Use of flow, electrical, and mechanical stimulation to promote engineering of striated muscles

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Abstract

The field of tissue engineering involves design of high-fidelity tissue substitutes for predictive experimental assays in vitro and cell-based regenerative therapies in vivo. Design of striated muscle tissues, such as cardiac and skeletal muscle, has been particularly challenging due to a high metabolic demand and complex cellular organization and electromechanical function of the native tissues. Successful engineering of highly functional striated muscles may thus require creation of biomimetic culture conditions involving medium perfusion, electrical and mechanical stimulation. When optimized, these external cues are expected to synergistically and dynamically activate important intracellular signaling pathways leading to accelerated muscle growth and development. This review will discuss the use of different types of tissue culture bioreactors aimed at providing conditions for enhanced structural and functional maturation of engineered striated muscles.

INTRODUCTION

Since the first successful organ transplantation in 1954, transplant rejection and organ donor shortage rendered the transplant therapies an imperfect solution to organ failure and tissue loss (1, 2). Over the past two decades, tissue engineering (TE) has evolved as a potential strategy for de novo design of replacement tissues and organs. Furthermore, TE techniques have been utilized for the development of in vitro microphysiological systems or 'organ-on-a-chip' platforms for pre-clinical drug screening and studying of microtissue physiology and pathology (3–6). Engineering and therapeutic use of striated (cardiac and skeletal) muscle tissues, in particular, while promising, has met with difficulties in replicating aligned cellular organization and adequate functional output characteristic of native muscle. Thus, significant research in the field has been aimed at providing more favorable culture environments to aid survival, growth, differentiation, alignment, and functional maturation of engineered striated muscles in vitro (7–9). In particular, mechanical (stretch, shear) and electrical stimulation as well as medium perfusion have been the key biomimetic signals applied to promote and support functional myogenesis in vitro.

Specifically, mechanical loading has been known to profoundly affect different cells in the body, generally termed mechanocytes, including osteocytes, chondrocytes, cardiomyocytes, and skeletal muscle cells (10, 11). The cellular responses to mechanical stimuli are mediated

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by mechanotransduction pathways that can either promote tissue development and function or lead to pathological changes (12). For example, mechanical overload of skeletal muscle during exercise can enhance its force generating capacity in vivo (13). Conversely, pressure or volume overload in the heart can lead to physiological or pathological hypertrophy depending on how the stress is applied (12, 14). Similar to mechanical stimulation, electrical stimulation influences the survival, proliferation rate, hypertrophy, and other properties of muscle cells. In vitro, the use of electrical stimulation is aimed at mimicking the action of the motoneurons (in skeletal muscle) or pacemaker cells (in cardiac muscle) found in native physiological environment. Denervated skeletal muscles are known to undergo a decrease of fiber diameter (atrophy) as well as become prone to injury and degeneration (15–17). The application of electrical signals may enable long-term survival and continued differentiation of muscle cells in vitro as well as prepare engineered muscle tissues for electromechanical integration following implantation in vivo.

The need to create a more biomimetic cell culture environment compared to previously used static and unstimulated conditions led to the development of different types of tissue culture bioreactors. Nowadays, the use of bioreactors has become a standard tissue engineering practice to provide a tightly controlled environment for long-term tissue growth as well as to test the acute responses of tissues to external stimuli (18, 19). While the bioreactor types used range widely from simple tissue culture plates to high-end multimodal systems, they are all designed with one or more of the following functions: (1) Maintenance of sterility, (2) Exchange of gases and nutrients, (3) Control of operating conditions (pH, temperature), and (4) Delivery of tissue-specific biophysical and biochemical cues. Bioreactor design is particularly challenging for tissues such as striated muscles that exist in high stress environments, are metabolically active, and perform vital functions in vivo. Between skeletal and cardiac muscle, design requirements for tissue culture bioreactors are comparable owing to the similarities in their cellular density, metabolism, function, and biomechanical growth laws (20). This review will mostly focus on the environmental conditions and biophysical stimuli provided by tissue culture bioreactors that can enable efficient structural and functional maturation of engineered striated muscles in vitro.

USE OF SCAFFOLDING MATERIALS TO PROMOTE ENGINEERED MUSCLE STRUCTURE, DIFFERENTIATION, AND FUNCTION

The ultimate goal of any tissue engineering effort is to generate a completely biomimetic tissue substitute that would structurally and functionally integrate with the host tissue after implantation at both micro- and macroscopic levels. In general, host-donor differences in tissue architecture may hinder graft integration, prevent healing and regeneration, and even compromise safety of the therapy (e.g. by causing cardiac arrhythmias). In the case of striated muscle, for the most efficient and safe repair, engineered tissue grafts should possess structural properties characteristic of native muscle including high cell density, alignment, and proper cell-type distribution (21). Furthermore, striated muscle constructs should be able to support macroscopic tissue contractions and not only generate high active (contractile) stresses but also sustain dynamic changes in passive tension that occur during normal muscle function and exercise.

