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Key Words:	Skeletal muscle bioengineering, muscle stem cell, muscle hypertrophy, electrical stimulation, mechanical overload

REVIEW

Mimicking exercise in three-dimensional bioengineered skeletal muscle to investigate cellular and molecular mechanisms of physiological adaptation.

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Abstract

Bioengineering of skeletal muscle *in-vitro* in order to produce highly aligned myofibres in relevant three dimensional (3D) matrices have allowed scientists to model the *in-vivo* skeletal muscle niche. This review discusses essential experimental considerations for developing bioengineered muscle in order to investigate exercise mimicking stimuli. We identify current knowledge in the use of electrical stimulation and co-culture with motor neurons to enhance skeletal muscle maturation and contractile function in bioengineered systems *in-vitro*. Importantly, we provide a current opinion on the use of acute and chronic exercise mimicking stimuli (electrical stimulation and mechanical overload) and the subsequent mechanisms underlying physiological adaptation in 3D bioengineered muscle. We also identify that future studies using the latest bioreactor technology, providing simultaneous electrical and mechanical loading and flow perfusion *in-vitro*, may provide the basis for advancing knowledge in the future. We also envisage, that more studies using genetic, pharmacological and hormonal modifications applied in human 3D bioengineered skeletal muscle may allow for an enhanced discovery of the in-depth mechanisms underlying the response to exercise in relevant human testing systems. Finally, 3D bioengineered skeletal muscle may provide an opportunity to be used as a pre-clinical *in-vitro* test-bed to investigate the mechanisms underlying catabolic disease, whilst modelling disease itself via the use of cells derived from human patients without exposing animals or humans (in phase I trials) to the side effects of potential therapies.

Introduction

In this review the authors discuss how recent advances in skeletal muscle bioengineering systems *in-vitro* may provide a physiologically relevant model to investigate adaptation to exercise. Recent evidence suggests that *in-vitro* bioengineered systems can provide relevant three-dimensional (3D) human models of skeletal muscle via the use of cells derived from human muscle biopsies seeded within biological scaffolds under tension that mimic native skeletal muscle more accurately (Martin et al., 2013; Powell et al., 2002). These novel systems may also provide an experimentally pliable environmental niche *in-vitro*, for example via genetic modification and/or pharmacological manipulation, to investigate underlying molecular mechanisms of physiological adaptation than is currently available from *in-vivo* human study. Furthermore, in conjunction with the advances in the use of bioreactors (Donnelly et al., 2010; Huang et al., 2005; Player et al., 2014), bioengineering can provide technological advancements that enable the physiological simulation of skeletal muscle contraction by means of electrical stimulation and/ or mechanical loading for sustained/repetitive periods. The potential advantage of this *in-vitro* experimentation is that studies are not limited by infrequent sampling due to animal number or repeated bioptic sampling in humans. Furthermore, together with the use of bioreactors, experiments could potentially be higher-throughput, avoid ethical constraints of animal or human study *in-vivo* and therefore help elucidate fundamental mechanisms of physiological adaptation in relevant human systems.

Overview

This review will therefore include a discussion of the current advances in the bioengineering of skeletal muscle, outlining important experimental research considerations when attempting to create the most physiologically relevant 3D skeletal muscle constructs *in-vitro*. The use of electrical stimulation to provide a surrogate neural input for muscle contraction, in order to create the most native skeletal muscle phenotype and optimize contractile properties will also be of focus. Furthermore, we will outline the current understanding in the use of electrical stimulation to mimic chronic exercise and the assessment of the mechanisms leading to adaptation in 3D bioengineered skeletal muscle. Also, we will touch upon recent advances in the co-culturing of motor-neurons with

muscle derived cells in 3D bioengineered skeletal muscle. Where investigations have attempt to form neuromuscular junctions and establish the optimal environmental niche *in-vitro* that mimics contractile properties and displays the characteristics of mature adult skeletal muscle *in-vivo*. Furthermore, we will provide an overview of the use of mechanical overload/stretch regimes in 3D bioengineered skeletal muscle. Finally, we suggest that the use of novel bioreactors allowing simultaneous mechanical and electrical stimulation will potentially provide the most physiologically relevant *in-vitro* exercise systems in future study. Furthermore, 3D bioengineered skeletal muscle could be used as a pre-clinical *in-vitro* test-bed to investigate the cellular and molecular mechanisms underlying catabolic disease without exposing animals or humans (in phase I trials) to the side effects of drugs/therapies and therefore the potential identification of novel therapeutic strategies to counteract muscle wasting.

