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Engineered skeletal muscles for disease modeling and drug discovery

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Abstract

Skeletal muscle is the largest organ of human body with several important roles in everyday movement and metabolic homeostasis. The limited ability of small animal models of muscle disease to accurately predict drug efficacy and toxicity in humans has prompted the development *in vitro* models of human skeletal muscle that fatefully recapitulate cell and tissue level functions and drug responses. We first review methods for development of three-dimensional engineered muscle tissues and organ-on-a-chip microphysiological systems and discuss their potential utility in drug discovery research and development of new regenerative therapies. Furthermore, we describe strategies to increase the functional maturation of engineered muscle, and motivate the importance of incorporating multiple tissue types on the same chip to model organ cross-talk and generate more predictive drug development platforms. Finally, we review the ability of available *in vitro* systems to model diseases such as type II diabetes, Duchenne muscular dystrophy, Pompe disease, and dysferlinopathy.

Keywords

Human muscle; Muscular dystrophy and disease modeling; Tissue engineering; Organ-on-a-chip; Induced pluripotent stem cells; Regeneration

1. Introduction

Of the three major muscle types, skeletal muscle is the most abundant by mass accounting for approximately 40% of human body weight. The primary function of skeletal muscle is to generate contractile forces that enable respiration, posture and locomotion and thus skeletal muscle function is critical for human health. Healthy skeletal muscle is capable of multiple cycles of regeneration in response to small tears that occur during daily activity and exercise due to the presence of myogenic stem cells termed satellite cells (SCs) [1]. Upon injury, SCs activate, proliferate and fuse to either form new or repair damaged muscle fibers [2]. Skeletal muscle regenerative ability and function can become impaired with aging [3], acquired diseases (such as cancer and large traumatic volumetric muscle loss (VML) injury), and monogenic neuromuscular or metabolic diseases such as Duchenne muscular dystrophy

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and Pompe disease, respectively. Currently, treatment options for these debilitating myopathies have limited therapeutic efficacy and generation of novel therapeutics is a large driving force for the fields of skeletal muscle biology, stem cell biology, and biomedical engineering.

Traditionally, drug discovery, development, and toxicity profiling are performed and validated in 2D cell culture and small animal models before use in clinical trials. However, only 11.8% of drugs entering clinical trials become approved resulting in the average cost of ~$2.5 billion total for a newly approved drug [4]. This low efficacy is due in part to animal disease models not truly replicating human diseases and differential drug response and toxicity between animals and humans. The limitations of the classical drug discovery-to-clinic pipelines have driven the development of *in vitro* human tissue mimetics for drug testing, with three-dimensional (3D) engineered tissues and organ-on-a-chip (OOC) microfluidic devices representing some of the most promising advances [5–9]. Over the last 30 years, *in vitro* tissue-engineered skeletal muscle models have progressed significantly to replicate key aspects of skeletal muscle function including isometric contractile properties [10–12], regeneration from cardiotoxin injury, and native pharmacological responses [13–17]. Recent advances in the optimization of 3D culture conditions and hPSC technology have permitted the generation of the first functional tissue-engineered human muscle constructs made of primary myoblasts or fusion-competent hPSC-derived muscle progenitors [12,13]. Additionally, smaller scale muscle-on-a-chip platforms offer the capability to study muscle biology and drug screening in a high-throughput fashion [18]. These systems have the potential to increase the predictive power of *in vitro* drug development systems by replicating the complex inter-organ crosstalk found *in vivo* by integrating multiple tissue types within a single microfluidic platform.

In this review, we first describe development, structure, and function of native muscle and the cell sources and culture systems utilized for modeling muscle physiology *in vitro*. We then review the progress made in increasing the developmental maturity and regenerative ability of *in vitro* muscle models as well as generating the models of functional neuromuscular junction. We further discuss the need to model muscle crosstalk with other organs to better replicate the *in vivo* systemic environment and develop improved *in vitro* disease models. We end by discussing the future utility of *in vitro* engineered skeletal muscle tissues for modeling muscle regeneration and disease, and predicting drug outcomes for improved therapy.

2. Skeletal muscle development, structure, and function

2.1. Skeletal muscle development and differentiation

All skeletal muscle cells in the body originate from muscle precursor cells derived from the somites [19]. Upon induction of transcription factors Pax3/7, the proliferating muscle progenitors migrate from the dorsal medial lips (DML), ventrolateral lips (VLL), and lateral edges of dermomyotome to form the myotome, the first muscle mass formed in the embryo. Distinct biochemical signals from the neural tube, notochord, and ectoderm including FGFs, BMPs, Wnts and sonic hedgehog support myogenesis. Muscle progenitors in the epaxial and hypaxial myotome generate the muscles of the deep back and body wall, respectively.

Muscles of the limb originate from progenitors of the VLL of the dermomyotome, which delaminate and migrate to the developing limb bud [19]. The commitment, differentiation, and formation of skeletal muscle is regulated by the myogenic regulatory factor (MRF) family of transcription factors Myf5, MyoD, myogenin, and MRF4 that function synergistically with myocyte enhancer factor 2 (MEF2). Myogenic commitment is specified first by sequential expression of Myf5 and MyoD which permit the proliferation and generation of sufficient numbers of myogenic precursor cells to generate mature skeletal muscle [20]. Terminal differentiation is dependent upon myogenin and Mef2, which function to direct cell cycle exit, differentiation, and fusion of myogenic progenitors to form multinucleated myotubes. A subset of myoblasts do not commit to terminal differentiation, lose expression of Myf5 and MyoD, and express the transcription factor Pax7, entering a quiescent state alongside the developing muscle fibers. These dormant myoblasts, termed satellite cells (SCs), function as muscle specific stem cells that, in response to injury, proliferate and generate new myoblasts for muscle regeneration.

2.2. Skeletal muscle structure

Skeletal muscle is composed of multinucleated myofibers that are densely packed with contractile material and range in length from millimeters to centimeters. Myofibers consist of highly organized myofibrils comprised of repeated sarcomeric units that enable muscle contraction. The lateral boundaries of the sarcomeres are defined by the Z-lines, which anchor the thin actin filaments via α-actinin. The actin filaments are interdigitated with the myosin thick filaments, which are cross-linked at the M line and fixed to the Z-line through the elastic protein titin. From longitudinal transmission electron micrograph sections, the myofibril organization gives a striated pattern, such that the dark A bands delineate the myosin thick filaments and the I bands contain solely the thin filaments. At the junction of the A and I bands are the transverse tubules (T-tubules), a branched network of membrane invaginations that run along the whole length of the myofiber that facilitate efficient delivery of calcium from the sarcoplasmic reticulum (SR) to the sarcomere for contraction.

2.3. Skeletal muscle contraction

Skeletal muscle contraction and thus movement is regulated by motor input through neuromuscular junctions (NMJs), specialized synapses between the presynaptic motor neuron terminal and the motor end plate of the postsynaptic muscle fiber. When a nerve impulse reaches the axon terminal, depolarization of the presynaptic membrane triggers a rapid influx of calcium ions. This influx initiates conformational changes in the SNARE complexes, which facilitate exocytosis of acetylcholine-filled vesicles into the synaptic cleft. Diffusing across the synaptic cleft, acetylcholine binds to nicotinic acetylcholine receptors (nAChRs) located within the motor endplate, triggering depolarization at the sarcolemmal membrane and consequent muscle contraction [21]. To prevent continuous activation of the acetylcholine receptors and muscle tetany, the motor end-plate contains high levels of acetylcholinesterase, which rapidly breaks down acetylcholine to permit muscle response to new impulses.

Activation of nAChRs present on the sarcolemma stimulate rapid sodium ion entry and muscle fiber depolarization. The depolarization wave is propagated through the T-tubules,

where voltage-gated channels (dihydropyridine receptors) directly open the ryanodine receptors (RyRs) and release calcium from the sarcoplasmic reticulum (SR) into the cytosol. The calcium ions bind to troponin C located on the thin filaments, inducing conformational changes to tropomyosin that enable myosin to bind to actin. When actin is free from steric hindrance by tropomyosin, myosin binds the actin (forming a cross-bridge) and engages in the power stroke. With each cross-bridge cycle, the myosin "steps" along the actin, in effect moving towards the Z-line, reducing the I bandwidth, and shortening the sarcomere. When muscle stimulation concludes, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) actively pumps calcium back into the SR, reducing the intracellular calcium ion concentration and returning tropomyosin to its inhibitory state.

Skeletal muscle force output is predominantly regulated by the neural input, specifically impulse frequency and motor unit recruitment. Impulse frequency controls force output through summation whereby successive action potentials that arise before muscle relaxation prevent calcium re-uptake into the SR and allowing continued cross-bridge cycling and greater force generation until a fused tetanus and maximal force is generated. Motor unit recruitment regulates force generation by the motor cortex recruiting greater numbers of motor units and thus numbers of contracting muscle fibers [22]. Skeletal muscle force generation is also regulated in part according to the sliding filament theory, which states that the active tension produced is proportional to the number of cross-bridges formed [23]. Thus, when there is maximal overlap between actin and myosin, the tension generated from cross-bridge cycling will also be maximal. This gives rise to the length-tension relationship, where excessive shortening or lengthening of the sarcomere reduces the number of cross-bridges that can form thus decreasing force generation.

2.4. Skeletal muscle extracellular matrix

Skeletal muscle function also requires efficient transmission of force from the sarcomere to the surrounding extracellular matrix (ECM) and ultimately to tendon and bone. Myofibers are surrounded by a basal lamina composed of non-fibrillar, network-forming collagen IV, laminin, heparin sulfate proteoglycans, and glycoproteins. The basal lamina not only provides sites for myofiber attachment but also a supportive ECM layer that contributes to muscle progenitor quiescence, proliferation, and differentiation. The muscle cell membrane, or sarcolemma, directly interacts with the basal lamina through integrins and the dystroglycan complex (DGC) [24]. The DGC links intracellular cytoskeletal proteins to the extracellular matrix and plays a role in both cell anchoring and force transmission. Surrounding the muscle fibers and basal lamina are the intramuscular connective tissue (iMCT) layers that are predominantly comprised of stress-bearing collagen I and III that confer tensile strength to skeletal muscle [25]. The iMCT is organized into three distinctive layers, the endomysium that surrounds individual muscle fibers and the basal lamina, the perimysium that encloses multiple muscle fibers forming a fascicle and the epimysium that encloses the entire muscle body and is continuous with the tendon.

3. Cell sources for engineering human skeletal muscle

3.1. Primary human muscle stem cells

As myonuclei within mature myofibers are post-mitotic, satellite cells (SCs) represent the only robust source of expandable primary myogenic cells in skeletal muscle. For use in *in vitro* studies, SCs can be isolated by two methods: 1) explant culture and 2) enzymatic digestion. In explant culture, myofibers are gently separated from the surrounding ECM by dissection and placed inside ECM-coated dishes on which SCs subsequently activate, migrate, and proliferate. In enzymatic digestion, SCs are released from their niches by enzymatic and mechanical dissociation and filtered to obtain a single cell suspension that can be subsequently plated and expanded. Both isolation procedures result in a mixed population of human myogenic cells and fibroblasts, but fibroblast numbers can be minimized by pre-plating which takes advantage of quicker adhesion kinetics of fibroblasts compared to myoblasts onto tissue culture polystyrene. If a pure myogenic population is desired, myoblast clones can be identified and expanded [26]. Alternatively, human satellite cells can be sorted out (using fluorescently or magnetically labeled antibodies) based on their expression of surface markers NCAM (CD56) and β1-integrin (CD29), and can be further separated from hematopoietic and endothelial cells based on the lack of expression of CD45 and CD31, respectively [27].

Once isolated, primary human myoblasts rapidly proliferate in high serum media, and readily fuse to form multinucleated myotubes when switched to low serum media. However, with serial passaging these cells become senescent: proliferation rate decreases, DNA damage accumulates [28], ability to terminally differentiate decreases [29], and potential to engraft into the SC niches *in vivo* is lost. Furthermore, myotubes derived from passaged myoblasts exhibit reduced insulin-stimulated glucose uptake, glycogen synthesis, and carbohydrate and lipid metabolism [30].

3.2. Human pluripotent stem cells (hPSCs)-derived myogenic cells

To overcome the passaging limitations and ethical considerations of utilizing primary cells from healthy donors and patients, methods have been developed to generate muscle progenitor cells (MPCs) from human pluripotent stem cells (hPSCs). The hPSCs, including human embryonic stem cells (hESCs) [31] and induced pluripotent stem cells (hiPSCs) [32] can serve as near-unlimited cell sources capable of differentiation into all three germ layers of the early embryo. In the past 10 years, significant progress has been made in the derivation of hPSC-MPCs for use in cell-based therapies and to study muscle development and disease. As described below, the hPSC-based myogenic induction strategies from can be divided into two groups: 1) transgene-dependent and 2) transgene-free.

3.2.1. Transgenic overexpression of MyoD—The first report of transdifferentiation to the skeletal muscle lineage occurred by treating fibroblasts with the demethylating agent 5-azacytidine [33]. Subsequently, Weintraub et al. demonstrated that this was due to the demethylation and consequent induction of MyoD and that overexpression of MyoD alone could convert multiple cell lines into myogenic cells [34]. The first successful generation of skeletal muscle from hESCs utilized lentiviral tetracycline-inducible MyoD overexpression,

which generated a highly pure population of myotubes 10 days following MyoD induction [35]. However, the degree to which MyoD overexpression activates the muscle transcriptional program depends on the chromatin state, with hiPSCs typically unable to completely differentiate into skeletal muscle with lentiviral overexpression of MyoD [36]. MyoD-mediated activation of myogenesis in hESCs requires BAF60C, a component of the chromatin remodeling complex switch/sucrose non-fermentable (SWI/SNF) [37]. Alternatively, reducing H3K27me3 through histone demethylase JMJD3 overexpression significantly increases the efficiency of synthetic MyoD mRNA transfection mediated myogenesis in hiPSCs [38]. To address potential genomic integration issues with lentiviral methods, a piggyBac transposon tetracycline-inducible MyoD-induction system has been used to generate hiPSC-derived myoblasts and myotubes with high efficiency (70–90%). This approach has been used to study Miyoshi myopathy [39]. Duchenne muscular dystrophy [40]. Pompe disease [41] and carnitine palmityoltransferase-II deficiency [42] with several expected disease phenotypes observed. However, although contractile myotubes can be quickly and efficiently formed with MyoD overexpression [35]. MyoD induction causes rapid cell cycle exit due in part to upregulation of the cyclin-dependent kinase inhibitor p21 [43]. The diminished expandability of these cells significantly limits their application for studying or inducing muscle regeneration due to the inability to generate satellite cells *in vitro* or repopulate the SC niche *in vivo*.

3.2.2. Transgenic overexpression of Pax7—The transcription factor Pax7 is required for the generation of satellite cells [44] and adult skeletal muscle regeneration *in vivo*. Importantly, Pax7 expression occurs prior to MyoD expression during muscle development and regeneration. In contrast to MyoD, which is restricted to skeletal muscle, Pax7 is expressed during development of both skeletal muscle and neural crest that originate from the mesoderm and ectoderm, respectively [45]. Thus, to generate a highly pure population of myogenic cells by Pax7 overexpression, while preventing induction of neural crest progenitors, requires initial mesoderm specification through either embryoid body formation [46] or small molecule differentiation [12,47,48]. After mesoderm specification from hPSCs, doxycycline (dox)-induced Pax7 expression can generate expandable muscle progenitors called iPax7 cells [46]. Terminal muscle differentiation of iPax7 cells *in vitro* requires withdrawal of doxycycline and downregulation of Pax7 to allow expression of MyoD and subsequently myogenin [49], yielding the formation of multinucleated myotubes expressing myogenin, dystrophin, and myosin heavy chain (MHC). Upon implantation into dystrophic (NSG-mdx) mice, iPax7 cells can fuse with host myofibers, replenish the satellite cell compartment, and increase contractile function [46]. Recently, iPax7 cells were used to generate the first hPSC-derived functional tissue-engineered skeletal muscle, which displayed physiological force-length and force-frequency relationships and the ability to contract in response to acetylcholine [12]. Compared to 2D cultures, the engineered tissues displayed greater maturation level as evidenced by increased myotube diameter and expression of mature myosin isoforms and calcium handling related genes [12]. The ability to generate and study functional human muscle tissues *in vitro* holds promise for development of personalized therapies for patients with severe genetic myopathies.