Biomaterial scaffolds represent a component of tissue engineering design that is critical for the proper structural organization and functional maturation of engineered muscle tissues as well as for their ability to sustain chronic biophysical stimulation in vitro and in vivo. Both synthetic and natural scaffolds have been previously utilized for striated muscle tissue engineering. Up to date, naturally derived hydrogels have proven more capable of producing constructs with high cell density (due to cell-mediated gel compaction), uniform cell distribution, and better cell alignment than synthetic polymeric scaffolds (21–23). Hydrogels can be also micropatterned to reproducibly generate specific tissue structures (21, 24) and can be used to deliver mechanical stimuli to cells when anchored at opposite ends via porous materials, metal wires, or elastomeric posts (25–27). On the other hand, hydrogel-based as well as scaffold-free tissue patches usually fall short of supporting uniform 3D cell alignment over a large tissue area (28–30). Cylindrically-shaped constructs can support both high cell density and alignment (31–33), however, they do not allow local control of the degree or direction of cell alignment. The existing methods to generate uniform 3-D orientation of cells (over multiple cell layers) in cardiac and skeletal muscle tissue patches involve the use of 3-D scaffolds with aligned pores (34, 35), mesoscopic hydrogel molding (25, 36, 37), and stacking of aligned tissue sheets (38). Still, the ability to engineer 3-D biomimetic striated muscle tissues that are soft, free-standing, relatively large, and possess stable aligned architecture and physiological density of muscle cells is yet to be demonstrated.

In addition to being able to support high cell density and alignment, biomaterial scaffolds need to provide a biophysical environment that is supportive of striated muscle differentiation and function. In particular, Discher and others have shown that myogenic cells can sense and react to stiffness of the surrounding matrix and optimally grow and differentiate on 2D substrates with a stiffness similar to that of native muscle (39, 40). Similarly, 3D hydrogels of different stiffness have been shown to distinctly affect gene expression and function of cardiac progenitor cells or neonatal cardiomyocytes (41, 42). More recent studies however suggest that the stem cell fate and thus the process of striated muscle differentiation and maturation may not be directly governed by the substrate stiffness per se, but instead depend on the type, density, and distribution of cell adhesion receptors which are in turn regulated by the hydrogel chemistry and, potentially, the dynamics of muscle cell contractions (43, 44). Furthermore, biophysical and biochemical properties of the surrounding matrix will directly determine how efficiently: 1) individual muscle cell contractions integrate at the macroscopic tissue level, and 2) externally applied mechanical stimuli transmit to individual cells. In general, improved understanding of cell-biomaterial interactions (for both muscle and supporting non-muscle cells) will be required for the design of advanced 3D scaffolds for tissue engineering of functional striated muscles.

USE OF FLOW TO ENHANCE MASS TRANSPORT WITHIN ENGINEERED MUSCLE TISSUES

Traditional cell culture systems such as tissue culture plates and flasks maintain cells in static condition whereby gas exchange that occurs via surface aeration and mass transfer is diffusion-limited (45). In static systems, engineered skeletal and cardiac muscle tissues

develop a necrotic core due to low mass transfer rates and high metabolic demand of cells (30, 31). In an attempt to mimic physiologic delivery of oxygen, nutrients, and chemical signals by blood flow, researchers have designed tissue culture bioreactors with continuously mixed media that provide significantly higher mass transfer rates compared to static cultures. These bioreactors enabled engineering of thicker tissue constructs, however, physiologically dense (10^8–10^9 cells/ml of tissue) engineered striated muscle tissues still remain limited in thickness to 120–150 μm (28, 46). Examples of dynamic tissue culture bioreactors (e.g., spinner flasks, rotating wall vessels (RWVs), perfusion systems) are listed in increasing order of their mass transfer rates in Figure 1. Characteristics of the bioreactors utilized for seeding and cultivation of engineered skeletal and cardiac muscle tissues are summarized in Table 1. In addition, different microfluidic systems can be used to facilitate drug screening studies in striated muscle microtissues by minimizing the fluid volume needed to perfuse such organ-on-a-chip platforms (47, 48).

Stirred Flasks

Stirred spinner flasks were among the first bioreactors employed to promote circulation of media and supply of oxygen to 3-dimensional cell cultures (19). Although media mixing in these bioreactors yields an increase in overall cell viability compared to static cultures (49), the use of rotating stirrer bars also creates a turbulent shear stress environment which can negatively affect health and viability of shear-sensitive cells such as cardiomyocytes (46, 49).

Rotating Wall Bioreactors

For shear-sensitive cells, such as cardiomyocytes, an ideal culture environment would involve both the presence of high mass transfer rates and low shear stresses. NASA was the first to introduce a tissue culture bioreactor that satisfied these requirements, a rotating wall vessel (RWV). Through control of rotation speed, the RWVs maintain a free-falling ("microgravity") tissue environment in which cells experience low shear stress but still receive sufficient supply of oxygen and nutrients (50). There are several adaptations of the RWV currently in use, including slow lateral turning vessel (STLV), high aspect ratio vessel (HARV), and the rotating wall perfusion vessel (pRWV) (19). STLVs and HARVs are cylindrical vessels that differ in the base-to-height aspect ratio and size and position of the gas exchange membrane. In STLVs, the gas exchange membrane wraps around an inner cylinder whereas in HARVs, the membrane is flat and positioned at the base of the cylinder (51). In pRWVs, the media perfuses through the rotating cylinders. These bioreactors have been utilized to promote formation of functional skeletal and cardiac muscle tissues. Specifically, Molnar et al. cultured satellite cells on microcarrier beads in HARVs and observed that cells experiencing microgravity showed better cellular organization than those in static cultures (52). Interestingly, application of microgravity improved cell proliferation without the anticipated muscle atrophy. Papadaki and Bursac et al. observed significant improvements in cell viability, hypertrophy, expression of cardiac proteins, and electrical function when engineered cardiac tissues were cultured in HARVs compared to spinner flask bioreactors (46, 53).