Experimental research considerations for deriving skeletal muscle cells and bioengineering 3D skeletal muscle

Adult muscle fibres past birth are terminally differentiated or post-mitotic. As a result, muscle turnover, regeneration and repair processes are dependent upon residing adult muscle stem cells/satellite cells and their ability to undergo cellular division. Under various biochemical, mechanical and hormonal cues, satellite cells are activated (their immediate progeny - termed myoblasts). They then proliferate, migrate, and fuse to muscle fibres to provide additional/replace myonuclei and repair the existing fibres [reviewed in (Sharples and Stewart, 2011; Sharples et al., 2016)]. It is therefore possible to isolate these cells from bioptic sampling via explant (Martin et al., 2013), enzymatic digest (Danoviz and Yablonka-Reuveni, 2012), or a combination of the two methods (Owens et al., 2015). Cells can subsequently be expanded under high serum conditions and stored for long periods under liquid nitrogen for future experimentation. Studies have also shown that once skeletal muscle stem cells have been isolated from primary animal (Juhas and Bursac, 2014; Mudera et al., 2010; Smith et al., 2012) and human tissue (Martin et al., 2013; Powell et al., 2002) and placed under uniaxial tension within representative extracellular matrices, multinucleated fibres can form in an aligned structure in parallel (Breslin and O'Driscoll, 2013; Martin et al., 2013). This is in

comparison with monolayer cultures that have no mechanical stimuli/tension, and myotubes form in seemingly spontaneous and even swirling formations as they become larger (Figure 1 A & B). Furthermore, as skeletal muscle contraction is fundamental to skeletal muscle function, both spontaneous (Bursac et al., 1999) and electrically induced contraction of monolayer cultures (Brevet et al., 1976; Kawahara et al., 2007; Thelen et al., 1997; Wehrle et al., 1994) results in non-uniaxial force production, as the fascicles are not aligned in parallel, whereas 3D culture systems allow for the functional measurement of force in aligned skeletal muscle fibres (Dennis and Kosnik, 2000; Khodabukus et al., 2007; Martin et al., 2015). Furthermore, contractions (spontaneous or induced) can cause the myotubes to pull off monolayer cell culture dishes and therefore time in culture and/or maturation of myotubes can therefore be enhanced in bioengineered skeletal muscle as the myotubes are supported by a 3D matrix that allows contractile activity. It has therefore been hypothesised that *in-vitro* monolayer models may therefore fail to accurately biologically mimic *in-vivo* muscle, whereas 3D muscle systems *in-vitro* may replicate the structure and niche of *in-vivo* skeletal muscle more closely (Seale and Rudnicki, 2000; Sharples and Stewart, 2011). However, despite the more relevant cellular architecture and extra-cellular matrix composition in 3D bioengineered skeletal muscle constructs, there are still only a few direct comparisons with two-dimensional monolayer cultures with respect to the cellular and molecular adaptation to exercise related cues such as electrical stimulation or mechanical stretch. Indeed, there have been several studies investigating electrical stimulation in isolation in monolayer cultures only (Brevet et al., 1976; Kawahara et al., 2007; Thelen et al., 1997; Wehrle et al., 1994), as well as mechanical stretch (Chang et al., 2016; Chen et al., 2013; Kook et al., 2008; Kumar et al., 2004; Pardo et al., 2011; Vandenburgh and Kaufman, 1979). There have also been studies to investigate electric stimulation in 3D bioengineered skeletal muscle (Cheng et al., 2016; Donnelly et al., 2010; Hinds et al., 2011; Huang et al., 2006; Juhas et al., 2014; Langelaan et al., 2011; Park et al., 2008; Rangarajan et al., 2014; Stern-Straeter et al., 2005) and 3D studies that include mechanical stretch (Heher et al., 2015; Player et al., 2011; Player et al., 2014; Powell et al., 2002; van der Schaft et al., 2011). To the authors knowledge, there are only few comparisons of monolayer versus 3D bioengineered skeletal muscle with the most notable study suggesting that after electrical stimulation, high voltage stimulation (2-2.5 V/mm) could