3.2.3. Small molecule/transgene-free differentiation—Although the transgene overexpression systems described above can model key aspects of multiple muscle diseases *in vitro* and contribute to regeneration *in vivo*, they require use of genomic integrating vectors that may result in incomplete reprogramming process and random epigenetic changes unsuitable for potential clinical applications. To overcome this limitation, transgene-free small molecule differentiation protocols that mimic *in utero* muscle development have been established by multiple groups. During muscle development, the paraxial mesoderm gives rise to the somites including the sclerotome, myotome, and dermatome. In the somites, multipotent mesenchymal cells receive a range of signals (Sonic hedgehog, BMP, TGF-β, Notch, FGF) that direct cell lineage selection [50]. For example, Sonic hedgehog (Shh), BMP-2, and TGF-β inhibit myogenic differentiation [50,51]. Conversely, BMP inhibitor noggin, Wnt1 and Wnt3A, and Shh promote myogenesis [50,51]. During myogenesis, Pax3/7 [52] cells in the myotome enter a proliferative, self-renewing phase regulated by Notch [53] and Sprouty1 [54], followed by FGF signaling dependent differentiation and expression of myogenic determination factors (Myf5, MyoD, and Mrf4) [54]. Transgene-free protocols aim to mimic these complex developmental events starting from hPSCs and using sequential application of small molecules and growth factors.

The first transgene-free myogenic induction of hPSCs was reported by Barberi et al. [55] in 2007 who differentiated hESCs into mesenchymal precursors with insulin, transferrin, and selenium followed by $CD73^+$ selection and expansion in serum-containing media. Resulting $MyoD^+$ progenitors were further enriched from 2-10% to 60–80% by sorting for NCAM (CD56). $CD73^+$ /$NCAM^+$ cells were capable of generating myotubes that expressed the terminal differentiation markers myogenin and MHC. Despite the promise of this method, only a small percentage of cells expressed Pax7 and differentiation and fusion efficiency were low. To achieve more efficient myogenic induction, 2400 chemicals were tested for the ability to accelerate muscle development in zebrafish. The screen identified three compounds, BIO (GSK3 inhibitor), FGF2, and forskolin (PKA activator) that in turn synergistically improved myogenic differentiation of hiPSCs [56]. Separately, treatments with CHIR99021 (GSK3 inhibition) and FGF2 were found to induce paraxial mesoderm specification and expansion of myogenic cells that could be further differentiated into multinucleated myotubes [57]. Enrichment for $CXCR4^+$ /$C\text{-}MET^+$ cells selected for progenitors that were able to retain robust differentiation ability following serial passaging and cryopreservation.

Myogenic cells generated by transgene-free protocols were also shown to engraft in host muscle when transplanted *in vivo* [56] suggesting potential utility in regenerative therapies. Still, current transgene-free protocols suffer from long differentiation times (> 30days) [47] with low efficiency, resulting in the generation of small, developmentally immature myotubes. The lack of quantitative measurements of muscle function makes it difficult to accurately compare the quality of hPSC-derived myotubes among different protocols. The most consistently used metric for evaluation is fusion index [58], which mainly reflects the purity of myogenic cells rather than their maturity. Further improvements in the differentiation and maturation of both transgene and transgene-free hPSC-derived myotubes

will be required to overcome their embryonic-like MHC transcription profile, smaller myotube size, and lower force generating ability compared to primary human myotubes.

4. *In vitro* skeletal muscle models

Traditionally, skeletal muscle cells have been cultured in 2D monolayers where proliferating myogenic progenitors can be induced to fuse and terminally differentiate by shifting from mitogenic, high serum-containing media to low serum conditions. The resulting multinucleated myotubes are contractile, express the terminal muscle differentiation transcription factor myogenin, and can be used for rapid, high-throughput drug screening studies. However, in comparison to adult muscle, the resulting myotubes are immature and consequently of limited physiological relevance. This developmental immaturity partially stems from use of stiff culture substrates (~1 GPa) favoring bonelike gene expression [59]. When myogenic cells are cultured on substrates with a more physiologically-relevant stiffness (12–18 kPa), muscle differentiation and structure is improved [59]. Additionally, non-compliant substrates such as tissue culture plastic do not support concentric muscle contractions, eventually resulting in myotube detachment that prevents long-term culture required for advanced tissue maturation. These limitations have restricted 2D studies to short term drug treatments on developmentally immature myotubes [60]. Furthermore, in 2D monolayers the primary clinical goals such as improved force production and/or fatigue resistance, cannot be directly measured. For use as predictive pre-clinical drug testing platforms, *in vitro* skeletal muscle models must have the ability to: 1) sustain spontaneously contractile myotubes for prolonged periods of time (> 1 month), 2) closely mimic the *in vivo* mechanical and biochemical milieu, 3) allow measurements of contractile and metabolic function, 4) accurately model congenital and acquired diseases, and 5) exhibit expected responses to known therapeutics. Compared to traditional 2D cultures, 3D engineered muscle models permit longer term culture, direct assessment of contractile function, and enhanced maturity. In this section, we will discuss how 3D cell culture techniques are being utilized to improve the utility of *in vitro* skeletal muscle systems for disease modeling and drug screening.

4.1. Tissue-engineered 3D muscle

To date, scaffold-based and scaffold-free approaches have been the two major strategies for engineering 3D skeletal muscle tissues. Scaffold-based approaches involve embedding and culturing cells within a 3D matrix that serves to replicate the native mechanical environment. Scaffold-free approaches involve seeding myogenic cells and fibroblasts in 2D monolayers under conditions that promote the synthesis of sufficient ECM followed by self-assembly of cells into a 3D tissue. While synthetic polymers such as polyurethane, poly-L-lactic acid, and poly (lactic-co-glycolic) acid can be used to generate 3D muscle tissues [61,62], the most common biomaterials used in scaffold-based approaches are collagen and fibrin, as discussed below.

The first 3D engineered muscle tissues utilized collagen hydrogels on top of cultured avian myotubes. As the myotubes detached from the tissue culture plastic, they became encapsulated within the collagen gel and were subsequently kept under tension between

nylon support structures for 3 weeks [63]. The extended 3D culture of muscle cells under tension increased their DNA, protein content, and expression of MHC compared to 2D cultures, indicating improved differentiation and maturation [63]. Separately, Okano et al. directly molded a mixture of C2C12 cells and collagen gel in molds of defined geometries, and found that uniaxial mechanical tension improved cell alignment [64]. These methods have been subsequently applied to 3D cultures of primary rodent and human muscle cells, resulting in the formation of multinucleated myotubes, but no functional studies have been performed in these reports [65].

Despite collagen being the key structural component of native muscle ECM [25] and thus the initial material of choice for 3D muscle cell culture, fibrin has become the gold standard biomaterial for engineering skeletal muscle tissues with the highest specific forces to date [66–69]. This inferiority of collagen for muscle engineering could be attributed to its higher stiffness and thus greater likelihood to rupture, as well as lack of α7 and αv integrin binding motifs [69]. Moreover, *in vivo*, excess collagen is typically associated with fibrosis and muscle dysfunction stemming from faulty muscle regeneration [70]. On the other hand, human myoblasts differentiated in a fibrin matrix generate tissues with an elastic modulus of 12 kPa, similar to that of native muscle and known to promote myogenic differentiation [71]. Additionally, fibrin sequesters growth factors—such as FGF2, VEGF, and IGF-1—that promote myoblast migration, proliferation, and differentiation [72,73]. Furthermore, fibrinolytic enzymes secreted during muscle differentiation that are essential for myoblast fusion also support matrix turnover and tissue remodeling which may enable improved contractile function [74]. Fibrin-based tissues made from primary rat myogenic cells exhibit a positive force-frequency relationship, normal length-tension relationship, twitch:tetanus ratio close to that of native muscle, and a specific force amounting to ~13% of the adult muscle values [75]. Further improvements in contractile strength of fibrin-based engineered tissues have been achieved by utilizing fibrin-Matrigel composite hydrogels, mesoscopic molding, and dynamic culture [5,16,76]. Building on these conditions, we have generated the first functional human skeletal muscle tissues, termed myobundles, from both primary and hPSC-derived cells [12,13]. The resulting tissues exhibited normal length-tension and force-frequency relationships (FFRs), physiological calcium-handling, functional acetylcholine receptors, and continued myotube hypertrophy over 4 week culture – suggestive of progressive muscle maturation (Fig. 3A–E) [13]. While neonatal rat-derived engineered muscle tissues exhibited specific forces of ~40 mN/mm^2, human hPSC- and primary cell-derived tissues generated specific forces of ~3 and 10 mN/mm^2 respectively, indicating a less mature cell phenotype.

The first scaffold-free engineered muscle tissues were formed by differentiating myogenic avian cells on collagen-coated Saran wrap with multiple stainless-steel pins as anchor points to generate star-shaped tissues [77]. Following myotube formation and sufficient ECM deposition, the myotube monolayers detached from the culture surface and self-assembled to be held under tension between the steel pins. After 37 days of culture, these 3D tissues had increased expression of the MHC compared to 2D cultures and exhibited identifiable epimysium, perimysium, and endomysium layers similar to those found in native muscle [77]. Another type of self-assembled 3D muscle tissues (“myooids”) fabricated by culturing cells for 35 days on dishes coated with laminin (the most abundant protein of the basal

lamina) generated specific forces ~1% of those of native muscle and exhibited a positive FFR and normal length-tension relationship [69]. Improvements in the differentiation of self-assembled muscles have been reported by using aligned micropatterned surfaces to guide muscle alignment and promote fusion [78]. Detachment of cultured muscle cells was also achieved by using dishes coated with the thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAM). Upon cooling the dish below the polymer's lower critical solution temperature (~32 °C), PNIPAM undergoes a phase transition to a swollen hydrophilic state, detaching cells and proteins. Culturing micropatterned human myoblasts on PNIPAM produced aligned myotubes that could be detached as sheets and layered either together or interlaced with sheets of vascular endothelial cells to form vascularized muscle tissues [79]. Still, no contractile force measurements have been performed in these studies.

4.2. Skeletal muscle-on-a-chip

While 3D engineered tissues increase muscle maturation compared to 2D cultures, most approaches are not amenable to high-throughput drug screening and automation. A newly emerging approach to address this limitation is the use of organ-on-a-chip devices, which take advantage of microfluidic technology to minimize cell numbers and media volumes while multiplying drug testing throughput. In addition, a wide range of technologies in these microphysiological systems have been developed to track muscle's contractile function, metabolism, and biomarker secretion. Additionally, muscle-on-a-chip devices can be coupled with other microtissue systems to investigate multi-organ drug effects. In this section, we will discuss how muscle-on-a-chip platforms can be applied to model and asses skeletal muscle function and screen candidate therapeutics.

4.2.1. Sensors for muscle-on-a-chip assessment—For utility as a pre-clinical drug discovery tool, muscle-on-a-chip systems must have online, non-destructive measures of skeletal muscle function and metabolism such as contractile force generation, calcium oscillations, oxygen consumption, and/or fatigue response. Passive and active tension can be non-destructively calculated in real-time based on displacements of mechanically characterized substrates or posts (Fig. 1A). For example, optically measured deflections of PDMS posts of known height and stiffness serving to anchor contracting muscle microtissues can be used to calculate muscle contractile force based on the beam theory [80]. Similarly, contractile function of single myotubes can be estimated by measuring deflection of mechanically characterized silicon cantilevers used for myotube attachment (Fig. 1B) [81]. Complementary or alternative to these measurements, contractile function of engineered muscles can be non-destructively assessed by fluorescent imaging of calcium transients. Specifically, normalized fluorescent signal amplitudes from contracting muscle cells expressing genetic indicators of intracellular calcium have been used as a relative measure of calcium transient amplitude and have been shown to correlate with force generation in both rodent and human engineered tissues (Fig. 1C and D) [12,13,16]. Furthermore, myotube hypertrophy can be estimated through electrical impedance. When C2C12-derived myotubes were treated with hypertrophic IGF-1 or atrophic dexamethasone, the expected changes in myotube diameter correlated with changes in electrical impedance [82]. Several metabolic parameters can also be assessed non-invasively including: changes in oxygen consumption using oxygen-sensitive dyes [83], glucose uptake and metabolism

using glucose sensors [84] and genetic GLUT4 reporters [85], pH sensing with light-addressable potentiometric sensors [86], and media lactate concentration using two-frequency phase modulation (Fig. 1E) [87,88]. Finally, the diverse myokines or injury biomarkers can be also sampled and assessed by coupling the microfluidic device to a mass spectrometer [89]. Overall, increasing numbers of new optogenetic tools and genetic reporters of cellular functions are likely to find their use in non-destructive assessment of various microphysiological platforms including muscle-on-a-chip [90].

4.2.2. Considerations for human skeletal muscle-on-a-chip—During the last decade, muscle-on-chip technologies have evolved from the use of C2C12s and primary rodent cells towards human myogenic progenitor cells that better model the genotypic diversity underlying physiology and disease. Encouragingly, cultured human myoblasts appear to maintain disease-specific epigenetic changes as shown for type II diabetes, where cultured cells exhibit impaired insulin-stimulated glucose uptake and oxidative metabolism [91,92]. Contractile function of primary human myotubes can be assessed using cantilever chips [93] and allow further coupling in multi-organ systems for drug toxicity screening [94]. Still, optimal differentiation of human myotubes in muscle-on-a-chip systems remains to be achieved and is likely to require 3D tissue environment mimicking that of native skeletal muscle. For example, matching substrate stiffness to that of native muscle (12–15 kPa) and using the basement membrane extract Matrigel has been shown to enhance the differentiation of human primary myoblasts over laminin or fibronectin alone [95]. However, the use of Matrigel limits translational relevance, suffers from significant lot-to-lot variation, and may alter calcium-handling of engineered muscle tissues [67]. The use of defined, xeno-free and serum-free conditions to culture and engineer human muscle tissues would be necessary to minimize variations both within and between laboratories, some of which may arise from using same sera types at different continents [68,96–98], while biomimetic electrical and mechanical stimulation could serve to further improve muscle structure, function, and maturity [99].