Perfusion Bioreactors

Perfusion bioreactors have been developed to allow continuous flow of culture medium and provide high transfer rates of gases and nutrients, stabilization of pH, and effective removal of toxins, all of which are expected to result in better structural organization and functional properties of engineered striated muscles. Specifically, the use of perfusion through porous polymer scaffolds has been shown to improve seeding efficiency leading to increased density and more uniform 3-D distribution of skeletal muscle cells and cardiomyocytes (54–56). In addition to more uniform cell seeding, perfusion of cardiac and skeletal muscle tissues can promote their viability and long-term maintenance in vitro, as shown by Chromiak et al. for avian skeletal muscle organoids (57). In recent studies, Cheng et al. analyzed the individual and combined effects of IGF and bidirectional perfusion on engineered cardiac tissues and showed reduced cell apoptosis, increased connexin-43 expression, stronger contractions, and decreased excitation threshold (58). Similarly, Shansky et al. employed a perfusion bioreactor to study paracrine effects of IGF-1 on avian skeletal muscle constructs and observed overexpression of muscle myosins (59). Brown et al. compared pulsatile and static perfusion of engineered cardiac tissues and showed that pulsatile flow led to improved contractile properties and excitability of cardiomyocytes (60). While these and other studies suggest beneficial roles of in vitro perfusion on the structure and function of engineered striated muscles, for physiologically dense muscle tissues, high shear stresses during interstitial perfusion may negatively affect viability of shear-sensitive muscle cells. Furthermore, removal of perfusion conditions at the time of implantation is likely to result in rapid tissue ischemia and death in vivo.

Recently, Okano's group reported a novel "vascularization" strategy whereby an underlying vascular bed was used to generate perfused vessel networks within engineered cardiac and skeletal muscle tissue sheets (61, 62). Specifically, co-cultured engineered sheets of neonatal rat cardiomyocytes and endothelial cells were laid on top of either perfused collagen micro-channels or an ex vivo vascular bed. The co-cultured cells showed high viability, formation of vascular structures under perfusion, and increased connectivity between underlying vascular bed and tissue sheets in the presence of bFGF. Theoretically, these vascularized cardiac tissues could be continually 'grown' by the repetitive stacking of additional cell sheets. Upon implantation, these tissue patches connected to host vasculature, resulting in increased viability compared to non-vascularized controls (62). While promising, it remains to be studied if this approach can enable in vitro growth of dense striated muscle tissues thicker than 150 μm.

USE OF BIOPHYSICAL STIMULATION TO PROMOTE ENGINEERED MUSCLE MATURATION AND FUNCTION

Engineering of highly functional striated muscle tissues will likely require extensive understanding of the muscle development, structure, physiology, functional adaptation, and injury repair mechanisms. Ideally, advanced bioreactors for muscle tissue engineering are expected to provide active monitoring of tissue viability, metabolism, and function, as well as controlled application of important biophysical cues present in healthy innervated muscle. In particular, controlled electrical stimulation (pacing) to induce active muscle contraction

and mechanical stimulation (stretch) to alter passive muscle loading are utilized to mimic the most important aspects of striated muscle work. Overview of bioreactor systems with capabilities for biophysical stimulation utilized for cardiac and skeletal muscle tissue engineering are listed in Table 2.

Mechanotransduction in Native and Engineered Muscle Tissues

Different regimes of mechanical loading have been shown to cause profound alterations in muscle structure and function by the activation of a variety of intracellular signaling cascades. In general, any change in the mechanical stress experienced by striated muscle cells leads to one of the following outcomes: cell elongation (preferential sarcomere addition in series), thickening (preferential sarcomere addition in parallel), elongation and thickening (sarcomere addition in both series and parallel), or apoptosis and death. In skeletal muscle, chronic active overload may lead to myofiber thickening (hypertrophy), while chronic passive stretch leads to myofiber elongation (63). In cardiomyocytes, the mechanosensitive response varies depending on the type of overload, pressure or volume, and can result in either physiological growth (characterized by both cell elongation and thickening), physiological or pathological concentric hypertrophy (cell thickening) and physiological or pathological eccentric hypertrophy (cell elongation) (14).