evoke considerable cell death in the monolayer mouse myoblast C2C12 cell line, whereas 2.5 V/mm was shown to be optimum versus 1.25 and 5 V/mm for improved contractile properties (greater peak force and excitability) within 3D bioengineered muscle (Donnelly et al., 2010). Despite this, electrical stimulation regimes in monolayer have broadly mimicked a cell signalling response to exercise, such as increased AMPK (Nedachi et al., 2008), as well as enhanced glucose metabolism (uptake and oxidation) and complete fatty acid oxidation (Nikolic et al., 2012) in response to continuous low-frequency stimulation. High-frequency stimulation (to C2C12 myotubes) also results in an increase in protein synthesis (Donnelly et al., 2010), both of which are well defined responses observed in skeletal muscle tissue following *in-vivo* aerobic (AMPK) and resistance exercise (protein synthesis) respectively. Therefore, the most suitable *in-vitro* model warrants further investigation before monolayer cultures are simply discarded for the determination of the mechanisms underlying exercise mimicking stimuli over 3D *in-vitro* systems. This is particularly important given the greater time and cost required for creating such 3D systems. Additionally, there are considerable technical challenges requiring unique expertise to create and undertake exercise mimicking stimuli in both monolayer and 3D bioengineered muscle *in-vitro.* An in-depth discussion of the response of skeletal muscle cells in 3D culture to electrical and mechanical overload stimuli are detailed later in this review.

Although animal models may allow for elucidation of underlying molecular mechanism due to the ability to knock-out or overexpress specific target genes (Bodine et al., 2001; Jørgensen et al., 2005; Leick et al., 2009; Leone et al., 2005; Shima et al., 1998), 3D bioengineered 'mini-muscles' may be of further advantage in certain situations. Indeed, animal studies can be extremely expensive, have relatively low throughput, and can sometimes be criticised for the lack of relation to human populations. Equally, although there is often limited practicality of such trials, human studies have been utilised to investigate mechanisms of adaptation to exercise (Coffey and Hawley, 2007; Drummond et al., 2008; Holloszy, 1973; Hood, 2009; Joseph et al., 2006). However, these experiments may also be extremely costly, time consuming, and are somewhat limited to fixed quantity biopsy collections (due to ethical considerations), therefore limiting the number of time points for sampling and therefore important information on the temporal regulation may not be

achievable. In addition, once specific cellular and molecular targets associated with exercise adaptation *in-vivo* are determined, the regulatory pathways responsible for the precise control of these systems cannot always be investigated, as genetic modification or pre-clinical drug therapy cannot be undertaken *in-vivo* in human participants. However, muscle derived cell isolation procedures from human muscle biopsies are becoming more routine under local anaesthetic within academic research environments. Therefore, once these techniques are well established, 3D muscle systems can make use of cells expanded from one or two biopsy events, and allow for development of several constructs to examine multiple time points as oppose to taking numerous biopsies over an acute time course from a single human subject. This is despite isolated primary human muscle cells being programmed to undergo minimal rounds of proliferation before they senesce due to their drive to differentiate (O'Connor et al., 2009). However, even after 4-5 passages, enough primary human cells can be derived from a single biopsy (approximately 5-15 million cells dependent on biopsy size due to technique e.g. needle biopsy yields 20-30 mg whereas a conchotome biopsy can yield 100-250 mg), with bioengineered human muscles only requiring 200-400 thousand cells seeding density to make mature and aligned 3D muscle fibres *in-vitro* over a 2-3 week period (Martin et al., 2013). The use of bioengineered systems may also permit the deeper investigation into mechanistic properties (as discussed above) and responses to various simulated exercise regimens in tightly control environmental conditions (e.g. prior nutritional status) through mechanical and/or electrical stimulation.

Muscle cells must first proliferate then physically fuse to differentiate into multinucleated fibres and importantly be orientated in fascicles that will allow uniaxial contraction. In order for this to occur, the temporal requirements of nutrients, growth factors (both endogenous and exogenous), matrix type and composition and the mechanical signal given by the custom designed culture chamber are of primary concern when bioengineering skeletal muscle and have been extensively reviewed recently (Khodabukus and Baar, 2016) therefore not discussed in the current review. The majority of literature within this area looking to investigate mechanistic responses to exercise stimuli utilize either rodent self-assembling fibrin or pre-assembled collagen gels, using electrical stimulation and ramp, static or

