturation and function of the resulting myofibres [[1,83,89,102]]. In parallel, other laboratories have focused on bypassing the stimulus provided by motor neuron innervation by providing direct electrical stimulation to the human muscle constructs.

Although more than 200 years have passed since the discovery of "animal electricity" by Italian physician and polymath Luigi Galvani, his classical experiments on frog's muscles continue to inspire discoveries using synthetic human muscle tissues [[51]]. In homeostasis, skeletal muscle tissues undergo cycles of contractions and relaxations that are initiated by electrical stimuli and continued by mechanical movement. However, reproducing this seemingly simple process using human bioengineered muscles has required significant efforts. In native skeletal muscle tissue, excitatory stimuli are delivered to muscles by motoneuron axons which branch to innervate multiple muscle fibres and further reach NMJs to transmit the electrochemical impulse. In contrast, *ex vivo* electrical stimulations are applied to induce contractions directly in *in vitro* 3D human skeletal muscle models. However, although some engineered skeletal muscle constructs are able to contract upon stimulation (often to a less extent than their murine counterpart), they often are locked in a fetal/perinatal stage of maturity compared to native adult skeletal muscle tissues.

Aiming to improve tissue maturation and functionality, a recent study applied chronic intermittent electrical stimulation at various frequencies on the engineered skeletal muscle tissues. Following treatment, the engineered muscles demonstrated improved maturation in terms of increase in myotube size, sarcomeric protein expression and force generation. [[69]]. Alternatively, direct mechanical stimulation can also promote maturation of the engineered muscle tissues. To this aim, Heher *et al.* established a custom-made bioreactor system which generated cyclic or static mechanical forces to mimic muscle contractions on cells embedded in fibrin matrix. The stimulated skeletal muscle constructs demonstrated improvement in both number and diameter of aligned myotubes compared to unstimulated constructs [[62]]. Thus, both mechanical and electrical stimulations are promising strategies to improve morphology, maturation and function of engineered skeletal muscle tissues.

5. Current challenges, emerging technologies and future perspectives

There are a number of hurdles in developing high-fidelity disease models and clinical applications of artificial muscle tissues that will need to be addressed by tissue engineering approaches in the future, including (but not limited to): 1) limited structural and functional maturation of the engineered skeletal muscle tissues; 2) lack of cellular heterogeneity and cell-to-matrix interactions as opposed to the *in vivo* skeletal muscle tissues; 3) need for vascularisation to facilitate suboptimal *in vitro* scaling up.

One of the key obstacles that results in the unfulfilled recapitulation of adult tissue features of the engineered skeletal muscle constructs is their incomplete maturation profile. Biofabrication strategies such as those based upon porous structures may facilitate the recapitulation of the mechanical and electrophysiological features to tackle this issue [[122]]. Nonetheless, this challenge could be less relevant for *in vivo* applications as the native/endogenous environmental cues may act as a "bioreactor" to facilitate maturation of the transplanted tissues. With the aim to improve myoblast maturation by modulating structural organization, Gilbert-Honick and colleagues employed electrospun fibrin scaffolds that can provide topological alignment for myoblasts during differentiation [[56]]. In another study, Xu *et al.* developed a photolithographic strategy for micropatterning to create a range of micrometre-sized grooves which can promote parallel alignment of iMPC-derived myotubes to model DMD diseases [[146]]. As outlined in the previous section, other relevant strategies to enhance *in vitro* maturation include the possibility to integrate electrical stimulation either directly [[69]] or by incorporating innervating motoneurons, or by direct mechanical stimulation of the constructs [[62]]. Another general limitation associated with engineered human tissues/organs is their stability and function after implantation, which is often a consequence of scaling up the size of the constructs *in vitro* due to limitations in nutrient and oxygen diffusion secondary to the absence of a vascular system [[54]]. Although discussing this topic lies outside the remit of this review, it is exciting to note that efforts in this area are being made to exploit promising technologies such as spatially patterned scaffolds and intravital bioprinting to overcome this limitation [[98,137]]. These and other emerging technologies, such as 3D bioprinting that aims to customize scaffold manufacture, tissue and/or organ-a-chip devices which facilitate *in vitro* scaling up and organoid cultures which can further introduce model complexity, are poised to advance the field by bypassing the current bottlenecks and fabrication hurdles, will be briefly discussed in the following sections (Fig. 3).