Mechanical loading affects muscle cell growth, differentiation, and morphology via various intracellular molecular pathways, some of which are depicted in Figure 3. In particular, static and cyclic stretch have been shown to upregulate secretion of different autocrine and paracrine factors (64, 65) including insulin-like growth factor I (IGF-I), adenosine mono- and tri-phosphate (AMP, ATP), reactive oxygen species (ROS), and prostaglandins (PG) (65, 66). In striated muscle cells, these cytokines activate a number of downstream effectors among which Phosphoinositide 3-kinase (PI3K), G-protein, Mitogen activated protein kinase (MAPK), calcium, and AMP activated kinase (AMPK) play the most important roles in muscle mechanotransduction (63, 65, 67, 68). Specifically, phosphoinositide 3-kinases play a major role in cell metabolism and directly regulate cell proliferation and differentiation. These enzymes can be activated by autocrine/paracrine action of IGF-I and other growth factors upon stimulation by mechanical stress (69). G-proteins (heterotrimeric guanine nucleotide binding proteins) associate with the cell-surface receptors and increase transcription of muscle-specific genes in cardiac and skeletal muscle cells subjected to mechanical strain to mediate cell growth and hypertrophy (11, 70, 71). Stretch-induced IGF-I and other cytokines act via G-protein coupled receptors (GPCRs) to activate mitogen activated protein kinases (MAPK) pathways known to play integral roles in muscle mechanotransduction (72, 73). MAPKs are divided into c-Jun NH2-terminal kinase (JNK), extracellular regulated kinase (ERK), and p38 families. The JNK family is also known as stress-activated protein kinases. Increased ERK and JNK phosphorylation was observed in response to mechanical stress in skeletal muscle cells, while p38 phosphorylation was either found to be unaffected, or increased for eccentric but not concentric muscle loading (67, 74). Positive growth and differentiation effects of IGF-I on skeletal and cardiac muscle were recapitulated in vitro where addition of IGF-I to culture medium improved structural and contractile properties of engineered muscle tissues (58, 59).

G-protein receptor activated phospholipase C (PLC) pathway is another major contributor to the mechanotransduction signaling in striated muscles that integrates with both the Ras-Raf and Calcium-mediated pathways (75). PLC activation of protein kinase C (PKC) isoforms has been shown to stimulate expression of cytoskeletal protein β-MHC leading to cardiomyocyte hypertrophy (76). Muscle mechanotransduction is also governed by intracellular calcium levels that are mainly regulated by calcium influx through stretch-activated channels (SAC), active force generation, and phospholipase pathways (65, 77, 78). Increased intracellular calcium levels can stimulate activity of PKC and Calmodulin-dependent protein kinase (CaMK) and profoundly affect proliferation and hypertrophy of developing cardiomyocytes (79). Stimulation of the calcium/calmodulin pathway can also activate calcineurin to induce translocation of cytoplasmic nuclear factor of activated T cells (NFATc) into the nucleus and enhance the transcription of a number of muscle-specific genes (e.g. MHC isoforms) leading to cardiac and skeletal muscle hypertrophy (64, 65, 80).

Mechanical Stimulation by Stretch

Due to essential roles that mechanical forces play in muscle growth, differentiation, and function, the ability to apply controlled patterns of mechanical stimulation is critical for promoting functional myogenesis in vitro. A variety of mechanical stimulation regimes have been utilized for muscle tissue engineering including static stretch (i.e., a single step of stretch at the beginning of culture), ramp stretch (i.e., continuous or discrete, step-by-step increase in stretch amplitude), and cyclic stretch (i.e., a periodic train of stretch pulses with a given shape, amplitude, frequency, and duration of intermittent pauses). In theory, these temporal regimes of mechanical stimulation can be applied along one (uniaxial), two (biaxial), or all planar tissue axes (multiaxial) (81) (Fig. 2), with uniaxial stimulation being the simplest and mostly utilized stimulation regime for engineering of striated muscle.

Studies that utilized mechanical stimulation for cardiac and skeletal muscle tissue engineering are summarized in Table 3. Vandenburgh et al. were among the first to utilize bioreactors with mechanical stimulation for culture and testing of cylindrically shaped striated muscle constructs. (82, 83). They and others (84, 85) found that both uniaxial static and cyclic stretch have beneficial effects on engineered muscle survival, alignment, hypertrophy, and differentiation, as well as glucocorticoid-induced skeletal muscular atrophy (86, 87). In a study by Candiani et al., application of a biomimetic stretch regime (with a 2-day steady ramp stretch to 3.3%, followed by cyclic 5-pulse, 0.5 Hz, 3.4% burst stretches for 2 min with 28 min intermittent breaks over a period of 10 days) to C2C12 cells induced an 8-fold increase in the expression of myosin heavy chain (MHC) protein compared to static control (88). More recently, Machingal et al. engineered skeletal muscle constructs using mechanically preconditioned muscle progenitor cells (by 10% stretch, 3 times per minute for the first 5 min of every hour, for 1 week). Two months post-implantation in a volumetric muscle loss injury model in mice, preconditioned constructs had superior biomechanical properties and induced accelerated and prolonged functional recovery following implantation (89, 90).

Interestingly, application of different mechanical stimulation regimes has been shown to affect the switch between the two skeletal muscle fiber phenotypes, namely, slow oxidative

(type I), which is economical in energy consumption and fatigue resistant, and fast anaerobic (type II), which is used when there is a large but short physical demand. Specifically, stretch or mechanical load upregulated type I and downregulated type II muscle fiber genes (e.g. slow MHC), and when the load was removed or reduced, the fibers reverted back to type II along with a reduction in slow MHC expression (11, 91). It is possible that the application of appropriate mechanical stimulation regimes within tissue culture bioreactors could allow engineering of skeletal muscle tissue with the desired fiber type composition, with the goal of better matching the native muscle phenotype present at the injury site.