cyclic mechanical stretch to investigate the cellular and molecular response to acute contraction or mechanical overload. There are numerous studies investigating the use of non-biological, non-biodegradable or synthetic materials/scaffolds. However, biological scaffolds such as collagen or fibrin have more *in-vivo* like mechanical properties (e.g. stiffness) (Heher et al., 2015) and therefore have been used much more frequently to investigate physiological adaptation to electrical and mechanical stimuli to mimic *in-vivo* muscle contraction. Furthermore, even synthetic scaffolds that are biodegradable are generally (but not exclusively) used for engraftment of bioengineered muscle to treat muscle injury or disease and therefore the use of non-biological scaffolds are not discussed in the present manuscript and are reviewed elsewhere (Mertens et al., 2014). The majority of studies investigating exercise mimicking stimuli include *in-vitro* models that are pre-cast/assembled matrices or self-assembling constructs. As alluded to above, the former includes biological scaffold materials (such as collagen), where cells are seeded into the collagen matrix often in pre-cast molds, which are then chemically polymerised and cast into position under tension (Figure 1E-G). Where self-assembling constructs, originally known as myooids (Dennis and Kosnik, 2000) use laminin and/or fibrinogen pre-polymerised matrices, where contraction from cells adhering to the matrix grown in high serum (e.g. 10-20%) media for a period of 2 days, lift and roll the thin polymerised fibrin/laminin gel from the surface of a silicone coated plate into a muscle 'bundle' towards the points of tension that are supplied by pre-prepared aligned sutures under strain from opposing aligned sterilised stainless steel pins (Figure 1C, D). Together with this mechanical stimulus, upon initial self-assembly cells are switched to low serum media (typically 2% sometimes with exogenous IGF-I in primary/human cells, or without in the C2C12 cell line that spontaneously differentiate following serum withdrawal) to enable differentiation. After 2 days of myotube formation, fibrin constructs are usually switched to 7% serum for a further 8-14 days to enable myotube maturation (Khodabukus and Baar, 2009).

The early use of electrical stimulation to investigate skeletal muscle contraction *in-vitro*

Both monolayer and tissue engineered muscle myotubes have been shown to spontaneously twitch during formation (Bursac et al., 1999). To enable sustained and repeated contraction, researchers have

utilised electrical stimulation of skeletal muscle cultures in monolayer (Brevet et al., 1976; Kawahara et al., 2007; Thelen et al., 1997; Wehrle et al., 1994) and 3D bioengineered constructs (Cheng et al., 2016; Donnelly et al., 2010; Hinds et al., 2011; Huang et al., 2006; Juhas et al., 2014; Khodabukus and Baar, 2012; Langelaan et al., 2011; Park et al., 2008; Rangarajan et al., 2014; Stern-Straeter et al., 2005). Indeed, in 1976, Brevet and colleagues first developed a monolayer model of electrical stimulation to model skeletal muscle contraction *in-vitro*. They isolated primary cells from the breast of 12-13 day old chick embryos. Cells were grown to confluence and differentiated for 4-5 days in low serum media to enable the fusion of the muscle derived cells into myotubes. They subsequently provided electrical stimulation over a 34h period (0.6 s train of 10- to 20-ms biphasic pulses, every 4 seconds making the stimulation approximately 7 hours in total with the remaining time as rest) (Brevet et al., 1976). Even though the authors did not directly measure the effects of electrical stimulation on morphological changes (myotube number and size), and the stimulatory regimes in frequency and duration were more analogous to slow twitch fibre recruitment patterns, electrical stimulation was sufficient to increase total myosin accumulation by an average of 39% and contractile protein synthesis by 21% versus unstimulated controls. To the authors knowledge, it was not until nearly 2 decades later that the effects of alternative chronic electrical stimulatory regimes on fibre type properties within skeletal muscle cells isolated from newborn rat hindlimbs (encompassing mixed fibre types) was conducted in an attempt to mimic both slow and fast twitch activation patterns (Naumann and Pette, 1994). Specifically, electrical stimulation duration was maintained for 20 days at bursts of 250 ms, every 1, 4 and/or 100 s with pulse frequencies of either 15, 40 and 100 hz. Indeed, it was suggested that 250 ms duration of stimulatory bursts, regardless of pulse frequency (15 Hz, 40 Hz or 100 Hz), repeated either every 1 or 4 s (burst frequency) induced an increase in the abundance of slow myosin heavy chains whereas high frequency 250 ms bursts, even at 100Hz, but with bursts repeated less often (every 100 s), enhanced the expression of faster myosin heavy chains but not slow isoforms. The authors therefore suggested that the increased bursts rather than pulse frequency resulted in a slower muscle phenotype and higher pulse frequencies with less bursts were advantageous for evoking fast fibre formation (MYHCI). With the exception that, following an increased number of bursts (every 1 to 4s) at higher pulse frequencies (100 Hz), slower fibre