5.1. 3D bioprinting

3D bioprinting is an emerging biofabrication technology which combines cells, proteins and biomaterials to manufacture tissues and/or organs for regenerative medicine. In 3D bioprinting, generally the scaffold components, growth factors and in some cases the cells are combined together into a bioink which is then used as the fabrication material for complex scaffolds [[148]]. Different formulation of bioinks can be tailored for supporting cell proliferation, maintenance, and

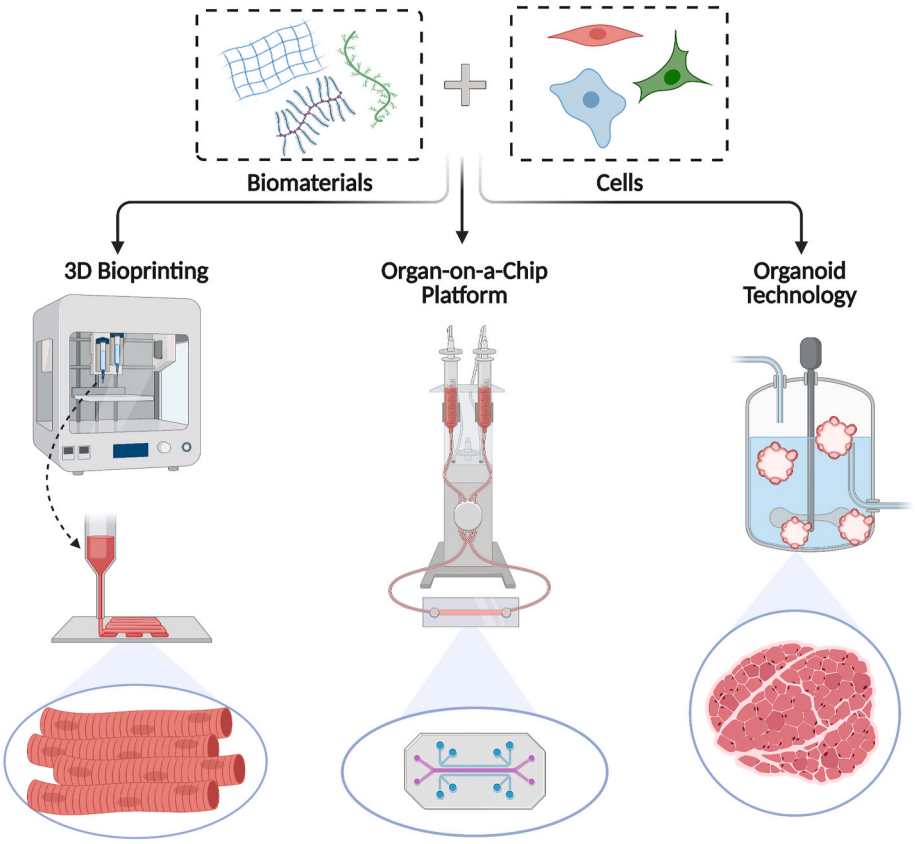

Fig. 3. Advanced bio-fabrication techniques of human skeletal muscle tissue. Cutting-edge techniques combine biomaterial scaffolds and multilineage cellular components to produce biomimetic human skeletal muscle tissues. Figure created with BioRender.com.

differentiation [[73]]. In particular, bio-inks have been developed from either natural ECM components or synthetic polymers, and beside the specific modifications necessary to accommodate the different fabrication dynamics of printing compared to - for example - casting hydrogels in molds, the vast majority of the biomaterials described in the sections above would be suitable to form bioinks. The fabrication of defined structures with bioprinting can be achieved by several techniques (e.g., nozzle extrusion, droplet-based deposition, light-induced polymerisation) and a detailed discussion of these different techniques goes beyond the scope of this review; nonetheless, we redirect the reader to a recently published article that categorises the different types of bioprinting currently adopted in tissue engineering and regenerative medicine approaches [[31]].

The complex structural assembly of muscle fibres requires a robust and precise fabrication technique that can generate skeletal muscle tissues with ability to reflect the physiological function of native skeletal muscle tissues. As such, 3D bioprinting allows to generate large 3D constructs with complex architecture in which the constituents are already mixed with both cells and bioactive components, simplifying the fabrication process [[103]]. For example, Choi *et al.* fabricated 3D engineered muscle tissues using tissue-specific ECM as bioink for volumetric injuries [[34]]. In another study, Kim *et al.* demonstrated the importance of biochemical and topographical cues in influencing myogenic differentiation by optimizing tissue-specific geometry [[75]]. Excitingly, novel bioprinting technologies such as intravital 3D bioprinting now enable direct fabrication of 3D constructs within tissues to minimize drawbacks related to invasive surgical interventions for tissue replacement. Using this approach, Urciuolo and colleagues showed *de novo* formation of myofibres in mice following intravital 3D bioprinting of muscle-derived stem cells under the epimysium [[137]].