Similar to studies with engineered skeletal muscle tissues, beneficial effects of mechanical stimulation on tissue structure, differentiation, and function have been also demonstrated for engineered cardiac muscle. Fink et al. investigated the effects of mechanical stimulation on engineered neonatal rat and chick cardiac muscle and found that 6 days of 20%, 1 Hz uniaxial cyclic stretch improved cardiomyocyte alignment, elongation, and hypertrophy yielding a 2–4 fold increase in contractile force amplitude (85). Akhyari et al. subjected human neonatal heart cells seeded in gelatin foam scaffolds to continuous cyclic stretch (20%, 1.3 Hz) for 14 days using the Bio-Stretch apparatus (81) and showed significant improvements in cell spreading, distribution, and proliferation (92). Birla et al. designed a novel bioreactor for stimulation of engineered cardiac tissues but was unable to show any positive effects of a short-term cyclic stretch (10% at 1 Hz up to 24 hours) on cardiac function (93). Recently, Tulloch et al. co-cultured endothelial cells with human pluripotent stem cell-derived cardiomyocytes and applied cyclic stretch (5% at 1 Hz) for 4 days. They found that synergistic action between mechanical loading and endothelial cell presence led to increased cardiomyocyte proliferation (94).

Of note is however that not all regimes of mechanical stimulation have positive effects on engineered striated muscle differentiation, as found by Boonen et al. who showed a decrease in myogenic regulatory factors and delay in sarcomerogenesis upon application of 2% uniaxial ramp stretch followed by 4% uniaxial intermittent dynamic stretch to C2C12 cells (95). Similarly, although not widely used, specific regimes of biaxial stretch have shown increased cell proliferation along with delayed activation and differentiation of satellite cells (96, 97). Taken together, while mechanical loading plays a vital role in myogenic growth and development, the optimal stimulation regimes for structural and functional maturation of engineered muscle remain to be determined and are likely to differ for different muscle types, scaffolds, and culture media formulations.

Electrical Stimulation

Continuous contractile activity of the beating heart muscle (initiated by firing of pacemaker cells) or working skeletal muscle (initiated by release of acetylcholine from motor neurons) is of critical importance for the maintenance of striated muscle cell viability, muscle tone and mass, and, ultimately, human mobility and life. The ability to replicate this activity in vitro requires that appropriate electrical stimulation regimen is applied to cultured cells or engineered tissues in a controlled and biomimetic fashion. Over the last 30 years, large numbers of in vitro and in vivo studies have shown beneficial effects of electrical stimulation on skeletal muscle cell fusion, alignment, hypertrophy, expression of contractile

proteins and myogenic markers, electrical excitability, and force generation (98–103). While most of the in vitro studies involved the use of standard monolayer cultures, few tissue culture bioreactors have been designed for long-term stimulation of 3D engineered muscle. For example, Donnelly et al. designed a tissue culture bioreactor with the ability to vary electrical stimulation parameters including frequency, pulse amplitude, and pulse width (104). At higher stimulation frequencies, they found that 2D monolayers of C2C12 cells showed higher rates of protein synthesis, while 3D muscle constructs displayed improved force generation. On the other hand, large amplitude electrical stimuli of more than $6 \times$ threshold value caused significant electrochemical damage in engineered muscle (103).

Specific patterns of muscle activity have been shown to determine the skeletal muscle fiber phenotype in seminal studies by Buller et al., who converted the fast into slow muscle fibers by cross-innervating them with slow fiber neurons (105). In vitro, Naumann et al., showed that different regimes of chronic electrical stimulation can modulate expression of MHC isoforms in primary rat myotubes and that changes from fast to slow fiber phenotype occur during stimulation with high frequency bursts (106). The fast-to-slow fiber type conversion has also been observed during application of chronic low frequency stimulation (CLFS) (107), presumably due to its similarity to neuronal stimulation patterns present in slow muscle fibers (108). The reversal of the fast-to-slow fiber type switch could be achieved by removing stimulation, denervation, administration of tetrodotoxin, stimulation at high frequency, and shortening of the muscle length (109). Still, slow-to-fast fiber type transition is more difficult to achieve due to lower work-to-rest ratio and random firing pattern of the fast muscle fibers (110). Lately, Guo et al. developed a functional human neuromuscular junction system by co-culture of human stem cell derived motoneurons and primary muscle cells (111). Such systems could be adapted to incorporate electrical stimulation, and when miniaturized, used for high-throughput drug and toxicology screening, disease modeling, and studies of neuromuscular synaptogenesis.

Similar to skeletal muscle cells, electrical stimulation of cardiomyocytes has been shown to promote cardiomyocyte ultrastructural organization, hypertrophy, alignment, excitability, and electrical coupling (112, 113). In 2D neonatal rat cardiomyocyte cultures, chronic electrical pacing (1–3 Hz for 2–4 days) stabilized action potential duration and conduction velocity as well as altered expression of ion channel and gap junctional proteins (114, 115). In 3D engineered neonatal rat cardiac tissues, 1 Hz electrical stimulation for 8–9 days yielded increase in cell elongation, volume fraction, expression of gap junctional protein connexin-43, excitability, and maximum tissue capture rate (112, 116). Sauer et al. and Chen et al. have shown that short term (90 s) and chronic (4 day) electrical stimulation can also promote cardiac differentiation of mouse ESC-derived embryoid bodies (EBs) through the generation of intracellular reactive oxygen species (ROS), suggesting the potential utility of electric stimulation for enhanced functional maturation of stem cell-derived cardiomyocytes (113, 117). Recently, Lieu et al. applied cyclic electrical stimulation (2.5V/cm, 1Hz for 14 days) to hESC-derived cardiomyocytes starting at 24–28 days of differentiation and showed compelling evidence for structural and electromechanical maturation of cells including appearance of robust sarcomeres, increased expression of excitation-contraction coupling and calcium cycling genes, increased potassium currents, hyperpolarization of resting potential, and increased calcium transient amplitude (118). Similarly, in recent studies by