formation was still predominant. These stimulatory regimes somewhat mimicked what is observed in *in-vivo* skeletal muscle after aerobic exercise (e.g. more repetitive/continuous contraction, akin to 1 or 4 s bursts in 3D muscle) where lower force contractions (affiliated to pulse frequencies of 15 or 40 Hz *in-vitro*) elicit slow fibre type adaptation vs. less frequent (bursts every 100s) but more forceful contractions (e.g. 100 Hz pulse frequency) that elicit faster fibre formation mimicking resistance type exercise. This group then went on to investigate the effects of higher number of bursts within the regimes (14 days of 250 ms trains, 40 hz, every 4 s) on muscle cells isolated from different adult rat muscle groups possessing different starting fibre-types (*soleus,* slow; *tibialis anterior,* fast) (Wehrle et al., 1994). The protein abundance for the MYHCI isoform, was found to be highest in cells derived from slow-twitch soleus muscle and lowest in the cells derived from fast-twitch tibialis anterior. Therefore, this early monolayer work *in-vitro* suggested that the fibre type of the originating muscle cells and the electrical stimulation regime would be an important consideration in enabling suitable electrical stimulation regimes in 3D bioengineered skeletal muscle. Since these studies it has now been observed in 3D bioengineered muscle that contractile properties and MHYC content are representative of the originating fibre type of the bioptic tissue (Huang et al., 2006). Fibrin self-assembling bioengineered skeletal muscle myooids using primary muscle derived cells originating from slow soleus or fast tibialis anterior muscle were matured over 14 days and contractile properties assessed via single twitch and tetanic electrical stimuli. Here, cells originating from slow soleus muscle had altered contractile dynamics, with 30% slower half-relaxation times (1/2RT) and time to peak tension (TPT), as well as reduced total MYHC content compared with muscle constructs using cells originating from the fast tibialis anterior (Huang et al., 2006). More recently, these data were confirmed in order to elucidate the biochemical and molecular mechanisms for these fibre type specific observations in 3D muscle. Following 10 days of construct maturation (2 days high serum, 8 days low serum), both single twitch and tetanic electrical stimulation was implemented to assess contractile properties. Here, TPT and 1/2RT were slower in constructs made with cells isolated from primary (C57BL/6) mice soleus muscles compared with the tibialis anterior (Khodabukus and Baar, 2015). These soleus 3D constructs were also more fatigue resistant and contained higher levels of the mitochondrial proteins (SDH and ATP synthase), the fatty acid transporter, Carnitine

palmitoyltransferase 1 (CPT-1) as well as increased content of beta oxidation enzymes (LCAD and VLCAD), alongside lower phosphofructokinase (PFK) activity, representative of lower glycolytic flux and oxidative slow fibres. Muscle-fibrin constructs made from cells isolated from the fast tibialis anterior had increased fast MYHC and fast troponin C, I, and T isoforms, together with increased levels of sex determining region (SRY)-box 6 (SOX6) and the six transcriptional complex (STC) proteins, EYA transcriptional coactivator and phosphatase 1 (Eya1) and Six4 (Khodabukus and Baar, 2015). Indeed, it has previously been observed that elimination of Six1, Six4, and the cofactor Eya1 can prevent fast-twitch muscle fibre formation (Grifone et al., 2004; Niro et al., 2010; Richard et al., 2011). Therefore, an increase in these proteins would serve to improve fast-fibre formation. Taken together, this data suggests that cells remember or possess a memory of the niche from which they were derived, in this instance the fibre type [reviewed in (Sharples et al., 2016)]. Therefore, originating fibre types and donor source of isolated muscle derived cells should be an important consideration when attempting to engineer skeletal muscle *in-vitro* in order to enable the investigation into the response to exercise stimuli *in-vitro*.