4.3. Electrical stimulation for enhanced engineered muscle function

The low specific forces of current engineered muscle tissues reflect their developmentally immature state and lessen their physiological relevance and utility for pre-clinical drug screening. Neural input is essential for complete muscle development, maintenance of both adult muscle mass and function [100], and regulation of muscle fiber-type [101]. In attempts to increase muscle maturation and induce specific muscle phenotypes, *in vitro* systems have been developed to imitate neural activity using electrical stimulation. Parameters such as stimulation pulse amplitude, width, frequency, total number of pulses delivered to muscle, and media antibiotics have been optimized to increase force production, minimize electrolytic damage of cells, and drive muscle cell adaptation (i.e. increase in force generation and partial fiber-type shifts) within 3D engineered tissues [102,103]. Chronic, 24-h low-frequency electrical stimulation mimicking neural activation patterns detected in whole muscle EMG recordings of the slow phenotype soleus muscle resulted in 2–3 fold increased force generation in engineered C2C12 tissues. Interestingly, total MHC protein levels were unchanged, suggesting that the increase in force production could be a result of cytoskeletal reorganization and improved force transmission [103]. Extending this electrical

stimulation regime to 2 weeks shifted engineered skeletal muscles to a slow phenotype based on their contractile kinetics and improved fatigue resistance and although MHC protein levels increased, no shift between MHC isoforms was observed [104]. To induce fast-to-slow shift in MHC and troponin isoforms, it was necessary to replicate single motor unit activity patterns of adult rat soleus muscle [102,104]. Recently, one-week electrical stimulation of engineered human muscle tissues ("myobundles") increased myotube diameter by 40% and tetanic force amplitude by 3-fold, yielding the highest specific forces (19.3mN/mm^2) reported to date for human engineered muscle [105].

Electrical stimulation can be also implemented at the microscopic level to measure muscle function [106], increase myotube alignment, and improve muscle maturity (Fig. 2A) [107]. Large-scale electrical stimulation of small muscle-on-a-chip systems poses significant technical challenges due to higher contamination risk as well as increased risks of hydrolysis and electrochemical damage due to smaller media volumes. To overcome these risks, the use of an interdigitated electrode arrays permits contactless electrical stimulation without the need for electrode immersion in media minimizing media hydrolysis, energy required, and joule heating [108]. Interdigitated electrode arrays stimulating C2C12 cells increased expression of myogenic genes (MyoD, MHC, sarcomeric alpha actinin, and MEF2C) and myotube size [108]. Alternatively, optogenetic stimulation of C2C12 myotubes expressing the light-sensitive channelrhodopsin-2 has been shown to induce cell hypertrophy and a ~3.5-fold increase in force production (Fig. 2B) [109]. However, optogenetic stimulation of primary human engineered muscle tissues failed to increase force generation but did stimulate mitochondrial biogenesis and increase cellular respiration [110].

4.4. Mechanical stimulation of engineered muscles

Muscle mass and size are also regulated by mechanical load, with increased mechanical load by synergistic ablation, resistance exercise, or bone lengthening during development inducing muscle hypertrophy and decreased mechanical load through hindlimb suspension or low gravity environments inducing muscle atrophy [111]. To date, *in vitro* mechanical stimulations of engineered muscle have aimed to replicate: 1) the progressive bone growth that stretches muscle tissue during development (ramp stimulation) and; 2) muscle strain during exercise bouts experienced in adulthood (cyclic stimulation). Ramp stretch of avian engineered muscle mimicking the elongation rate of the developing chick limb increased myoblasts proliferation, differentiation, and myotube length [112]. Cyclic stretch-relaxation of the engineered avian tissues increased glucose uptake and lactate efflux, suggesting increased metabolic activity [113]. An eight-day application of combined ramp and cyclic stretch to human bioartificial muscles (HBAMs) made from primary human myoblasts increased myofiber area and diameter by 40% and 12%, respectively (Fig. 2C and D) [65]. In small muscle-on-a-chip models, myotubes cultured on a thin, flexible PDMS membrane have been stretched by applying vacuum (Fig. 2E) [114]. Additionally, mechanical stimulation to 3D cultures on a chip can be controlled magnetically (Fig. 2F) [115]. Similar to electrical stimulation, current mechanical stimulation protocols do not induce developmental maturation and significant increase in tissue size. Longer-term electro-mechanical stimulation may be required to generate more developmentally mature tissues [116].

4.5. Drug testing in engineered skeletal muscles

The ability to generate engineered muscle tissues from human primary and pluripotent stem cells can enable personalized tests of human muscle function and metabolism in response to various drugs *in vitro*. For use in preclinical pharmacological tests, these tissues must respond to drugs in a reproducible manner and similar to native muscle. For example, acute treatment with IGF-1, known to stimulate muscle growth and hypertrophy *in vivo* [117], was shown to increase contractile function by 50% in tissues engineered from primary rat myoblasts [75], while chronic IGF-1 treatment increased force production 3-fold in engineered mouse muscle [118]. Similarly, atorvastatin (a drug with known myotoxic effects) eliminated force generation in tissues engineered from mouse myoblast [118]. Tissues made from dystrophic mouse myoblasts allowed identification of 11 compounds (out of 31 tested) that increased tetanic force [119]. Importantly, human myobundles—which displayed functional acetylcholine receptors and a positive force-frequency relationship (Fig. 3A–E)—have also demonstrated physiological responses to diverse-acting drugs (Fig. 3F–I). For example, in response to growth-promoting β_2-agonist clenbuterol, myobundles displayed increases in force production and myofiber diameter (Fig. 3F). Known myotoxic agents, cerivastatin and lovastatin, decreased force production and induced lipid buildup in myobundles made from 3 donors (Fig. 3G–H). Similarly, treatment with chloroquine, an inhibitor of autophagosome-lysosome fusion and inducer of myopathy [120], resulted in autophagic buildup and reduction in force and sarcomeric protein expression (Fig. 3I) [13]. A significant portion of drugs fail during clinical trials due to mitochondrial toxicity [121], and human myobundles have been shown to elicit physiological responses to mitochondrial toxicants. Myobundles treated with rotenone, a complex I inhibitor, exhibited decreased fatigue resistance, oxygen consumption and force generation [122]. Collectively, microphysiological tissue-engineered models of functional skeletal muscle show promise as a novel platform for studies of human muscle disease and predictive drug development.

5. Innervation

As described previously, motor neurons conduct electrical impulses and chemically transmit them to muscle fibers to initiate muscle contraction (Fig. 4A). In addition to inducing muscle contraction, the neuromuscular interface plays complex and incompletely-understood roles in muscle and nerve development, adaptation, and survival [123]. Defects in NMJ development and function are implicated in debilitating or fatal diseases such as Myasthenia Gravis (MG), Amyotrophic Lateral Sclerosis (ALS), Lambert-Eaton syndrome, Bulbospinal muscular atrophy, as well as in sarcopenia [124]. Furthermore, a range of animal venoms such as those from spiders (latrotoxin), snakes (e.g. notexin and bungarotoxin) and bacteria (e.g. tetanospasmin) can impair NMJ function and cause paralysis or muscle damage [125]. Additionally, NMJ structures are primary or incidental targets of numerous therapeutic drugs including muscle relaxants, paralytics, cholinesterase inhibitors, and anticholinergics, as well as weaponized nerve agents. Thus, the development of functional neuromuscular interfaces *in vitro* is critical for studying neuromuscular biology, disease, and pharmacology.

5.1. Motor innervation of 2D skeletal muscle cultures

The foundational work in neuromuscular interface engineering was performed 40 years ago by Anderson et al., who demonstrated that co-cultures of Xenopus muscle progenitors and embryonal neurons resulted in clustering of acetylcholine receptors (AchR) in the membranes of the developing myocytes – a marker of muscle maturity [126]. These findings were replicated in murine co-cultures of embryonic motor neurons with primary myocytes, which resulted in histological evidence of neuromuscular synaptogenesis and maturation reflected by increased sarcomeric organization and MHC transition from fetal to neonatal isoforms, suggesting that innervation may offer a tool for advancing *in vitro* muscle maturation [127,128]. In each of these studies, however, the identified NMJs appeared morphologically immature, and often AchR clustering was not co-localized with nerve terminals, similar to the pre-patterning phases of embryonic NMJ formation *in vivo* [129]. Furthermore, the early neuromuscular co-culture systems did not offer direct evidence for functional communication through the NMJ.

While these initial studies from primary animal cell cultures demonstrated that primitive NMJs can form *in vitro* and increase certain markers of muscle maturation, they were not conducive to modeling human disease. In a two-species co-culture, human fetal spinal cord stem cell-derived motor neurons and rat myocytes showed AchR clustering and the formation of NMJs, similar to rodent co-cultures. For patient-specific disease modeling, human motor neurons must be derived from hiPSCs because adult lower motor neurons are post-mitotic and cannot be isolated routinely and safely from patients [128]. Recent advances in the derivation of motor neurons from hPSCs have provided a potentially unlimited source of neural progenitors for studying neurodegenerative diseases [130]. Of note, patient-specific hiPSC models of ALS and spinal muscle atrophy have been developed, yielding motor neurons capable of excitation, pathological interactions with disease-affected glia, and innervation of myotubes in 2D culture [131,132]. However, protocols for motor neuron differentiation from hiPSCs remain limited by poor differentiation efficiency and incomplete control over rostral-caudal and limb-medial column subtype differentiation [133]. Improved differentiation methods are necessary because neurodegenerative diseases differentially target regional subtypes [133].

To date, several studies have successfully demonstrated co-cultures of hiPSC-derived motor neurons with primary or hiPSC-derived myotubes in which AchR clustering was noticed around the axon terminals and myotube contractile activity was increased after innervation, the effect that was abolished by anticholinergic drug administration [134]. In a recent report, functional NMJ development was demonstrated using hiPSC-derived motor neurons expressing light-sensitive ion channel (channelrhodopsin2, ChR2 [90]) that were co-cultured with primary human myotubes. Transgenic expression of ChR2 permitted specific activation of only the motor neurons by blue light and true identification of functional NMJs that mediated muscle contraction, which could be blocked with vecuronium, a non-depolarizing muscle relaxant [135]. This system was further used to model myasthenia gravis (MG), a neuromuscular disease where autoantibodies targeting the postsynaptic AChRs activate the complement system and lead to progressive muscle weakness. Treating myotubes with IgG and active complement from MG patients reduced muscle contractility which could be

reversed with pyridostigmine, an acetylcholinesterase inhibitor used in myasthenia gravis treatment [135].

Additional neuromuscular pathologies have been successfully modeled *in vitro*, including muscle inflammatory disorders and myelopathies, peripheral neuropathies, and lipid handling disorders [135–137]. For utility as pre-clinical platform, these disease modeling systems need to be adapted for high-throughput drug screening with non-destructive functional readouts of both pre- and postsynaptic processes. Several tools have been recently developed to increase throughput with functional readouts of muscle contractility. Specifically, use of silicon cantilevers to culture rat myotubes functionally innervated with motoneurons allowed the simultaneous measurement of contractile force in multiple co-cultures [138] and permitted the testing of patient-specific NMJ drug-responses using human iPSC and spinal cord progenitor-derived motor neurons [139].

5.2. Motor innervation of 3D engineered tissues

While 2D micropatterned systems enable the site of NMJ formation to be more accurately controlled, they neither accurately replicate the 3D mechanical environment in which innervation occurs *in vivo* nor permit the ability to measure muscle function directly. Compared to the 2D culture setting, functional innervation of 3D engineered muscle is complicated by the greater distances that neurites must extend to form functional NMJs through multiple layers of myotubes. For successful innervation of 3D engineered muscle tissues *in vitro*, motor neurons are either: 1) directly incorporated with muscle cells at the time of 3D muscle formation or 2) cultured in a separate chamber and induced to sprout and grow towards a muscle tissue. The first study reporting a functional neuromuscular interface in 3D engineered muscles utilized rodent self-assembled myooids and embryonic spinal cord explants [140]. Over 2 weeks in culture, neurons projected out from the explants into the myooid improving force generation two-fold. Selective electrical stimulation of neurons resulted in tetanic contraction of muscle with the peak force amounting to ~25% of that produced by electrical field stimulation, indicating incomplete innervation of all myotubes within the myooid and/or immature NMJ function [140]. Primary motor neurons isolated from embryonic mice [141] or rats [142] were also shown to form neurite extensions and primitive NMJs with muscle cells yielding improved sarcomeric structure and doubled force generation of engineered muscle tissues. Building on this work, embedding hESC-derived motor neuron clusters with primary human myoblasts in a fibrin/matrigel matrix resulted in the formation of human NMJs that were maintained in culture for up to 3 weeks (Fig. 4B) [143]. Despite the formation of functional NMJs, only a subset of myotubes were innervated as indicated by direct muscle stimulation via acetylcholine that elicited over 2-fold greater myotube calcium transients than motor neuron stimulation via glutamate (Fig. 4C) [143]. When compared to 2D NMJs, a greater number of functional NMJs and increased expression of the mature AchR-ε subunit were identified in age-matched 3D tissues, suggesting increased NMJ maturity in a 3D culture environment [143]. Importantly, in all of these studies, selective activation of motor neurons by glutamate or light via ChR2 expression yielded muscle-specific calcium transients and/or contractions that were inhibited by the pre-synaptic blocker botox and/or the post-synaptic blocker D-tubocurarine indicating functional NMJ formation [141–143].

Unique studies of neuromuscular biology can be performed in microfluidic platforms due to their modular design that enables highly controlled 3D culture environment [144]. Specifically, a two-compartment microfluidic system was designed to culture mouse ESC-derived ChR2-expressing motoneurons and differentiated C2C12s in two separate but connected compartments. Neurites extended from the motoneuron compartments into the muscle compartment to form functional NMJs that could be controlled by light-induced activation of the motoneurons to elicit muscle contraction [145]. Recently, similar NMJ functionality was demonstrated using human microfluidic cell culture system where ChR2-expressing hiPSC-derived motoneuron spheroids successfully innervated a compartment with primary or iPSC-derived skeletal muscle tissues (Fig. 4D and E) [146,147]. Fewer functional NMJs were generated with iPSC-derived neurons from patients with amyotrophic lateral sclerosis (ALS), which could be partially rescued by treatment with clinical drug candidates rapamycin and bosutinib [146]. These systems also permit patient-specific studies of other NMJ pathologies including Myasthenia Gravis [143,147]. and may allow identification of muscle-secreted chemotactic factors that attract ingrowth of neurites.

Stimulating functional neuromuscular integration *in vivo* is a significant barrier to clinical utilization of engineered muscle tissues for treatment of volumetric muscle loss. To date, innervation has primarily been encouraged by transection of the femoral, common peroneal, or sural nerve and suturing the nerve into the implanted muscle construct [148–150]. One week post-implantation, contractile force of the implanted tissues doubled and AchR clustering and primitive NMJ synaptogenesis was observed; however, functional innervation from the host system was not detected [149,151]. Four-weeks post-implantation, implant force production increased five-fold and ~25% of this force could be elicited through direct neural stimulation and eliminated by curare administration, suggesting the development of a functional neuromuscular interface between the host and the engineered graft [148]. Furthermore, three months post-implantation neural stimulation resulted in detectable EMG signals from the implanted tissue indicating that functional innervation and host integration requires prolonged implantation times (> 1 month) [152]. Muscle innervation after VML injury treated with satellite cell-containing construct can be enhanced with exercise [153], suggesting a possibility that engineered muscle innervation could be accelerated and enhanced through physical therapy. Additionally, treatment of engineered muscle with agrin, a synaptogenic factor, increases AchR formation and clustering and contractile force generation 1.7-fold *in vitro* [154]. Implantation of engineered muscle tissues pre-treated with agrin *in vitro*, increases both the number of NMJs and angiogenesis within the implanted tissue, suggesting that biochemical signals can be used to improve functional integration with both the host neural and vascular systems [150]. Together, these studies demonstrate that implanted engineered skeletal muscle tissues have potential to integrate with host neuromuscular system, which is a critical step toward clinical translation and may permit pharmacological tests in grafted muscle *in vivo*.