Nunes et al., cyclic electrical stimulation of variable frequency (1–6 Hz increase for the first 7 days followed by steady 1 Hz for 14 days) promoted structural and functional maturation of human ESC-derived cardiomyocytes within 3D collagen-based tissue constructs (119). While it is almost certain that chronic electrical stimulation can provide significant benefits for differentiation and maturation of engineered striated muscles, the optimal stimulation regimes leading to physiological rather than pathological phenotypic changes remain to be identified (120).

Multimodal Stimulation

The native microenvironment of striated muscle is a complex source of various physical and biochemical signals, which to be fully reproduced in vitro, will require the design of multimodal bioreactors with the ability to simultaneously provide a combination of flow, electrical and mechanical stimuli. Feng et al. were the first to design a bioreactor capable of co-delivering electrical and mechanical stimulation to engineered cardiac muscle. They suggested that application of a 10 V electrical pulse at the beginning of each period of cyclic stretch (3.7%, 1 Hz) enhanced cardiomyocyte size and contractile properties (121). Using a multimodal bioreactor, Liao et al. simultaneously applied intermittent cyclic stretch (5% amplitude, 1 Hz for 1 h with 5 h of rest for total of 7 days) and electrical stimulation (4 V/cm) to tubular skeletal muscle constructs and measured higher fraction of cross-striated myotubes, expression of MHC proteins, and excitability compared to unstimulated cultures (122). In this particular case, combined electromechanical stimulation did not provide additional benefit compared to application of individual (electrical or mechanical) stimulation regimes. Barash et al. (123) and more recently Maidhof et al. (124) constructed bioreactors that delivered combined electrical stimulation (1 Hz) and media perfusion to engineered cardiac tissues and found that the bimodal stimulation improved cardiomyocyte structural and contractile properties. In a recent report, Kensah et al. designed a bioreactor that was capable of electrical stimulation, mechanical stimulation, perfusion, and real-time monitoring of tissue morphology and force production. In these bioreactors, collagen-based neonatal rat cardiac tissue constructs were subjected to mechanical stretch (10%, 1 Hz, for 7 days) and β-adrenergic stimulation. Expression of cardiac hypertrophy markers and contractile force amplitudes in stretched tissues were higher than those cultured in static conditions but lower than those observed under β-adrenergic stimulation alone or with mechanical stretch (125). Overall, multimodal bioreactors hold promise in providing the most biomimetic culture environment for growth and maturation of engineered striated muscles. However, with increased bioreactor complexity, susceptibility to failure increases and so do the difficulties in identifying the optimal regime of stimulation (110). In addition, translational potential of the entire approach may decrease due to potential regulatory and commercialization issues. All of these factors must be considered when designing a viable tissue engineering strategy for use in toxicology screening, drug development, and cell-based therapies.

CONCLUSIONS

Striated muscle tissue engineering has come a long way since the very first attempts to culture and maintain muscle cells in vitro. With better control of pH, temperature, and

sterility, improved mass transfer of nutrients and oxygen, application of electrical and mechanical stimulation, and ability to monitor cell differentiation and function in real time, sophisticated tissue engineering bioreactors will pave the way for generation of highly functional biomimetic muscle tissues. When developing a successful bioreactor system, one should have an in-depth knowledge of the native tissue biology and physiology and prioritize the design parameters in accordance with the aims of the study in order to strike a balance between the minimum required complexity and reliability. Understanding potential synergistic or antagonistic effects of different biophysical and biochemical stimuli on engineered muscle structure and function is of particular importance and is yet to be explored in detail. For the optimal tissue growth and maturation, operational parameters for different types of stimuli should be carefully selected and, if not physiologically or developmentally based, may be detrimental to cell health and/or cause compensatory pathological changes. These changes, often transient in nature and eventually followed by tissue deterioration, may be mistakenly interpreted as improved functional outcomes. Development of bioreactors that can support long-term tissue growth and provide continuous tuning of operational parameters based on real-time monitoring of tissue function and maturation could help avoid these issues. Recently developed tissue systems, such as microtissue systems or NMJ systems, show real promise in therapeutic applications, studying the physiopathology of tissue-specific diseases and even enhancing post-implantation integration and survival. We expect that in the years to come new advances in bioreactor technology will be critical for successful engineering of highly functional striated muscle tissues eventually leading to their clinical use for treatment of muscle disease and injury.