Acute electrical stimulation to assess basal contractile properties of bioengineered skeletal muscle *in-vitro*

Together with an appreciation for the origin of muscle derived cells and the subsequent maturation of 3D bioengineered muscle, researchers have also been required to validate the functional performance/contractile properties of the self-assembling 3D myooid constructs in order to establish their similarities and differences vs. *in-vivo* tissue and therefore their potential utility in future research to investigate chronic stimulation and subsequent physiological/molecular adaptation. The first studies to do this provided single twitch and tetanus electrical stimulation via custom-made transducers to self-assembling laminin myooids produced from primary rat muscle derived cells that had been allowed to differentiate and mature over 15 days and contractile properties assessed over the ensuing period of 50 days (Dennis and Kosnik, 2000; Dennis et al., 2001). It was shown that when the 3D muscle myooids contracted spontaneously, they produced approximately 25 μN of force. When

stimulated electrically they produced a peak twitch force of 320 μN and a tetanic force of 575 μN (Dennis et al., 2001). Furthermore, by increasing the proportion of myogenic cells to 88% vs. non-myogenic cells via a pre-plating technique during cell isolation from bioptic material (despite authors not stating the average myogenic purity without pre-plating, which is typically between 40-75%), it was later shown that after 14 days of maturation, these myooids with myogenically enriched cell populations could produce a tetanic force of 805 μN using fibrin as the matrix within the self-assembling constructs (Huang et al., 2005). Indeed, all of the authors above also observed qualitatively similar contraction profiles to adult muscle, specifically the general appearance of a positive force-frequency relationship (depicted in Figure 2) and normal length-tension relationships, as well as normal metabolic profiles (Baker et al., 2003; Dennis and Kosnik, 2000; Huang et al., 2005). Furthermore, where the enriched myogenic population were used, twitch-to-tetanus ratios of 2.5 were observed, which is close to adult muscle range of 3 to 5 (Huang et al., 2005). Also, the specific force (force relative to size of the muscle) of these constructs was 36.3 kN/m^2 which is similar to 44 kN/m^2 in the soleus muscle of 1-day-old Wistar rats (Close, 1964), albeit the specific force was not as large as 260 kN/m^2 that has been observed in adult skeletal muscle (Urbanchek et al., 2001). However, the specific force of 36.3 kN/m^2 reported by Huang et al., (2005) using myogenically enriched fibrin constructs was 12.5-fold higher than the 2.9 kN/m^2 previously described using laminin myooids unenriched with primary derived muscle cells (Dennis and Kosnik, 2000). Furthermore, myotube size in these constructs was typically 10 μm in diameter (Huang et al., 2005), similar to aneural rat primary myotubes (Wilson and Harris, 1993; Wilson et al., 1988). However, this was around 10-fold smaller than adult human muscle fibres which are approximately 100 μm. Indeed, it has previously been reported that when muscles are denervated *in-utero*, aneural myotubes fail to develop into fully adult myofibre phenotypes, perhaps contributing to this discrepancy (Fredette and Landmesser, 1991; Harris et al., 1989; Wilson and Harris, 1993; Wilson et al., 1988). Therefore, as the constructs are essentially aneural, it was suggested by these authors that perhaps more chronic innervation of the bioengineered muscle would enable maturation from aneural myotubes to more adult like fibres (Huang et al., 2005).

Chronic electrical stimulation of bioengineered skeletal muscle *in-vitro*

Following initial experiments described above exploring the contractile properties of 3D bioengineered muscle in response to acute electrical stimulation, investigators have implemented the use of electrical stimulation regimes over more chronic periods to enable more mature adult phenotypes and improve functional contractile properties. For example, after 14 days of maturation followed by 14 days of chronic low frequency electrical stimulation (5 pulses at 20 Hz every 4 seconds, pulse width 1.5 ms and voltage 5V) resulted in improved contractile dynamics with an average 15% longer TPT and a 14% increase in 1/2RT duration in the fibrin self-assembling myooid constructs with primary cells derived from the rat fast muscle tibialis anterior (Huang et al., 2006). However, this observation occurred without a change in the total amount of force produced. Furthermore, TPT and 1/2RT were not increased in 3D constructs made from cells derived from the slow rat soleus, although the same 14 day stimulation was able to increase the force produced in the slow soleus 3D bioengineered constructs by 80% (Huang et al., 2006). Furthermore, Donnelly *et al.,* (2010) undertook 7 days of electrical stimulation of bioengineered skeletal muscle using the same fibrin self-assembling myooids as above, yet with a combination of 90% myogenic population (C2C12's mouse cell line) and 10% 3T3 fibroblast cells to model a mixed population of enriched primary isolated muscle derived cells yet using commercially available cell lines. A regime of 4 pulses x 0.1 ms pulse width delivered in a 400 ms train followed by 3.6 s recovery for a 7 day period was undertaken at either 1.25 V/mm, 2.5V/mm and 5 V/mm. Indeed, 1.25 V/mm stimulation over 7 days resulted in increased force yet lower excitability, shown by an increased rheobase (where rheobase refers to the electric field required to produce 50% peak twitch force at a given pulse width, therefore, a decrease in rheobase is a measure of increased excitability). Stimulation at 2.5V/mm increased force production and excitability. However, force and excitability was decreased at 5 V/mm versus relevant controls. This suggested that slower-moderate voltage stimulation was required to improve functional performance of 3D bioengineered muscle and indirectly suggested that the improved maturation of 3D bioengineered muscle was as a consequence of electrical stimulation (albeit at lower voltages) versus controls. It is important to note that myotube diameter was not