Despite the described advances, NMJs in engineered muscles remain developmentally immature and lack the structure and function of adult NMJs. Detailed studies will need to be performed to thoroughly assess NMJ architecture at ultrastructural level, as well as to evaluate NMJ function with respect to fatigability, sensitivity to presynaptic action potentials, and calcium and acetylcholine handling. Additionally, the effects of secreted

neurotropic factors on engineered muscle structural and functional maturation, the limits of motor unit size and neuronal recruitment distance *in vivo*, changes in innervation throughout muscle engraftment and repeated injuries, and the synaptogenic roles of supporting cell types, such as Schwann cells and inflammatory cells warrant future investigations.

5.3. Sensory neurons

While motor input regulates the initiation of muscle contraction, muscle contraction and locomotion is also dependent upon sensory innervation which controls proprioception and reflex initiation via extension-detecting intrafusal muscle fibers coupled to type 1a sensory neurons [155]. Recently, *in vitro* protocols have been developed to generate intrafusal-type muscle fibers starting from primary human myoblasts using defined serum-free media conditions. These fibers were shown to form mechanosensory-like connections with co-cultured human proprioceptive sensory neurons and exhibit repetitive firing patterns typical of mechanosensory tissues [156]. A related study subsequently revealed functional neuromuscular interactions between intrafusal fibers and motor neurons *in vitro*, thus completing a model for both the afferent and efferent branches of the muscle spindle reflex arc [157]. This system may permit systematic studies of human proprioception physiology, although the evidence for sensory neuron response to changes in intrafusal fiber length and tension is yet to be reported.

6. Regeneration

Skeletal muscle is a highly regenerative organ, having the ability to functionally recover from ischemic, mechanical, and toxin-induced injury [158,159]. The regeneration process in mammals is critically dependent upon the muscle resident stem cell population, known as satellite cells (SCs) [1]. In healthy skeletal muscle, SCs lie in a quiescent state between muscle fibers and the basal lamina and express the transcription factor Pax7 but not the myogenic regulatory factor MyoD [160]. SC quiescence is actively regulated at the epigenetic, transcriptional, and post-transcriptional levels [161,162]. Loss of SC quiescence in transgenic mice results in an impaired regenerative response [163]. Disruption of the SC niche by tissue damage or stretch [164] initiates SC activation, which is characterized by cell proliferation and expression of the transcription factors Myf5 and MyoD (Fig. 5). Proliferating SCs have two fates: 1) commitment to differentiation by expression of myogenin [165] followed by fusion with other differentiating myoblasts or existing fibers to improve contractile function [166]; or 2) exit from the cell cycle to replenish the quiescent SC pool and contribute to future regeneration events [167].

While SCs are required for muscle regeneration, complete regeneration and functional recovery require the coordinated action of multiple cell types including: macrophages [168,169], fibroblasts [170], and fibroadipogenic progenitor cells [171,172] (Fig. 5). Chemoattractants—such as CCL2, fractalkine, and monocyte chemoattractant protein 1 (MCP-1)—released from damaged muscle, SCs, and tissue-resident macrophages attract bone marrow-derived monocytes/macrophages (Mo/MΦ) to the site of muscle injury [173,174]. In a somewhat simplified scenario, these macrophages adopt a pro-inflammatory M1 followed by pro-regenerative M2 phenotypes which support the SC proliferation and

differentiation, respectively [169,175]. During the early stages of injury, M1 macrophages secrete inflammatory factors (e.g. TNF-α, IFN-γ) and clear cell debris, which promote SC proliferation and inhibit premature differentiation [176]. Although the canonical M1 macrophage is known to release reactive oxygen species in inflammatory settings, this does not appear to be the case during skeletal muscle regeneration [177]. The M1 macrophages transition to M2 macrophages in response to IL-10 [178] and phagocytosis of apoptotic cells [179]. The M2 macrophages reduce myofiber apoptosis [174], secrete IGF-1 [176,180], stimulate differentiation of the proliferating SCs [178,181], and inhibit fibrosis [182]—all processes that are critical for completion of muscle regeneration. Failure of macrophages to transition from the M1 to M2 phenotype impairs regeneration [169]. Additionally, chronic activation of M2 macrophages results in prolonged secretion of TGF-β1 resulting in fibrosis [182]. *In vitro* studies on human myoblasts and human monocyte-derived macrophages suggest that macrophages reduce myoblast apoptosis [183], and that M1 and M2 macrophages enhance myoblast proliferation and differentiation, respectively [181]. These findings were validated with an *in vivo* cell transplantation model where co-injecting human myoblasts with pro-inflammatory macrophages increased the percentage of proliferating myoblasts and co-injection with anti-inflammatory macrophages increased differentiation and fusion with host fibers [184].

Fibrosis is the characteristic histological feature of impaired regenerative response seen in severe injuries, such as volumetric muscle loss (VML), or chronic muscle degenerative diseases such as Duchenne muscular dystrophy [1,170,185]. Classically, fibrosis was considered to be a consequence of fibroblast over-proliferation and excessive ECM deposition. However, in mild injuries where regeneration occurs, fibroblasts supports SC proliferation and partial ablation of murine muscle connective tissue (MCT) fibroblasts results in premature SC differentiation and reduced myofiber diameter [170]. In biopsies of human muscle damaged by eccentric contraction, SCs and fibroblasts were found to proliferate in close proximity. Additional *in vitro* studies showed that human myoblast differentiation and fusion were enhanced in the presence of fibroblasts [186]. MCT fibroblasts (marked by transcription factor 4, Tcf4) may overlap with another connective tissue cell type known to support muscle regeneration—PDGFRα$^+$ fibro/adipogenic progenitors (FAPs) [187]. Upon muscle injury, eosinophils recruited to the injury site secrete IL-4 that activates FAPs, which clear necrotic debris and promote SC differentiation through paracrine signaling [171,172]. However, for successful regenerative response, FAPs need to be activated only transiently. During aberrant repair, prolonged presence of macrophage-secreted TGF-β1 inhibits myogenesis and differentiates FAPs into collagen-producing myofibroblasts, leading to fibrosis [182].

The ability to accurately model muscle regenerative response *in vitro* would enable identification of important paracrine and cell contact mediated mechanisms of muscle repair, thus enabling the discovery of novel therapies for impaired muscle regeneration. Successful modeling of muscle regeneration *in vitro* would require: 1) the presence of a substantial pool of quiescent SCs and 2) an injury that induces SC proliferation, differentiation and return to quiescence as well as initial loss followed by recovery of contractile function. Currently, the major limitation in studying muscle regeneration *in vitro* is the ability to generate a sizeable quiescent SC pool due to the rapid activation of SCs upon *in vitro* culture. This rapid

activation of SCs results in a 10-fold loss in engraftment potential *in vivo* after only 72hrs of cell culture and prevents the expansion of SCs for use in clinical cell transplantation therapies. Much work has been done to prevent the loss of the *in vivo* regenerative potential of cultured SCs, including growing cells on laminin-coated substrates with muscle-like stiffness (12 kPa) [188,189] and modulating specific signaling proteins and pathways (e.g. p38 MAPK [190], Notch [191], JAK/STAT [192], Setd7). While these approaches improved the SC myogenic capacity, maintaining the full regenerative potential of SCs during culture would require reestablishing the native SC niches *in vitro* [2]. Towards this goal, the use of a defined "quiescence media" and growth on collagen I fibers coated with laminin and integrin α4β1 maintained the quiescence of human SCs for 3.5 days. However, culturing the quiescent SCs beyond 3.5 days made them senescent, suggesting additional cues are necessary for generating and maintaining large numbers of quiescent, regenerative SCs, needed for cell therapies. The first *in vitro* model to meet the desired characteristics of a regenerative muscle was the engineered skeletal muscle tissue made from neonatal rat myogenic cells. In this muscle-mimetic 3D culture environment, cross-striated myofibers were highly aligned and surrounded by basal lamina proteins, laminin and collagen IV. Abutting muscle fibers were quiescent $Pax7^+$ SCs that, upon cardiotoxin injury, rapidly activated, proliferated, and differentiated, eventually leading to near-complete recovery of ordered sarcomeric structure and contractile function within 10 days post-injury [16]. Importantly, a subset of $Pax7^+$ SCs returned to quiescence following injury indicating that this critical step of muscle regeneration (i.e., SC deactivation) can be modeled *in vitro*. These findings remain to be validated in human cells.

More biomimetic models of muscle regeneration will require the presence of additional cell types. Specifically, muscle co-cultures with macrophages have begun uncovering paracrine and cell contact-based signaling mechanisms crucial to understanding muscle repair [174,181]. Current 2D human co-culture models have examined myoblast response to macrophages activated by prototypical M1 (LPS/TNF-α and IFN-γ), M2a (IL-4/13), or M2c (IL-10) stimuli [181]. However, the resulting phenotypes do not recapitulate the complex polarization states that macrophages acquire during muscle injury and repair [193]. In a 3D, incorporation of bone-marrow-derived macrophages (BMDMs) within adult rat engineered muscle tissues significantly reduced the apoptosis and stimulated myogenesis to allow successful muscle regeneration after cardiotoxin injury [194]. *In vivo*, implanted muscle-BMDM tissues exhibited improved survival and vascular integration compared to muscle-only implants. Interestingly, unlike adult rat-derived engineered muscles, neonatal rat-derived muscles did not require the presence of macrophages for successful regeneration due to innate resistance to apoptotic injury [194].

Similar to the described rat 3D muscle-BMDM model, human macrophages and other immune system cells could be exposed to the relevant damage associated molecular patterns (DAMPs) from injured skeletal muscle [169,175] and examined for phenotypic and secretome changes. Such studies could identify novel proteins, microRNAs, and pathways that affect SC proliferation and differentiation as well as macrophage phenotype, fibroblast activation [170], and myofiber death and regeneration [174]. Overall, biomimetic *in vitro* models of human muscle regeneration could be instrumental in the development of novel drug-, gene-, or cell-based therapies for skeletal muscle injury and degeneration.

7. Modeling skeletal muscle cross-talk with other tissues and organs

Classically, one of the benefits of *in vitro* cell cultures is the ability to study a single tissue type in isolation to determine tissue-specific responses to experimental interventions. However, even when not in direct physical contact, organs and tissues in the body communicate through secretion of small proteins, peptides, and exosomes into the bloodstream. Skeletal muscle serves as a major endocrine organ, secreting numerous proteins in response to exercise, termed myokines, which signal to muscle to adapt to exercise [195], modulate systemic inflammation [196], and induce hippocampal neurogenesis [197]. The release of myokines during physical activity may also contribute to enhanced organ function leading to prolonged health span [198]. Several of these myokines (e.g. IL-6, IL-8, CXCL1, and TNF-α) have been also identified in culture media following electrical pulse stimulation (EPS) of human myotubes suggesting the potential utility of *in vitro* systems to study functional consequences of myokines on various tissues [199]. In this section we will describe direct and indirect interactions of skeletal muscle with other organs *in vivo* and demonstrate the potential for further exploring this paracrine cross-talk *in vitro* (Table 1).

7.1. Tendon

Each skeletal muscle in the body transitions into a tendon that physically attaches the muscle to bone and helps convert muscle contraction into movement. Tendons are primarily comprised of ECM, namely fibrillar collagen I, which is maintained by a population of endogenous fibroblast-like cells, called tenocytes. The highly specialized myotendinous junctions (MTJs) connect skeletal muscle and tendon into an integrated mechanical unit that permits efficient transmission of force from muscle to bone. Structurally, the MTJ consists of digit-like extensions from the tendon which penetrate the muscle belly and deep sarcolemmal folds that greatly increase the surface area of attachment between the two tissues, reducing stress concentrations at the MTJ. Additionally, efficient force transmission to the extracellular matrix at the MTJ is regulated by connections of sarcomeric actin to the tendon ECM via the dystrophin-glycoprotein complex (DGC) and the vinculin-talin-integrin system that connect to extracellular laminin-211 through the α7β1 integrin and utrophin which are specifically enriched at the MTJ compared to other sarcolemmal regions. Mutations in α7β1 and utrophin cause muscular dystrophies with a significant MTJ involvement in mice [200] and also result in human myopathies [201]. Additionally, MTJ injuries by trauma, repeated overloading, or overuse impair locomotion resulting in significant societal burden and associated economic costs.

The ability to engineer a functional muscle-tendon interface *in vitro* would provide a unique model for understanding the development and maturation of the MTJ. The engineering of a muscle-tendon interface is technically challenging due to the distinct biochemical and mechanical environments required to generate each of skeletal muscle, the MTJ, and tendon. The most advanced *in vitro* MTJs were engineered by first generating self-organized tendon tissues from rat tenocytes and then using them as anchors for engineering a self-organized muscle tissue [202,203]. This method formed a MTJ with embryonic-like tensile strength, but the majority of tissues still failed in the muscle belly. While promising, the use of

primary rat cells, long culture time (> 2 months), and immature structure of the resulting MTJ preclude utility of this system for large-scale drug tests. The ability to directly engineer co-cultured muscle-tendon interface within a hydrogel environment would provide a more efficient approach, however, generating a hydrogel scaffold with a non-uniform mechanical stiffness matching that of the native MTJ is technically challenging. Co-electrospinning of wo polymer blends (poly(ε-caprolactone)/collagen and the stiffer poly(l-lactic acid)/ collagen) onto different ends of a mandrel was one approach to generate a polymer sheet with a stiffness gradient similar to that of a native MTJ [204]. Despite the efficient method to generate mechanically biomimetic growth substrate, co-cultured myoblast and fibroblast lines did not form an MTJ. Engineering a mature, functional MTJ will likely require electrical and/or mechanical stimulation to place increased loading on cells as typical for native growth and development. Previously, cyclic stretch of tissue-engineered tendon increased the expression of a tissue-specific marker scleraxis, collagen I expression, and the ultimate tensile strength (UTS) [205].

While the existence of MTJs contributes to skeletal muscle biomechanical properties and force transmission, tendon and muscle also exhibit paracrine cross-talk to regulate function. During skeletal muscle development, the tendon primordia and myotome mass form autonomously [206], however, the maturation and segregation of tendon primordia into mature tendon requires muscle contractile activity which regulates tenocyte maturity in part through tension-dependent activation of FGF and TGF-β signaling at the primitive MTJ [207]. Additionally, muscle morphogenesis is regulated by tenocyte-secreted retinoic acid and muscle survival is dependent upon attachment to tendon [208]. In adult muscle, the paracrine cross-talk between skeletal muscle and tendon is poorly understood due to difficulties in discriminating the source of released signaling proteins. Similar to skeletal muscle, tendon releases IL-6 during exercise, although this secretion remains prolonged resulting in tendon hypertrophy and collagen synthesis [209]. Similarly, cyclic stretching of primary human tendon fibroblasts *in vitro* also increased secretion of IL-6 and prostaglandin E2 [210,211]. The ability to tissue-engineer mature biomimetic models of muscle and tendon *in vitro* can allow systematic studies of their paracrine cross-talk including the types, quantities, and effects of factors secreted in response to exercise or biomechanical stimulation.

7.2. Bone

Skeletal muscle and bone have an intimate relationship mediated through both mechanical stress and endocrine signaling. During all stages of development, mechanical forces exerted between muscle and bone regulate both muscle and bone growth and maturation. Increased muscle contractile activity and mechanical load placed upon both tissues result in muscle hypertrophy and increased bone mineral density. The mechanostat theory postulates that bone strength adapts to muscle forces, whereby muscle activity positively correlates with bone mineral density [212]. Mechanical forces applied to the bone are sensed primarily through osteocytes, which signal to matrix-synthesizing osteoblasts and bone resorbing osteoclasts to regulate bone mass [213]. In recent years, our knowledge of the communication between muscle and bone has moved beyond the role of mechanical forces towards appreciating the significant roles that secreted biochemical factors have in

regulating muscle growth and bone turnover [214]. Improved understanding of the endocrine signaling between muscle and bone may aid in the development of new therapies for osteoporosis and sarcopenia to reduce their increasingly negative socioeconomic impact on the aging population.