5.2. Organ-on-chip platforms

Organ-on-chip platforms are advanced biomimetic tissue constructs that aim to capture *in vitro* complex arrangements of cells, ECM and some of the mechanical properties of the target tissue in a miniaturised form, often suitable for medium-/high-throughput testing in preclinical setting [reviewed in Ref. [81]]. Organ-on-chip platforms usually consist of microfluidic systems that distribute nutrients to target cells mimicking the native microenvironment (e.g., blood flow) of the tissues/organs of interest [[3]]. Similar to other aforementioned bioengineering strategies, organ-on-chip platforms can support maintenance of balanced proportions of different cell populations to reconstruct the multilineage cellular environment and can be used to monitor responses of the engineered tissues under mechanical and/or electrical stimulation while minimising nutrient requirements.

Organ-on-chip platforms can also be useful in modelling muscle disorders *in vitro* as they incorporate key mechanical and biochemical features of muscle tissue and can help elucidating maturation and regeneration dynamics. Indeed, Kim and colleagues applied cyclic stretching of muscle fibres that were physically and chemically damaged in a microfluidic platform to study recovery dynamics of damaged skeletal muscle cells [[74]]. Microfluidic platforms can be designed to monitor and improve alignment of myofibers, maturity of the engineered muscle tissues and release of cytokines such as IL-6 and TNF-α which are involved in muscle regeneration [[101]]. Notably, micro-scaled chip-based *in vitro* models can be used to validate and reduce or replace preclinical essays in animals, as shown in a comparative study designed to assess dystrophin production and distribution by human myoblasts and mesoangioblasts before *in vivo* testing [[121]].

Organ-on-chip platforms can further combine different cell types to mimic pathologically relevant cell interactions. To this aim, a recent study established a protocol to combine motor neurons with muscle cells

in an optogenetically controllable microfluidic platform which enables analysis of real-time interactions between motor neurons and muscle cells at NMJs [[102]].

5.3. Organoids

Organoids are 3D self-organized structures composed of different cell types derived from stem or progenitor cells, with structural resemblances with the tissue of origin. They have been developed for a wide range of human tissues or organs for disease modelling [reviewed in Ref. [21]]. Organoid technology promises to bridge the gap between *in vitro* monolayer cell cultures and *in vivo* studies using animal models. However, it is debatable to what extent the organoid definition could be applicable to skeletal muscle, as it is a tissue (and not an organ) requiring mechanical cues such as tendon-mediated anchorage to bones to organize and function, hence not fulfilling the self-organizing criterium defining organoids. Nonetheless, recent studies have shown that meaningful and exciting data can be obtained even in the absence of structural cues and native tissue architecture. For example, Faustino Martins and colleagues generated self-organizing 3D human trunk neuromuscular organoids from neuromesodermal progenitors derived from pluripotent cells. The resulting 3D cultures displayed functional NMJs which modelled pathological features of myasthenia gravis [[46]].

Activation of skeletal muscle contraction is controlled by the cortico-motor pathway consisting of neurons in the cerebral cortex and hindbrain/spinal cord. A recent study combined human cortical, spinal and skeletal muscle spheroids into cortico-motor assembloids which could be subjected to glutamatergic or optogenetic stimulation and induce contractions in the skeletal muscle organoids [[7]].

Finally, as somitogenesis and early developmental myogenesis proceeds without the anchoring of skeletal muscle precursors to bones, it is possible that an organoid-based platform could be used to study and model this process in human without the necessity of complex structural scaffolds, as elegantly showed by Matsuda and co-workers [150]. Indeed, a recent pre-print reported the development of a 3D culture system that models human myogenesis from iPSCs, enabling the generation of uncommitted PAX7-positive progenitors alongside fibroadipogenic progenitors [[88]]. We foresee that the promising developments in this area will eventually converge with bioprinting, microfluidics and organ-on-chip technologies to generate advanced models capable of simulating complex processes such as gradient-induced developmental patterning.

6. Concluding remarks

In this review we summarised key components and recent advances in engineering human skeletal muscle, focusing on *in vitro* models and providing also an overview on current challenges and emerging technologies to tackle them. Promising strategies include exploiting the controllable proliferation and differentiation capacity of iPSCs to obtain isogenic and developmentally isochronic models, 3D bioprinting to finely control and mimic the complex skeletal muscle architecture and organ-on-chip platforms to closely resemble native physiology *in vitro* (e.g., vascularisation and innervation). These technologies promise to tackle key limitations of current platforms such as scaling up for tissue replacement *in vivo* whilst also delivering *quasi vivo* skeletal muscle models which will dramatically reduce the use of animals to study pathogenesis and develop therapies for neuromuscular and musculoskeletal disorders.

Author contributions

YJ and TT wrote the manuscript draft with contributions from SMM, AS and FST. AS and FST finalised the manuscript. FST coordinated and supervised the work, contributed to the draft, and acquired funding.

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