investigated within this study to ascertain if chronic electrical stimulation could produce increases above 10 μm observed in aneural bioengineered muscle discussed above, in order to move towards a more adult muscle fibre phenotype of closer to 100 μm fibre diameters. While this study established that 5 V/mm could cause decreased function as a consequence of electrochemical damage and established that 2.5 V/mm was optimum, researchers also wished to identify the optimum pulse widths especially given that *in-vivo* force of skeletal muscle increases as a greater number of motor units are activated (Henneman et al., 1965) and this can be modelled *in-vitro* by increasing pulse width or amplitude (Dennis and Dow, 2007; Dennis and Kosnik, 2000; Dennis et al., 2001). Indeed, even in the presence of tetrodotoxin induced-denervation that blocks voltage-gated sodium channels, pulse widths greater than 0.5 ms have been shown to lead to calcium influx suggesting that a longer pulse width, for example that of 1.5 ms used in the study above by Huang et al., (2006), may not be physiological (Cairns et al., 2007). Investigators in 2012 attempted to investigate the optimum pulse width to evoke increases in force production and excitability in 3D bioengineered skeletal muscle (90% all myogenic population C2C12's plus 10% 3T3 fibroblast cells) after 24 hrs of continuous electrical stimulation. Here, there was no significant increase or decrease in force when pulse widths were increased from 0.25 to 1, 4, 9 and 16 ms (Khodabukus and Baar, 2012). Furthermore, because excitability (refers to the ability to initiate and propagate depolarisation in skeletal muscle) is an important function of skeletal muscle contraction, investigators also wished to identify the excitability following different voltages and currents by determining rheobase (defined above). Indeed, acute single twitch and tetanus stimulation at a pulse amplitude greater than six-times rheobase, resulted in a reduction of 50% peak force (measure of maturity), impaired contractile dynamics shown by a 2.4-fold slowed half relaxation time (dynamics) and a 58% increase in fatigability. In comparison 4 x rheobase (pulse widths ranging from 1 to 4 ms) resulted in the largest tetanic force, improved dynamics and reduced fatigability without electrochemical damage (Khodabukus and Baar, 2012). Finally, continuous stimulation for 24 h induced increases in force production when the electric field was greater than 0.5 V/mm regardless of the pulse width i.e. electric fields at 0.7, 1 and 1.4 V/mm resulted in a 2.5-fold increase in force (0.30 vs. 0.67 kN/m^2) versus controls. In this investigation, the

mTOR inhibitor rapamycin was able to prevent 40% of the increase in force observed as a result of 24 hrs electrical stimulation. However, there were also no observable changes in downstream translation initiator, p70s6K activity, alongside no increase in total MYHC suggesting no changes in muscle size. Therefore, the increase in force observed after 24 hrs of stimulation was perhaps more likely due to cytoskeletal rearrangement rather than increases in muscle size. This data combined suggest overall that pulse widths ranging from 1 to 4 ms, voltages of 0.7-2.5 V/mm and electric fields at 4 x Rheobase are required to elicit the most advantageous maturity (assessed via force), improved force dynamics (assessed via TPT and 1/2RT) and improved resistance from fatigue in 3D bioengineered skeletal muscle.

Chronic electrical stimulation to mimic exercise regimes in 3D bioengineered skeletal muscle *in-vitro*

Khodabukus et al. (2015) was one of the first studies to attempt to mimic a continuous exercise stimuli and investigate the physiological, biochemical and molecular adaptation in 3D bioengineered skeletal muscle. This was achieved via altering contraction duration (0.6, 6, 60, and 600 sec) of 14 days of stimulation in 3D bioengineered muscles created from primary muscle derived cells from both the soleous and tibilias anterior of C57BL/6 mice, while using a constant pulse frequency of 10 Hz. This regime was an attempt to optimise slow fibre formation that would mimic more closely chronic endurance/aerobic exercise *in-vivo* (Khodabukus et al., 2015). The authors suggested that all contraction durations evoked a similar slowing of TPT and similar increases in total MYHC content and a reduction in total fast myosin. Specifically, the greatest reductions in fast MYHC IIx were observed with contraction durations of 60 and 600 seconds as well as slower 1/2RT's in these conditions. Finally, all regimes induced an oxidative fibre type phenotype via an increased capacity for glucose transport (GLUT4), metabolic activity (SDH and ATPsynthase), mitochondrial biogenesis (PGC, MEF2) as well as fat oxidation (CPT-1).