One of the first discovered signaling molecules co-regulating skeletal muscle and bone mass was IGF-1, which exists in two splice variants, the classical IGF-1Ea (IGF-1) and IGF-1Eb/c or mechanogrowth factor (MGF) [215]. Both IGF-1 and MGF are generated by muscle and bone and promote bone growth [216] through increased osteoblast proliferation and differentiation [217] and muscle hypertrophy through IGF-1R/PI3K/Akt signaling and activation of satellite cells. Aging is associated with decreases in IGF-1 circulating levels and the ability of skeletal muscle to express the MGF [218]. Given that polymorphisms that increase IGF-1 levels are associated with greater strength increase following resistance exercise in elderly [219], improved understanding of how IGF-1 splicing and secretion are regulated may aid treatment of osteoporosis and/or sarcopenia.

Myostatin or growth and differentiation factor 8 (GDF8) is a member of the TGF-β superfamily and is a potent negative regulator of muscle mass. Myostatin inhibits muscle growth through negative regulation of satellite cell proliferation, downregulation of the Akt/mTOR pathway leading to decreased protein synthesis and activation of forkhead box protein O1 (FOXO1) resulting in increased protein degradation. In response to exercise in rodents and humans, myostatin levels decrease – making myostatin an inverse myokine. In bone, the loss of myostatin leads to an increase in bone mineral density and strength by reducing osteoclast differentiation and bone resorption [220]. In a clinical setting, myostatin inhibitors such as ACE-011 have promisingly increased bone mineral density in osteoporotic women [221]. However, safety issues regarding ACE-011 treatment suggest that new targeting strategies may need to be developed.

IL-6 and IL-15, which are secreted by skeletal muscle after exercise, have dual effects on bone. The absence of IL-6 in mice (IL-$6^{-/-}$) results in lower bone mineral density and crystallinity and leads to delayed bone callus mineralization in response to fracture, suggesting that IL-6 enhances osteoblast precursor differentiation [222]. IL-6 mediated signaling also increases osteoclast differentiation and resorption [223]. Post-exercise release of IL-15 has hypertrophic effects on muscle and bone-resorptive effects by promoting pre-osteoclast formation [224]. FGF23, produced by osteocytes in the bone or post-exercise muscle, regulates bone mineral homeostasis [225] and protects against reactive oxygen species in muscle [226].

The ability to better investigate the relationships between human muscle and bone *in vitro* could significantly enhance our understanding of the etiology of diseases such as sarcopenia, rheumatoid arthritis, and osteoporosis. However, to generate high fidelity systems that more accurately replicate native muscle and bone significant advances in organ-on-a-chip bone cell culture systems are required. While the existing microfluidic models mimic the cellular heterogeneity of bone marrow [227], physiologically relevant devices to apply and study mechanical loading of osteoblasts, osteoclasts, and osteocytes remain to be developed. Promisingly, microfluidic chip models applying cyclic compression to human mesenchymal

stem cells [114,228] or shear stress to osteoblasts [229] are shown to stimulate osteogenesis. Future on-a-chip approaches would benefit from non-destructive assessment of tensile strength, co-culture of osteoblasts and osteoclasts derived from hiPSCs [230], use of more native-like ECM, and exploration of the paracrine signaling between bone and muscle microtissues under controlled levels and patterns of mechanical stimulation.

7.3. Liver

The liver is the largest visceral organ in the body which plays an essential role in the maintenance of whole-body homeostasis through the regulation of metabolism, digestion, blood coagulation, and immune surveillance [231]. Skeletal muscle and liver constantly interact to tightly regulate whole-body glucose and fatty acid metabolism. In the postprandial state, insulin stimulates glucose uptake in the liver and skeletal muscle for storage of glycogen [232]. In times of energy stress (e.g. fasting or prolonged exercise), glycogen stores in the liver are mobilized to maintain plasma glucose concentration and the muscle breaks down glycogen to meet energy demands. Fasting glucose levels are also supported by gluconeogenesis in the liver, which utilizes the amino acids alanine and glutamine that are by produced muscle proteolysis. To meet the increased energy demand during exercise, the liver increases glucose output through glycogenolysis and gluconeogenesis. A main substrate source for gluconeogenesis is lactate produced by contracting skeletal muscle which is cleared by the liver, metabolized to glucose and secreted back into the circulation in a process known as the Cori cycle. Liver gluconeogenesis is also regulated by the myokine IL-6, with circulating IL-6 levels directly correlating to exercise intensity and inversely correlating with plasma glucose levels [233]. IL-6 directly regulates hepatic glucose production [234], upregulates gluconeogenic gene expression, and increases secretion of the “liverkine” CXCL-1 which is involved in angiogenesis and anti-inflammatory processes [235]. In addition, nutrient status-dependent secretion of the myokine myonectin is known to regulate fatty acid uptake in the liver [236]. These observations have been reproduced *in vitro*, and serve as an example for potential metabolic adaptation due to cross-talk between the muscle and liver [236]. Similar to skeletal muscle, the liver also undergoes adaptations to exercise such as increased sensitivity to glucagon and insulin [237], increased fat oxidation and decreased *de novo* lipid synthesis [238]. Exercised rats were able to reduce liver triacylglycerol concentrations and prevent high fat induced hepatic steatosis [239]. Current *in vitro* models of exercise have yet to include the crosstalk between liver and muscle in a co-culture system, which is essential to more accurately model metabolic adaptations to exercise and provide a platform for the development of therapies for lipid accumulation and hepatic steatosis.

The current gold standard for modeling hepatic function *in vitro* are liver slices [240] which accurately predict drug responses, but require invasive isolation procedures and are limited to short experimental interventions due to rapid loss of metabolic activity [241]. The accurate modeling of hepatic function *in vitro* is technically challenging as the culture system needs to recapitulate the complex 3D architecture [242], biochemistry, and cellular heterogeneity of the liver [243]. Acinus, functional unit of the liver, contains parenchymal cells (hepatocytes) and non-parenchymal cells (NPCs)—namely stellate, Kupffer, and endothelial cells. Similar to skeletal muscle, the most advanced *in vitro* models of liver have

been achieved using 3D culture systems [244]. 3D co-cultures of primary human hepatocytes and NPCs at physiological mimetic ratios replicate several hepatic functions such as synthesis of key proteins (e.g. albumin, fibrinogen, and transferrin) and urea, inflammatory cytokine release in response to bacterial pathogens, expression of active cytochrome p450 proteins for drug oxidation, and insulin-stimulated glucose uptake and glycogen synthesis [245]. An artificial, microfabricated liver sinusoid has been generated that mimics the endothelial barrier, replicates the mass transport parameters of the hepatic microcirculation, and minimizes shear stress on the cultured hepatocytes [246]. Microfluidic devices have also successfully incorporated hiPSC-derived hepatocytes that maintained albumin production and CYP activity for over a month [247].

The development of high-fidelity *in vitro* liver tissue models is necessary to better predict drug toxicity and response in humans due to the critical roles that liver has in drug metabolism and detoxification. For example, processing of terfenadine in the liver microtissue module prevented its toxic effects on engineered human skeletal muscle, demonstrating potential for drug toxicity testing [248]. Similarly, Oleaga et al. developed a multi-organ microphysiological system interconnecting tissue-engineered liver, skeletal muscle, cardiac, and neuronal models in a common, defined culture media. Several known myotoxic drugs were tested in this system and produced the expected reductions in either skeletal muscle viability or contractile activity [94].

7.4. Adipose tissue

Adipose tissue functions as an energy store to maintain metabolic homeostasis, and its dysfunction is a key factor in the development of type II diabetes, cardiovascular disease, and cancer [249]. Adipocytes are present in two forms, brown and white, giving rise to brown adipose tissue (BAT) and white adipose tissue (WAT), respectively. BAT's main role is energy dissipation through non-shivering thermogenesis. Compared to white adipocytes, the brown adipocytes possess greater metabolic activity, mitochondrial mass, lipid droplets, vascularization, sympathetic innervation, and expression of uncoupling protein 1 (UCP1)—which uncouples oxidative phosphorylation to produce heat instead of ATP [249]. BAT is more abundant in lean individuals and is associated with improved insulin sensitivity and lipid homeostasis [250]. In contrast, WAT plays roles in lipid storage, hormone production, insulation, immune function, and exists in two main depots: visceral (vWAT) and subcutaneous (scWAT) that have differential effects on whole body metabolism [251]. Compared to scWAT, vWAT is more insulin-resistant and lipolytic, and possesses an elevated fraction of inflammatory cells [252]. As a result, excess visceral fat is associated with type 2 diabetes, atherosclerosis, dyslipidemia, and metabolic syndrome [253], whereas subcutaneous fat is associated with increased insulin sensitivity and reduced risk of developing type II diabetes [254]. The differential effects between subcutaneous and visceral fat are due to inherent differences among progenitor cells that comprise these tissues and different adipokine secretion [255]. ScWAT secretes higher levels of both insulin-sensitizing and anti-inflammatory adipokines such as adiponectin and leptin than vWAT [256]. In contrast, resistin [257], and retinol binding protein 4 [258] are secreted more abundantly by vWAT and are associated with insulin resistance and type II diabetes. The release of myokines and adipokines results in a reciprocal communication between skeletal muscle and

adipose tissue that regulates muscle fatty acid metabolism and influences insulin sensitivity in both tissue types [259]. Exercise decreases adipocyte size and lipid content in both vWAT and scWAT due in part to increased mitochondrial content and lipid oxidation in adipocytes [260]. Encouragingly, 2D monolayer co-cultures of differentiated myotubes and adipocytes have replicated several of the *in vivo* muscle-adipose tissue interactions. Specifically, the myokines IL-6, IL-15, and myonectin increase adipocyte lipolysis, fat oxidation, insulin-stimulated glucose uptake, and adiponectin secretion through SOCS and AMPK signaling both *in vivo* and *in vitro* [236,261–263]. Importantly, exercise can also induce the beiging of scWAT where UCP1 becomes expressed and scWAT adipocytes adopt a more metabolically active brown adipocyte-like state [264]. The myokines myostatin [265], myonectin [236]. B-aminoisobutyric acid [266], meteorin-like 1 [267], and FGF-21 [268] have been all implicated in the beiging of scWAT. The adipokines, leptin and adiponectin secreted primarily by scWAT and BAT increase skeletal muscle fatty acid oxidation, glucose uptake, insulin sensitivity and mitochondrial biogenesis both *in vivo* and *in vitro* [269,270]. In contrast, the vWAT associated adipokines (monocyte chemoattractant protein-1 (MCP-1) [271], chemerin [272], resistin [273], and TNF-α) impair insulin sensitivity or induce insulin resistance in both cultured and native skeletal muscle cells [274–276].

The ability to accurately model skeletal muscle-adipose tissue crosstalk has the potential to identify novel therapies for systemic metabolic diseases such as type 2 diabetes and metabolic syndrome and better replicate native skeletal muscle metabolism *in vitro*. Moreover, cultured primary human myotubes from diabetic patients were less sensitive to leptin and exhibited reduced AMPK activity and fatty acid oxidation [277]. In an independent study, treatment of cultured human myotubes with MCP-1 and chemerin (associated with insulin resistance) reduced their insulin-stimulated glucose uptake. These results suggest that basic metabolic responses to adipokines and myokines are conserved during 2D human muscle culture. Given that 3D adipocyte culture increases the expression and secretion of adipokines and generates more developmentally mature adipose tissue [278], the co-culture of 3D adipose and skeletal muscle tissues will likely provide more physiologically relevant system for studying lipid and muscle metabolism and related diseases [278].

8. Disease modeling

Despite their widespread use to study various muscle diseases, small animal models typically lack the ability to fully replicate the contractile and metabolic dysfunction and diverse epigenetic backgrounds that determine the severity of acquired and inherited myopathies in humans. The advancements outlined above in generating functional tissues from patient-derived primary cells and iPSCs can provide unique personalized platforms for drug discovery and understanding the molecular and genetic basis of disease. In this section, we will describe recent progress in the development of *in vitro* models for skeletal muscle disease phenotyping and therapeutic screening using the examples of type 2 diabetes and 3 monogenic disorders – Duchenne Muscular Dystrophy (DMD), Pompe disease, and dysferlinopathy.

8.1. Type II diabetes (T2D)

Type II diabetes (T2D) is a metabolic disease characterized by impaired glucose and lipid homeostasis in insulin-responsive organs such as skeletal muscle, liver and adipose tissue. In healthy individuals, elevated blood glucose (e.g. in the post-prandial state) results in pancreatic β-cell insulin secretion into the circulating blood, which reduces hepatic glucose production (HGP) [279] and increases whole-body glucose uptake. Skeletal muscle is a major peripheral site for glucose disposal and accounts for up to 90% of the body's insulin-stimulated glucose uptake [280]. Skeletal muscle glucose uptake occurs due to activation of the IRS-1/PI3K/Akt pathway and consequent translocation of the insulin-sensitive glucose transporter GLUT4 to sarcolemma. T2D develops first from peripheral insulin insensitivity, which is initially compensated by increased insulin secretion before a further reduction in insulin sensitivity as well as secretion [281]. T2D is a complex disease with a broad range of cellular defects including increased inflammation and decreased glucose uptake, glycogen synthesis, fatty acid uptake [279], mitochondrial function, and oxidative metabolism [282].

The majority of mouse models of diabetes develop the condition through genetic modifications that result in obesity and thus do not accurately mimic the progression towards insulin resistance and impaired glucose tolerance seen in humans [283]. The most common monogenic mouse models used are defective in leptin signaling, through loss of leptin ($Lep^{ob/ob}$) or the leptin receptor ($Lepr^{db/db}$), which causes hyperphagia and subsequent obesity, insulin resistance, and hyperglycemia. However, β-cell dysfunction is the underlying cause of T2D, not obesity [284], and plasma leptin level does not accurately predict disease severity in humans [285]. On the other hand, *in vitro* studies of human skeletal muscle strips show insulin-stimulated GLUT4 translocation to the plasma membrane and 4-fold lower insulin-stimulated glucose uptake if harvested from diabetic patients [286]. However, the inability to maintain muscle strip function and viability for several days or cryopreserve samples prior to testing precludes their use for large-scale *in vitro* disease modeling and drug testing.

Primary 2D human myotube cultures have been shown to display physiological responses to insulin such as increased glucose uptake, increased glycogen synthesis, and increased fatty acid uptake. Encouragingly, myotubes derived from diabetic patients display blunted insulin-stimulated glucose uptake [91], reduced glycogen synthesis [287], and impaired fatty acid uptake and oxidation [288]. Importantly, at the molecular level, T2D derived myotubes show blunted or failed activation of the PI3-K/Akt pathway and impaired IRS-1/PI3K binding, replicating *in vivo* results in patients [289]. Furthermore, myotubes derived from diabetic donors secrete higher levels of inflammatory cytokines (IL-6, IL-8, TNF- α, and IL-15) [290] suggesting that skeletal muscle contributes to the pro-inflammatory T2D environment. Together, these studies indicate that the environmental, molecular, and epigenetic changes believed to contribute to T2D are preserved during cell culture and support the use of *in vitro* models to identify novel anti-diabetic treatments.