Acknowledgments

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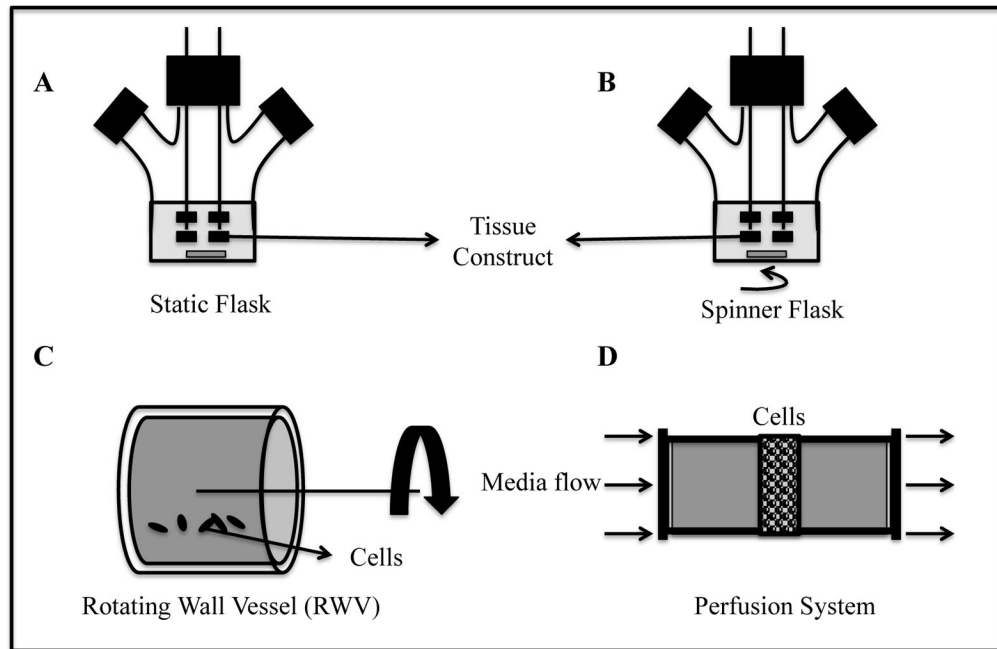
Fig. 1.
Bioreactors for engineering of striated muscle tissues. Static flask, A; Spinner flask, B. Tissue constructs are threaded onto stainless steel needles; Rotating Wall Vessel, C. Levitating tissue constructs experience "microgravity" condition. Arrow shows direction of vessel rotation; Perfusion bioreactor, D. Arrows shows the direction of media flow through tissue construct.
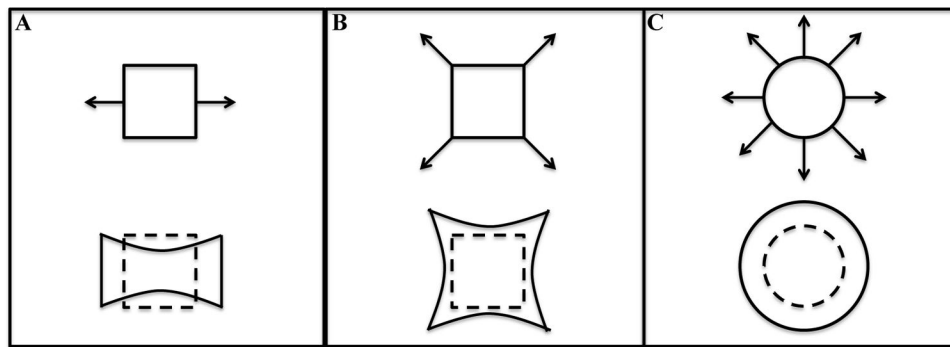
Fig. 2.
Examples of stretch regimes to be applied to engineered striated muscle: Uniaxial stretch, A;
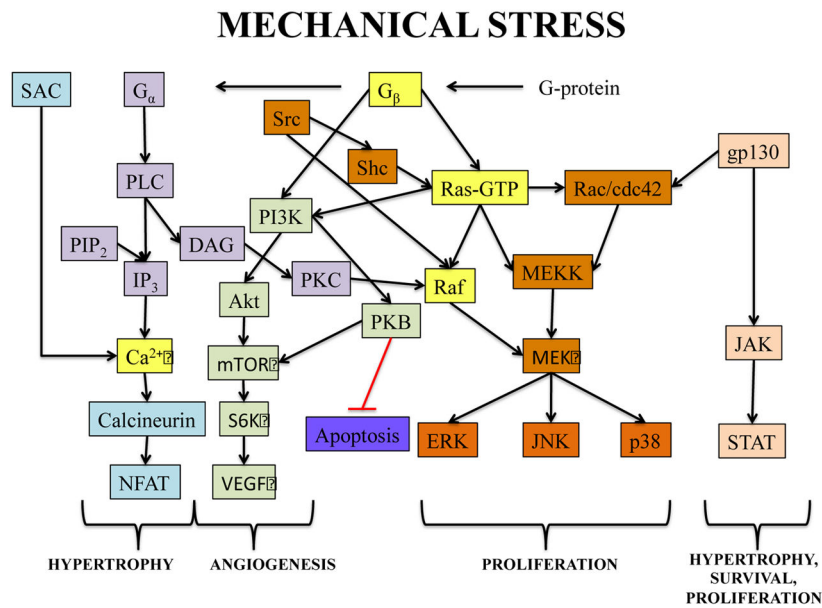Biaxial stretch, B; Multiaxial stretch, C.

MECHANICAL STRESS

Fig. 3.

Important mechanotransduction signaling pathways in striated muscle cells. Blue, Intracellular calcium-mediated signaling; Purple, Phospholipase C signaling; Orange, MAPK pathway; Green, PI3K pathway; Peach, JAK/STAT pathway; Yellow, Overlap between signaling pathways.