Despite their promise, cultured myotubes have greatly reduced insulin-stimulated glucose uptake compared to native muscle, which is further lessened with cell passaging [30]. The decreased glucose uptake rates can be attributed in part to the higher levels of GLUT1 compared to the insulin-responsive glucose transporter GLUT4 [291] and lack of mature T-

tubules that are key sites of insulin-stimulated glucose uptake [292]. Additionally, insulin-stimulated glucose uptake is higher in more oxidative fiber types [293] but muscle cell cultures predominantly utilize glycolytic metabolism to meet energy demands. Supraphysiological levels of glucose can be used to increase levels of GLUT4 protein in engineered muscle tissues but precludes physiologically-accurate modeling of healthy blood glucose levels [294]. Alternatively, replacing glucose with galactose causes a shift to oxidative metabolism and reveals the expected differences in oxidative metabolism between healthy and diabetic myotubes that are hidden when culturing in physiological glucose levels [295]. Finally, denervation greatly diminishes insulin-stimulated glucose uptake *in vivo* [296] and electrical stimulation in primary human myotubes increases insulin-stimulated glucose uptake [297]. In a 3D engineered rat muscle system, insulin stimulation increased glucose uptake by 30% but basal glucose uptake was approximately 5 times higher than that of native tissue most likely due to the 8-fold higher levels of GLUT1 protein [298]. Together, these studies indicate that improved modeling of insulin signaling and T2D phenotype in cultured muscle cells will require several methodological modifications with respect to the metabolic substrate utilization, electrical or neuronal stimulation, and incorporation of additional cell types that affect insulin sensitivity, such as adipocytes.

8.2. Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that affects approximately 1 in 5000 male births [299]. DMD results from mutations to the dystrophin protein, a key structural component of the dystrophin-glycoprotein complex (DGC) that functions to transmit forces to ECM and stabilize the plasma membrane during contraction. Dystrophin defects compromise membrane stability, resulting in progressive muscle weakness, loss of ambulation by age 12 and loss of life by age 20 due to respiratory failure [300]. Current standard-of-care treatments with corticosteroids (prednisone and deflazacort), ventilation, and physical therapy can improve life expectancy up to the fourth decade but act by delaying disease progression and are not curative [300]. Of the > 4700 mutations identified in the dystrophin gene, a majority result in frameshifts or the appearance of nonsense codons [301]. In addition to the specific gene mutation, disease severity and response to pharmacological treatment is also influenced by genetic and epigenetic modifiers. In particular, effectors of TGF-β signaling significantly impact disease progression and efficacy of corticosteroid treatment, and inhibition of TGF-β signaling has been shown to improve muscle function in animal models [302]. Thus, identification of effective therapies will likely require the development of personalized *in vitro* muscle models that recapitulate the diverse genetic landscape underlying the disease and pharmacological response.

The first reported *in vitro* 3D model of DMD muscle was made from immortalized dystrophic mouse myoblasts and utilized pharmacological testing to identify 11 compounds (out of 31 tested) that increased tetanic force [119]. In recent studies, 2D-cultured human myotubes differentiated from primary DMD myoblasts exhibited reduced nuclear anisotropy, decreased fusion, smaller final myotube size, and produced less force compared to healthy controls [303]. Given the ethical considerations with obtaining muscle biopsies, limited proliferative potential of primary myoblasts, and the large number of mutations

across DMD patients with unknown disease modifiers, DMD will likely be best modeled using human iPSCs. Specifically, DMD myotubes differentiated from MyoD overexpression in hiPSCs exhibited some known disease features including calcium overload, branched myotube morphology, and increased creatine kinase release [304]. The use of transgene-free myogenic differentiation methods has also replicated some of the expected disease phenotypes. Specifically, comparison of DMD myoblasts derived from 5 iPSC lines identified common disease markers such as elevated BMP/TGF-β signaling as well as increased secretion of IL-6, IL-8, and collagen III [48]. iPSC line-specific differences in the degree of aberrant BMP/TGF-β signaling, expression of the putative disease modifier osteopontin and responses to SMAD inhibition suggested that patient-specific phenotypes can be preserved and studied *in vitro*. Furthermore, accurate modeling of the neuromuscular component of DMD will require co-culturing of skeletal muscle with motor neurons. Maffioletti et al. showed successful NMJ formation between DMD iPSC-derived 3D skeletal muscle and healthy iPSC-derived spinal motor neurons [305]. Although promising, isogenic human co-cultures recapitulating NMJ dysfunction in DMD remain to be established.

Recent technological advances in gene therapies and genome editing, including use of engineered dystrophins and GALGT2, antisense oligionucleotides (AONs), CRISPR/Cas9, TALEN, and human artificial chromosomes (HACs), have given hope to correcting the underlying genetic defects causing DMD, thus preventing the need for lifelong pharmacological therapy [306,307]. Since the full length dystrophin gene exceeds the carrying load of traditional viral vectors, regions of the gene were deleted to produce shortened but still functional micro- and minidystrophins [308]. However, delivery of the miniaturized dystrophin via adeno-associated virus (AAV) elicited T-cell immunity [309] which can be abrogated with treatment of a DNA plasmid vaccine to dampen autoimmunity to both AAV and dystrophin [310]. Currently, Phase I clinical trials are underway to test the efficacy of microdystrophin (, and) and GALGT2 (). Alternatively, AONs have been identified that restore the reading frame of dystrophin by exon skipping to endogenously produce a truncated but functional protein. Initial treatments of DMD patients with eteplirsen, a phosphorodiamidate morpholino approved by the FDA for treatment of DMD by skipping of exon 51, resulted in improved walking ability with no observed adverse effects [311]. *In vitro*, treatment of iPSC DMD myotubes with exon-skipping AONs induced expression of dystrophin protein, decreased calcium levels, and reduced contraction-induced damage of sarcolemma [304]. However, AON treatment is expensive due to its short half-life and need for frequent re-administration, highlighting the need for stable gene therapy or genome editing approaches. Genome editing systems that utilize TALEN and CRISPR-Cas9 can induce permanent exon skipping, frameshifting, or exon knock-in to enable expression of truncated dystrophin. In mouse and dog models of DMD, single AAV delivery of CRISPR-Cas9 editors has shown great promise in restoring dystrophin expression [306,307,312]. One limitation of CRISPR-Cas9 and TALEN based approaches is the potential for off-site genomic insertions and deletions, although such effects were found to be minimal in DMD hiPSCs [313]. While the exon skipping approaches result in the formation of a truncated dystrophin protein that results in a shift to a more mild Becker Muscular Dystrophy (BMD) phenotype, the HAC approaches are able to deliver the full length dystrophin protein without genomic integration and to ameliorate muscular dystrophy

in vivo [314]. Furthermore, human DMD iPSCs carrying HACs that encode the full length dystrophin gene have been shown to express dystrophin, attenuate disease marker expression, and increase myoblast fusion [48].

Full-length restoration of dystrophin, sufficient dystrophin protein expression, and minimization of off-target and immunogenic effects will be important criteria for development of improved therapies. *In vitro* engineered models of human DMD muscle may allow head-to-head comparisons of the therapeutic efficacy of different candidate approaches in patient-specific fashion.

8.3. Pompe disease

Pompe disease is an autosomal-recessive metabolic disorder that affects ~1:40,000 individuals, and is caused by mutations in acid alpha-glucosidase (GAA) protein. GAA breaks down lysosomal glycogen; however, its dysfunction results in glycogen buildup in striated muscles, progressive muscle weakening, and respiratory failure [315]. Pompe disease exhibits a continuous disease spectrum but can be divided into two major types based on age of onset: infantile and late-onset [316]. Infantile-onset presents within the first months of life and is characterized by cardiomegaly, liver enlargement, neural abnormalities, skeletal muscle weakening, and a median lifespan of ~6 months [317]. Compared to infantile-onset patients who tend to possess very low enzymatic activity (< 12%) [316,318], late-onset patients tend to possess higher levels of GAA, but still experience muscle weakness, difficulties in ambulation, and respiratory insufficiency. The inability to degrade lysosomal glycogen in skeletal muscle leads to significant lysosomal enlargement, vacuolation, autophagosome buildup [319], and lipofuscin accumulation [320] that is hypothesized to physically disrupt myofibrillar architecture and impair contractile function. The current clinical treatment for Pompe disease is enzyme replace therapy (ERT), whereby delivery of recombinant human GAA (rhGAA) reduces glycogen levels and, in infantile cases, alleviates the hypertrophic cardiac phenotype. The ERT significantly improves patient survival and quality of life [321]; however, it is expensive (costing $300,000 per year), requires much higher dosages than therapies for other lysosomal storage diseases [322], can be compromised due to the generation of neutralizing antibodies [323], and is not delivered efficiently to skeletal muscle resulting in variable functional improvements [324]. Therefore, new therapies with enhanced biocompatibility and muscle targeting are needed to improve patient outcomes. *In vivo*, Pompe disease has been studied in GAA knockout (GAA-KO) mice that display a phenotype between that of the infantile and adult-onset, with glycogen accumulation and skeletal muscle weakening but only mild cardiac symptoms. These mouse models have been utilized for preclinical drug discovery and validation, and have highlighted the role of autophagy, lysosomal dysfunction, and impaired mTOR signaling in disease progression [325–327]. Still, disease severity in humans is significantly impacted by the genetic background and GAA-KO mice do not fully reflect the diversity of disease phenotypes documented in Pompe patients [328]. Therefore, patient-specific *in vitro* models are necessary to develop a complete understanding of Pompe disease, which may lead to more effective clinical therapies.

Several essential aspects of Pompe disease have been modeled *in vitro*. Both Pompe hiPSCs and hiPSC-derived myotubes generated by MyoD overexpression have shown reduced GAA activity accompanied by elevated glycogen levels [41]. Treatment of Pompe iPSC-derived myotubes with recombinant GAA [329] or lentivirus encoding GAA [41] reduced glycogen content. Well-described features of infantile and late-onset Pompe disease include lysosomal enlargement and autophagosome buildup, respectively [330]. However, it remains unknown why these phenotypes do not co-occur in the same patients. Development of high-fidelity disease models *in vitro* may improve understanding of Pompe pathology and design of more efficient therapies. For example, in cultured human primary myotubes, autophagic impairment correlated with disease severity in late- but not early onset cells [331]. These studies further suggested that autophagy and autophagosome buildup exacerbate the late-onset disease phenotype, partially through buildup of immature GAA. Infantile Pompe myotubes also display lysosomal enlargement [332], and overexpressing TFEB (a master regulator of lysosomal biogenesis and autophagy) reduced muscle glycogen levels likely through exocytosis of lysosomal glycogen. These results are consistent with *in vivo* findings [332] and could potentially be used to identify drug candidates for stimulating the nutrient-responsive TFEB (or TFE3). Recently, a transgene-free method was used to generate $Pax7^{+}$ myogenic progenitors from infantile Pompe iPSCs, which harbored the C.-32-13T > G mutation hotspot resulting in exon 2 skipping. Treatment of differentiated myotubes with carefully designed AONs increased exon 2 inclusion, mRNA levels of the normal GAA splice variant, and GAA enzyme activity [333]. Together, these studies demonstrate significant progress in developing *in vitro* muscle models of Pompe disease and their initial use in testing candidate therapeutics.

While the above described *in vitro* models may present a controllable environment to study Pompe disease alterations in glycogen content, autophagy [334], lysosome regulation [332], mTOR signaling [335], calcium homeostasis [336], metabolism [337], contractile force deficit in engineered Pompe disease muscles has not been reported. The generation of higher fidelity skeletal muscle models of Pompe disease may require extremely long culture periods to enable sufficient glycogen accumulation, autophagosome buildup, and lysosomal rupture to disorganize the contractile machinery. Alternatively, pharmacologically or genetically increased glycogen and/or perturbed lysosome stability or autophagic flux may accelerate engineering of biomimetic models that would permit discovery of novel mechanisms of contractile dysfunction in Pompe disease to inspire development of more effective therapies.

8.4. Dysferlinopathy

Mutations in the dysferlin gene result in the development of several progressive muscular dystrophies termed dysferlinopathies. Dysferlin is a transmembrane protein that localizes to the sarcolemma and is involved in membrane repair, membrane trafficking, and muscle regeneration. The two major dysferlinopathic phenotypes are Miyoshi myopathy (MM), which presents with distal weakness, and limb-girdle muscular dystrophy type 2B (LGMD2B), presenting with more proximal muscle weakness [338]. Whilst congenital and early adult onset cases have been reported [339], dysferlinopathy onset typically occurs between 18 and 20 years of age and is characterized by progressive weakness of the limb and pelvic girdle muscles, myofiber necrosis, high numbers of inflammatory cells, extensive

lipid deposition, and fibrosis [338,340]. The two most commonly used mouse models, A/J and SJL/J mice, possess retrotransposon insertion and in-frame deletions, respectively, and develop myopathy due to naturally occurring mutations in the dysferlin gene [341]. These dysferlinopathy mouse models show expected disease phenotypes such as myofiber necrosis, fibrosis and inflammatory cell infiltration, but typically do not display muscle weakness, indicating incomplete modeling of the human disease [342,343]. A more severe mouse model of dysferlinopathy has been produced by deletion of apolipoprotein E (ApoE) to increase circulating cholesterol and lipid levels [344]. Intriguingly, double dysferlin and ApoE knockout mice fed high-fat diet resulted in better replication of the severity of the clinical phenotype with increased intramuscular lipid deposition and severe myopathy resulting in loss of ambulation in 50% of animals by 11 months [344]. While promising, it is currently unknown if alterations in ApoE function or elevated circulating cholesterol and lipid levels are causative factors in disease severity and progression in patients and thus if this approach truly replicates the human disease.

The wide range of mutations, lack of mutational hotspots, and the poor genotype-phenotype correlation in human cohorts, which suggest significant involvement of disease modifiers in disease severity, make LGMD2B difficult to model in small animals [345]. These challenges make dysferlinopathy an ideal disease to study *in vitro* to discover novel biology and screen personalized therapeutics. Traditional 2D cell culture models utilizing human iPSCs [39,346], primary human myoblasts [347], and immortalized human primary myoblasts [348] have been used to study the role of dysferlin in skeletal muscle *in vitro*. These cell culture studies have shown that dysferlin expression increases with muscle differentiation [349], with dysferlin forming a complex with Fam65b and HDAC6 to upregulate myogenin expression [350] and dysferlin deficiency decreasing myogenin expression [347,349]. The role of dysferlin in myogenesis is disputed, with several studies suggesting that dysferlin-deficiency impairs myoblast fusion and muscle differentiation [347,351] and other studies suggesting the dysferlin does not impact muscle differentiation [352]. The reason for this discrepancy is unclear but is likely reflective of differences in culture conditions, cell origin, or cell donor age. Defects in muscle differentiation and fusion attributed to dysferlin deficiency are due in part to activation of NF-kappaB (NF-κb) inflammatory pathway and IL-1β secretion from myoblasts, with inhibition of both these pathways reversing fusion defects in primary mouse myoblasts [353]. Dysferlin-deficiency is also characterized by increased lysosome size, defects in lysosome trafficking and impaired lysosome fusion to cell membranes [351]. High levels of the IGF1 receptor have been shown to accumulate in dysferlin-deficient myoblasts and may contribute to impaired fusion in dysferlin-deficient cells and underlie the lack of hypertrophic response to IGF-1 in dysferlin-deficient mice [351].

Multiple *in vitro* and *in vivo* studies have demonstrated that dysferlin plays a key role in membrane resealing following membrane disruption/injury [354,355]. Dysferlin is required for lysosome fusion at cell membranes during membrane repair and interacts with MG53, annexin Al, and annexin A2 which have been individually implicated in membrane repair [356]. Delivery of sphingomyelinase, which is released from lysosomes during cell membrane repair, or recombinant MG53, have been shown to restore membrane resealing in dysferlin-deficient cells [352,357]. Dysferlin itself can contribute to membrane repair when

cleaved by calpain to form mini-dysferlinC72 which then fuses at sites of membrane injury [358]. Dysferlin has also been implicated in both T-tubule biogenesis [359] and stabilization of T-tubules following injury [356,360]. In response to osmotic shock, dysferlin-deficient fibers show decreased T-tubule disruption, a decrease in electrically-stimulated calcium transients, and concomitant increased intracellular calcium which can be prevented by the L-type calcium channel blocker diltiazem [360].

Currently, there is no pharmacological or gene-based therapies available for dysferlinopathy. Daily treatment of dysferlinopathy patients with the glucocorticoids prednisone or deflazacort, the standard of care for DMD patients, resulted in no functional benefit or induced a functional decline in patients [361,362]. In contrast to daily steroid treatments that activate muscle atrophy pathways and promote lipid deposition, weekly prednisone treatments improve muscle function in dysferlin-deficient mice [363]. While more infrequent prednisone treatment may improve muscle function, prednisone itself does not improve recovery to membrane injury in single fibers [364]. However, vamorolone, a novel glucocorticoid improves muscle membrane repair and stabilization in dysferlin-deficient mouse fibers [364]. Membrane resealing in single dysferlin-deficient fibers has also been improved by halofuginone that also improves muscle function and histology in dysferlin-deficient mice [365]. Like dystrophin, the large size of the dysferlin gene has hindered gene therapy approaches for dysferlinopathy. Single AAV delivery of mini-dysferlin [366] or dual AAV vectors to deliver full length dysferlin [367] have improved muscle histology and membrane repair in mice. To overcome the need for repeated AAV injections, the delivery of full length dysferlin by sleeping beauty transposon gene transfer into semi-transformed myoblasts [368] and direct injection of cDNA [369] have been successful at ameliorating histopathology in mouse studies. Recently, antisense oligonucleotide-mediated exon skipping in both human primary and immortalized myoblasts [370,371] has been shown to restore dysferlin protein levels and improve membrane resealing ability in dysferlin-deficient cells. Future studies will likely utilize hiPSCs to develop effective CRISPR-Cas9 or similar genome editing approaches for permanent restoration of dysferlin expression [346]. Overall, *in vitro* studies of dysferlinopathy, including 3D tissue engineering approaches, hold the potential for discovery of novel mechanistic insights into disease progression and identification of effective therapeutics. For improved predictability, future *in vitro* studies will also require the incorporation of additional non-muscle cell types, such as endothelial cells [372], monocytes and macrophages, which have been also implicated in contributing to muscle pathology in dysferlinopathy [373].

9. Future directions for *in vitro* skeletal muscle models

The described advances in optimization of ECM composition, substratum stiffness, or cellular makeup, among others, have resulted in engineering 3D muscle tissues and OOC models that greatly surpass traditional 2D monolayer cultures with respect to maturation state. Furthermore, the 3D systems have extended duration of muscle cell culture to over 2 weeks and added the ability to measure contractile function – the key parameter for evaluating drug efficacy *in vitro*. While the progress has been rapid, widespread use of these *in vitro* models as preclinical drug discovery tools or complements to animal models requires further improvements. First, the extensive future utilization of human primary

Pax7^{+} satellite cells would require the identification of *in vitro* conditions that permit significant cell expansion but enable the expanded cells to regain a functional quiescent state. Second, the field would highly benefit from reproducible and efficient methods to generate expandable transgene-free hPSC-derived muscle progenitors through improved directed differentiation to the myogenic lineage and cell surface marker selection. Third, the ability of these primary and hPSC-derived myogenic progenitors to generate mature myotubes that more closely match the structural and functional maturity of native muscle tissues is essential. Progress has been already made in advancing myotube maturation *in vitro* by introducing 3D culture environments, extending culture time, and applying mechanical, electrical, and biochemical stimulation [12,13,68,103,104]. However, additional work is needed to merge these technologies and to develop new methods to accelerate engineered muscle maturation *in vitro* [116]. Fourth, to accurately model native tissue regeneration, engineered muscles should contain a pool of functional satellite cells and other tissue-resident cells needed for structural and functional recovery in response to toxic, ischemic, or traumatic injury.

Addressing the above four challenges would permit the *in vitro* studies of engineered skeletal muscle as an isolated organ, however, it would be unlikely to truly replicate native muscle function and behavior or to accurately predict metabolic changes and drug responses. Achieving these objectives would first require integration of functional motor and sensory innervation to better model neuromuscular diseases, but might be also necessary to generate muscle tissues with adult levels of function and specific fiber types. Second, the incorporation of other engineered organ systems on the same *in vitro* platform will be essential for modeling the complex mechanical and endocrine cross-talk that mediates muscle adaptation, metabolism, function, pathological changes, and drug response. Ideally, these engineered multi-organ platforms should accomplish the following: 1) include a defined universal blood substitute media capable of maximizing function and maturation of all co-cultured cells, 2) provide adequate media perfusion and recirculation to mimic vascular transport and facilitate multi-organ cross-talk, 3) utilize human primary or iPSC-derived cells rather than immortalized cell lines, 4) scale various organ compartments in a metabolically relevant fashion, and demonstrate accurate pharmacological responses and perturbations to cell function predictive of drug safety and efficacy *in vivo*. Meeting these requirements will not only improve drug discovery research, but also permit identification of novel biomarkers and understanding of multi-organ involvements in complex diseases.

10. Conclusion

In this review, we have described significant progress in modeling key skeletal muscle functions and diseases using *in vitro* engineered tissues and microphysiological systems. These advances have already provided the scientific community with novel technologies to study human muscle biology, pathology, and drug response. Future developments in generating hiPSC-derived myogenic progenitors, biomimetically incorporating resident non-muscle cells in 3D muscle culture, improving the contractile and metabolic maturity of tissue-engineered muscle, and coupling the skeletal muscle with other organ-on-chip systems are anticipated to further improve the personalized nature and pathophysiological

relevance of these *in vitro* models and increase the likelihood of identifying novel, clinically effective therapeutics.

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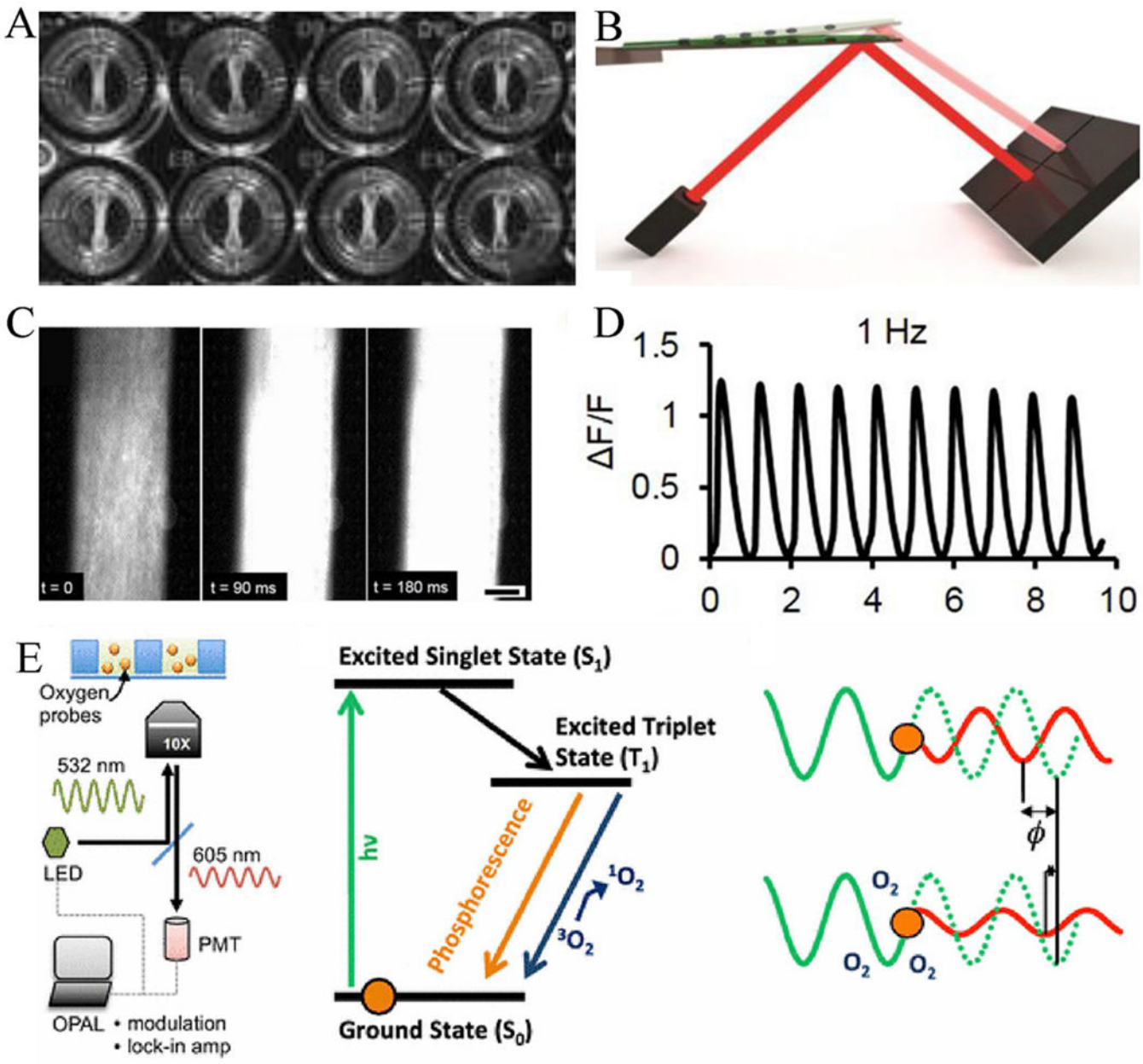

Fig. 1. Methods for *in situ* assessment of engineered muscles.

(A) Miniature bioartificial muscles formed between a pair of PDMS posts in a 96-well plate, where imaging of post displacement is used to calculate generated force.[80] Copyright 2010, Tissue Eng Part B Rev (B) Myotubes cultured atop silicon cantilevers, where laser-detected tip deflection during contraction is used to calculate contractile output. [81] Copyright 2013, Appl Phys Lett (C-D) Imaging of calcium transients in engineered muscle tissues transduced with a lentivirus coding a fluorescent calcium indicator, GCaMP6, and electrically stimulated at 1 Hz [13] Copyright 2015, Elife (E) Real-time tracking of oxygen concentration whereby a phosphorescent oxygen probe is excited by a 532 nm LED, amplitude and decay time of phosphorescence released during switch from the triplet to the ground state recorded, and the phase shift (ϕ) between incident and phosphorescent light

used to calculate oxygen concentration.[88] Copyright 2016, Arch Toxicol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

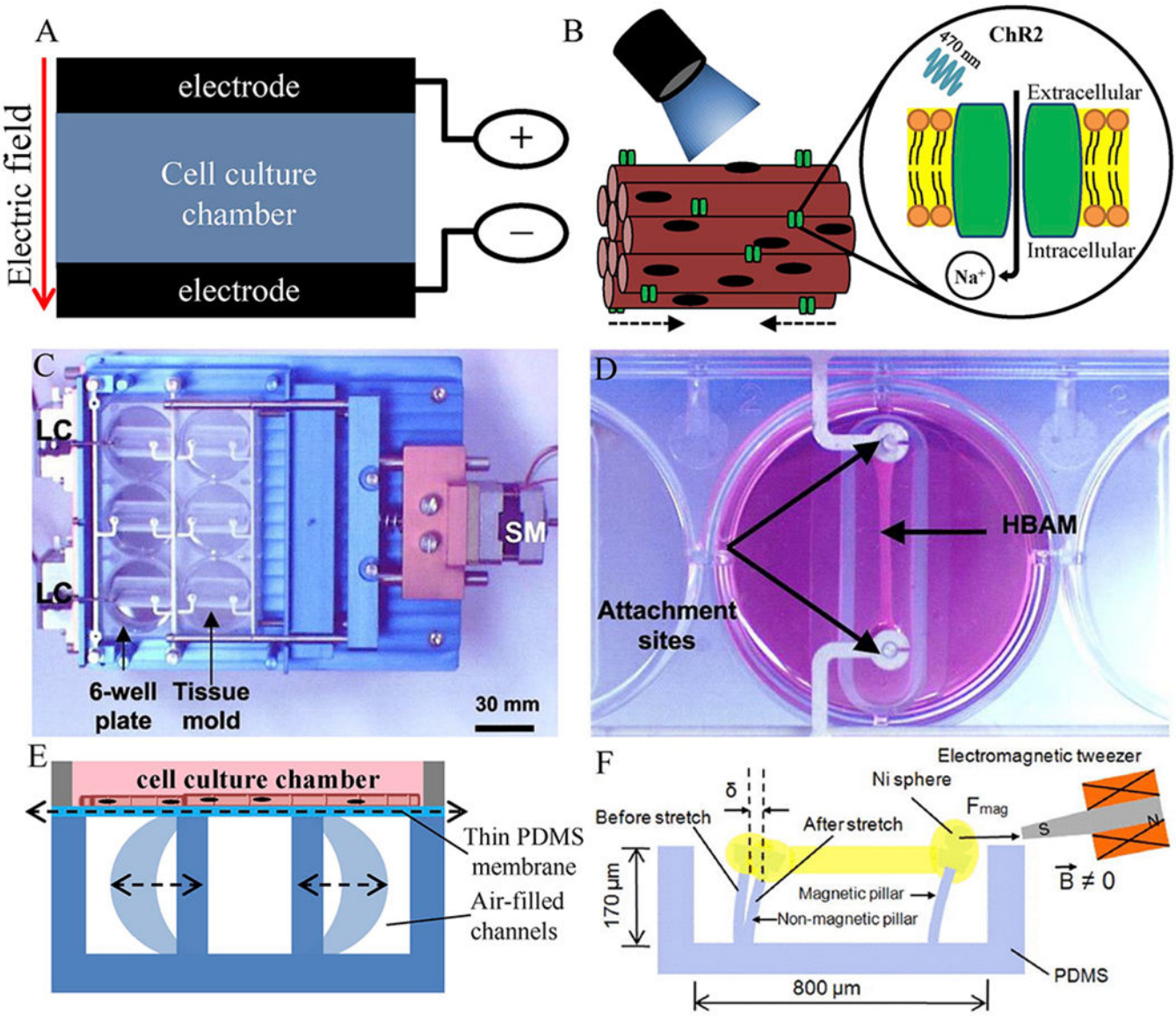

Fig. 2. Methods for chronic *in vitro* stimulation of engineered muscles.

(A) Electrical stimulation of muscle cells using corrosion-resistant electrodes directly in contact with the media. [108] Copyright 2013, Biomed Microdevices (B) Optical stimulation of engineered muscle expressing channelrhodopsin-2 (ChR2) whereby light-induced membrane depolarization triggers muscle contraction. (C) Mechanical stimulation of human bioartificial muscles (HBAMs), whereby a stepper motor (SM) strains tissues via the rightmost attachment site in each well, while the leftmost attachment site is either fixed or connected to load cells (LC) for tension measurement. (D) A single well of the setup in C displays a suspended 20 mm long HBAM fixed at the upper attachment site and lengthened/shortened by the lower attachment site. [65] Copyright 2002, Am J Physiol Cell Physiol.(E) Pneumatic mechanical stimulation of muscle cells by applying a vacuum through channels underneath cultured cells to bend the supporting beams and stretch the cells. (F) Magnetic mechanical stimulation of microtissues tethered between 2 PDMS pillars with one holding a

nickel sphere, whereby tissue strain is controlled by modulating the magnetic field and the force (Fmag) applied to nickel. [115] Copyright 2014, J Appl Phys.