Table 1

Bioreactors with media mixing for engineering of striated muscles

Vessel Type	Type of Flow	Seeding Mechanism	Mass Transfer	Engineered Muscle	Ref.
Spinner Flask	Stirring/turbulent	Statically or dynamically on microcarrier beads or polymer scaffolds	Convection (recirculation)	Skeletal	(126)
				Cardiac	(30, 49)
Rotating Wall	Laminar	Statically or dynamically on microcarrier beads or polymer scaffolds	Convection (recirculation)	Skeletal	(52, 127)
				Cardiac	(49, 53, 128)
Perfused Chamber	Laminar	Statically or dynamically on polymer scaffolds	Convection (perfusion)	Skeletal	(57, 129)
				Cardiac	(56, 58, 130)

Table 2

Bioreactors with biophysical stimulation for engineering of striated muscles

Cell Source	Electrical Stimulation	Mechanical Stimulation	Perfusion	Ref.
Bioreactors for Cardiac Muscle Tissue Engineering				
ECCMs, NRCMs	–	+	–	(131)
NRCMs	–	+	–	(132) (93)
NRCMs	+	–	–	(116)
NRCMs	+	–	+	(123)
NRCMs	–	+	+	(60)
NRCMs	+	+	+	(125)
Mouse iPSC-CMs	+	+	+	(133)
Human Pediatric CMs	–	+	–	(92)
Human ESC/iPSC-CMs	–	+	–	(94) (133)
Human ESC/iPSC-CMs	+	–	–	(118, 119)
Bioreactors for Skeletal Muscle Tissue Engineering				
C2C12 Line	+	–	–	(104)
C2C12 Line	–	+	–	(88)
C2C12 Line	+	+	–	(122)
Rat MPCs + MDCs	–	+	–	(89) (90)
Human Muscle Cells	–	+	–	(134)
Human Muscle Precursor Cells	–	+	–	(135)

CM: Cardiomyocyte; ECCM: Embryonic Chick Cardiomyocyte; NRCM: Neonatal Rat Cardiomyocyte; hESC-CM: Human Embryonic Stem Cell-derived Cardiomyocyte; iPSC-CM: Induced Pluripotent Stem Cell-derived Cardiomyocyte; MPC: Muscle Progenitor Cell; MDC: Muscle Derived Cell.

Table 3

Use of bioreactors for mechanical stimulation of engineered striated muscle

Cell Source	Stretch Regime	Strain (%)	Duration	Outcome	Ref.
ECCMs, NRCMs	1.5 Hz cyclic	20	6 days	Hypertrophy, improved contractile function	(131)
NRCMs	1.34 Hz cyclic, continuous or 15 min/h	1, 3.5, 7	7 days	Increased tensile strength, CM and EC marker expression	(132)
NRCMs	1 Hz cyclic for 2h, 6h, or 24h per day	10	1 day	No functional differences among groups	(93)
Human Pediatric CMs	1.34 Hz cyclic	20	14 days	Improved CM proliferation, distribution, mechanical strength	(92)
Human ESC/iPSC-CMs	1 Hz cyclic	5	4 days	Increased CM proliferation, hypertrophy	(94)
Mouse + Human iPSC-CMs	1 Hz cyclic	10	7 days	Improved tissue formation, CM hypertrophy, contractile function	(133)
	Static ramp with 2% every 2nd day	0–23	14 days		
C2C12 Line	1 Hz for 1 hour every 6 hours	5 or 10 with 5% static pre-strain	2–14 days	Increased cell alignment, contractile proteins	(122)
C2C12 Line	5-pulse 0.5 Hz bursts for 2 min, then 28 min rest	6.7	10 days	Increased MHC expression in myofibers	(88)
Adult Rat MPCs/MDCs	0.05 Hz for 5 min every hour	10	7 days	Improved host recovery upon implantation	(89) (90)
Human Skeletal Muscle Cells	5-pulse 0.5 Hz bursts for 2 min, then 28 min rest	5, day 8–10 10, day 10–12 15, day 12–16	8 days	Increased myofiber diameter and density, decreased tissue stiffness	(134)
Human Muscle Precursor Cells	0.05 Hz for 5 min every hour	10	5–21 days	Improved force generation post-implantation	(135)

Table 4

Use of bioreactors for electrical stimulation of engineered striated muscle

Cell Source	Cyclic regime	Duration	Outcome	Ref.
NRCMs	2 ms pulses 5 V/cm, 1 Hz	8 days	Improved CM ultrastructure, alignment, contraction, excitability	(112)
NRCMs	2 ms pulses 4 V/cm, 1 Hz	9 days	Increased CM volume fraction, elongation, connexin-43 expression	(116)
Human ESC-CMs	2.5 V/cm, 1 Hz	14 days	Enhanced CM sarcomerogenesis, Ca handling, electrical properties	(118)
Human ESC/iPSC-CMs	3–4 V/cm, 1–6 Hz for 7 days, then 1 Hz	7–21 days	Promoted cell organization, conduction velocity, Ca^{2+} handling	(119)
C2C12 Line	5 V/cm, 1 Hz for 1 hour every 6 hours	7 days	Enhanced formation of striations, contractile protein expression	(122)
C2C12 Line	12.5, 25, 50 V/cm, 4 pulses at 10 or 100 Hz, then 3.6 s rest	7 days	Higher pulse amplitude increased force production and excitability, higher pulse frequency increased the rate of protein synthesis	(104)