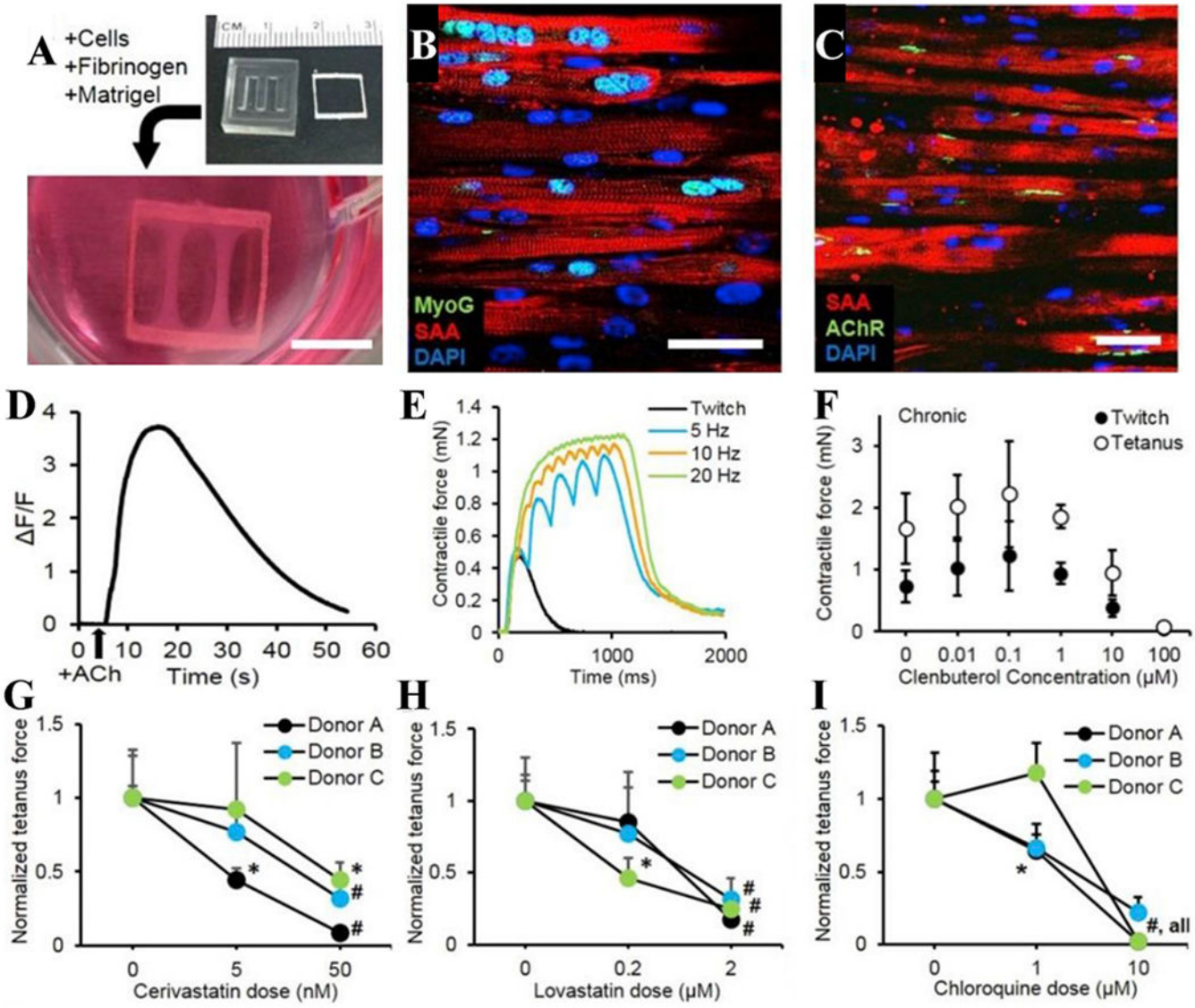

Fig. 3. Structure, function, and drug response of engineered human skeletal muscle (myobundle).

(A) Myobundles were made from a mixture of primary human muscle cells, fibrinogen, and Matrigel attached to a porous polymer frame. (B-C) Resulting 3D tissues contained aligned, multinucleated myofibers expressing myogenin (MyoG), sarcomeric alpha actinin (SAA), and bungarotoxin-labeled acetylcholine receptors (AChR; scales, 50 μm). (D-E) Myobundles respond with a large GCaMP6-reported calcium transient to a bolus of Ach (D) and exhibit a positive force-frequency relationship in response to electrical stimuli of increasing frequency (E). (F-I) Dose-dependent responses of myobundles to clenbuterol (β2-adrenergic agonist), cerivastatin, lovastatin, and chloroquine (anti-malarial agent). Treatment with statins or chloroquine induced the expected myopathy along with lipid accumulation or autophagic buildup, respectively.[13] Copyright 2015, *Elife*.

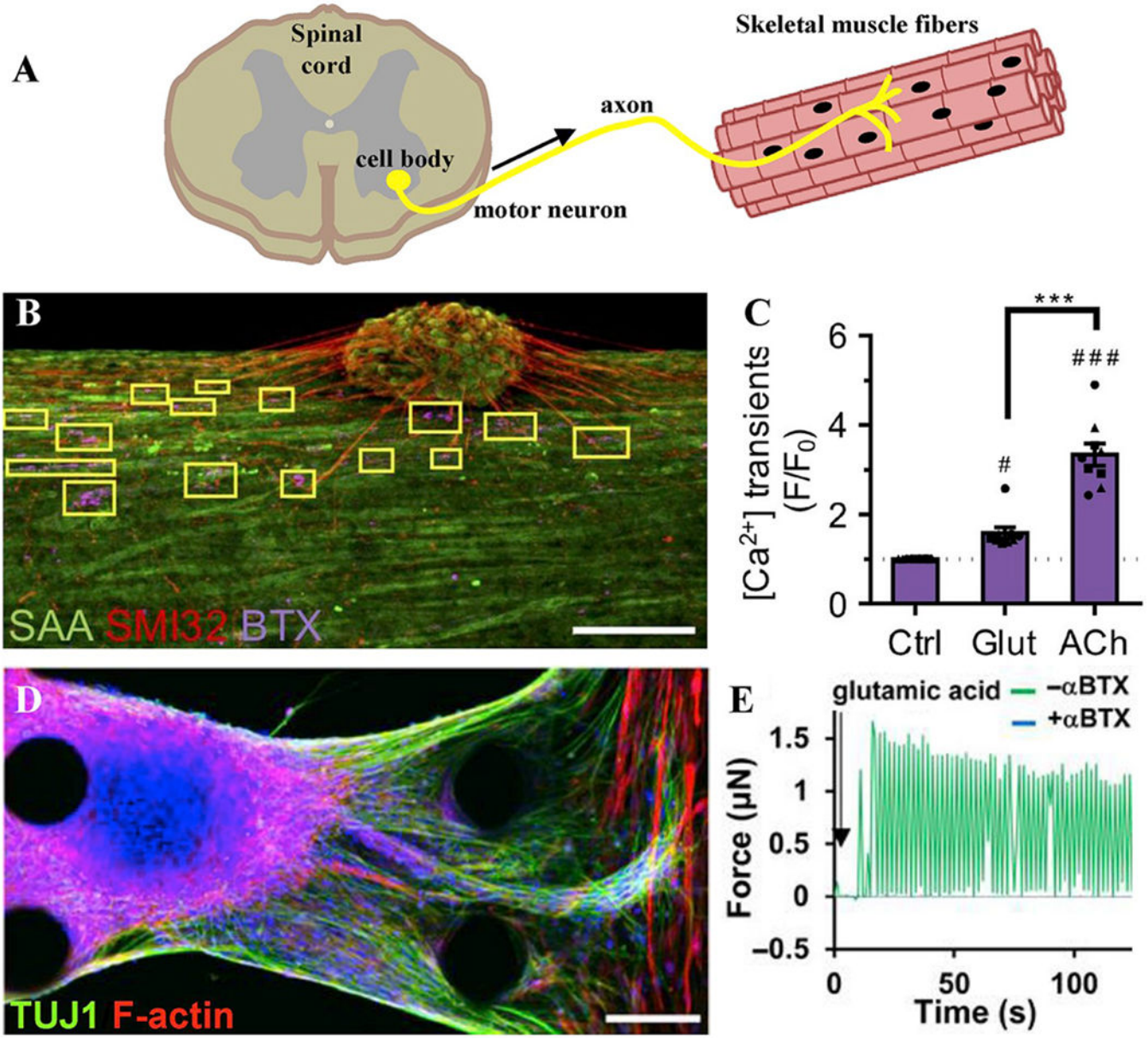

Fig. 4. *In vitro* skeletal muscle innervation.

(A) Schematic showing a somatic motor neuron carrying nerve impulses from the spinal cord to a bundle of skeletal muscle fibers *in vivo*. (B) 3D co-culture of human muscle fibers (SAA+) and clusters of human iPSC-derived motoneurons (SMI32+) results in formation of neuromuscular junctions (NMJs, BTX+). (C) In this system, glutamate stimulation of the motoneurons increases calcium transient amplitude in the muscle fibers; however, only a fraction of the fibers are functionally innervated.[143] Copyright 2019, *Elife*. (D) In a microfluidic device, human ESC-derived motoneuron spheroids (TUJ1+) extend neurons to form NMJs with the 3D-cultured human muscle fibers (F-actin+). (E) In this system, glutamate stimulation of motoneurons induces muscle contractions. [146] Copyright 2018, *Science advances*.

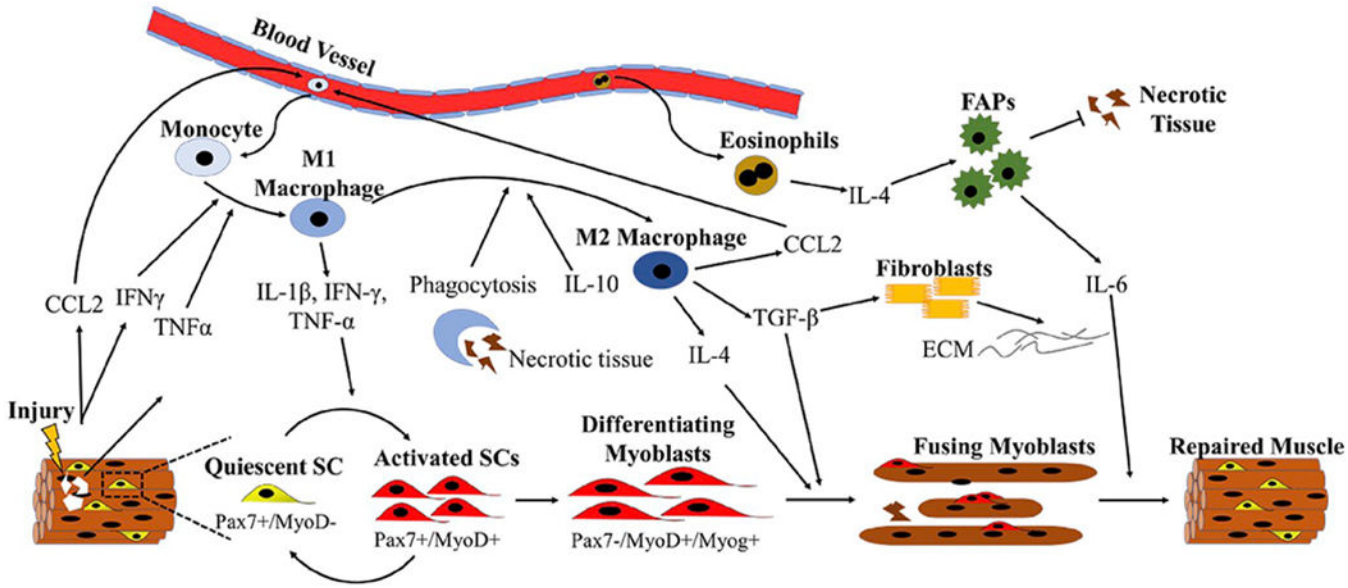

Fig. 5. Schematics of skeletal muscle regeneration.

In response to skeletal muscle injury, quiescent satellite cells (SCs) activate, proliferate, differentiate, and fuse to repair the damaged fibers. Cytokines released from damaged muscle recruit circulating monocytes which are polarized to become M1-like, then M2-like macrophages. Macrophages phagocytose necrotic tissue and secrete cytokines that promote SC activation, proliferation, and differentiation, and resolve inflammation. Fibro-adipogenic precursors (FAPs) are stimulated by eosinophil-secreted IL-4 to proliferate, clear necrotic tissue, and secrete cytokines that aid in muscle repair. Finally, TGF-β-stimulated fibroblasts deposit new ECM in the damaged area.

Table 1

Direction of Interaction	Biochemical factor	Effects modeled *in vitro*	Ref.
bone ⬌ muscle	IGF-1	• Stimulated osteoblast proliferation, differentiation, and mineralization	[75,80,217]
		• Increased contractile force in engineered 3D muscle	
tendon, bone ➡ muscle	PGE-2	• Increased COX-2 gene expression in fibroblasts	[211, 374]
		• Increased myoblast proliferation	
tendon ⬌ muscle	IL-6	• Mechanical stretching induced IL-6 release in tendon fibroblasts	[210]
bone ⬌ muscle	MGF (IGF-1Eb/c)	• Increased myoblast proliferation and differentiation	[375–377]
		• Promoted osteoblast-like cell proliferation	
muscle ➡ bone	IL-6	• Simultaneous IL-6 and soluble IL-6R induced osteoclast-like cell formation	[378]
muscle ➡ bone	IL-15	• Stimulated preostoclast formation and osteoclastogenesis	[224]
bone ⬌ muscle	myostatin	• Induced osteoclast formation	[220, 379]
		• Inhibited human myoblast differentiation and reduced myotube diameter	
adipose ⬌ muscle	acute IL-6	• Increased fatty acid oxidation, basal and insulin-stimulated glucose uptake, and glycogen synthesis	[261, 380,381]
		• Increased basal and insulin-stimulated glucose uptake in adipocytes	
adipose ⬌ muscle	chronic IL-6	• Impaired insulin signaling in adipocytes	[382]
scWAT,BAT ➡ muscle	adiponectin	• Increased mitochondrial biogenesis, palmitate oxidation, and citrate synthase activity in human myotubes	[383]
adipose ➡ muscle	leptin	• Stimulated glucose transport and glycogen synthesis	[277, 384]
		• Increased fatty acid oxidation	
vWAT ➡ muscle	resistin	• Reduced palmitate uptake, fatty acid oxidation, basal and insulin-stimulated glucose uptake, and glycogen synthesis	[273, 385]
vWAT ➡ muscle	MCP-1	• Impaired insulin sensitivity	[271]
WAT ➡ muscle	chemerin	• Induced insulin resistance	[272]
vWAT ⬌ muscle	TNF-α	• TNF-α was overexpressed in myotubes of insulin resistant and diabetic patients relative to insulin-sensitive patients	[386,387]
		• Suppressed IGF-II and IGFBP-5 production in myoblasts, leading to apoptotic death	
muscle ➡ liver	IL-6	• Inhibited glycogen synthesis and increased glucose production in hepatocytes	[388,389]
muscle ➡ liver	myonectin	• Increased fatty acid uptake and suppressed autophagy in the hepatocytes	[236, 390]

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