Therefore, chronic low frequency stimulation of contraction durations of 60 and 600 seconds appear most advantageous to evoke similar metabolic adaptation to what occurs *in-vivo* after aerobic

exercise. However, at present, there are limited chronic stimulation studies in 3D bioengineered skeletal muscle longer for periods longer than 14 days, and/or that mimic resistance exercise type stimulation regimes via higher frequency contractions, in order to investigate subsequent adaptation with respect to anabolic/catabolic signaling pathways or gene regulatory systems. It is worth mentioning that Donnelly et al. (2010) undertook higher frequency contractions in myotubes (100 Hz) in monolayer and observed a greater increase in total protein synthesis following higher 100 Hz stimulation vs. lower 10 Hz using the same stimulation protocol (pulses delivered in 400 ms trains with 3.6 s recovery). Therefore, future experimentation in 3D bioengineered skeletal muscle should investigate higher frequency stimulation protocols interspersed with recovery to mimic chronic resistance exercise to evoke increases in muscle size *in-vitro*.

Co-culture of neurons in aneural 3D bioengineered skeletal muscle

Co-culture of neurons with engineered muscle have also been observed to improve muscle development and maturation. However, the formation of functional neuromuscular junctions (NMJs) within bioengineered tissues are rare (Larkin et al., 2006). Nonetheless, functional NMJs have been identified when bioengineered skeletal muscle (generated using C2C12 myoblasts embedded within Matrigel) are co-cultured with mouse neural stem cells, as evidence by the myotube contractile response to chemical neuronal activation and the subsequent dampening following NMJ chemical blockade (Morimoto et al., 2013). More recently, co-culture of rat embryo (gestational age E14) motor neurons taken from the ventral horn, seeded within a 3D type-I collagen matrix 4 days after the primary muscle derived cells from neonatal rat muscle, were shown to enhance mRNA expression of a number of markers of skeletal muscle maturation (Smith et al., 2016). Overall, suggesting that the addition of a neural input may drive the contractile maturity of bioengineered skeletal muscle. Indeed, further evidence for enhanced skeletal muscle phenotype as a result of innervation within bioengineered skeletal muscle has been generated in 3D fibrin self-assembling constructs. Again, using embryonic rodent motor neurons co-cultured with neonatal primary muscle derived cells, it was established that the presence of motor neurons can improve contractile properties after 18 days of total time in culture (Martin et al., 2015). Indeed, following electrical field stimulation (1.2 ms pulse width, 100 Hz impulse frequency train at 3.5 V/mm), the addition of embryonic motor neurons

promoted increased maximal twitch and tetanic force. Furthermore, cytoskeletal organization improved, assessed via immunohistochemistry analysis of desmin and MAP2 to highlight the muscle intermediary protein filament and motor neurons respectively, and revealed myotube formation was in a fascicular arrangement and that there was neurite outgrowth from motor neuron cell bodies toward the aligned myotubes. Furthermore, chemical antagonism of the acetylcholine receptor (AChR) using D-tubocurarine negated the increase in twitch frequency that was improved in the presence of motor neurons, suggesting the presence of NMJ formation was indicative of successful innervation of the 3D engineered skeletal muscle constructs (Martin et al., 2015). At present, the use of these motor neuron muscle derived cell co-cultures have not been used to investigate the role of acute or chronic exercise mimicking stimuli, however these data may suggest that these models represent even more physiologically relevant innervated muscle phenotype vs. aneural constructs and therefore their response to chronic stimulation would be an interesting area for future investigation. This would be especially pertinent given that whilst contractile function was improved following motor neuron addition, no hypertrophy was observed (Martin et al., 2015), yet it maybe hypothesised that chronic electrical stimulation of these cultures may evoke hypertrophy versus aneural cultures.

Mechanical loading of 3D bioengineered skeletal muscle

Mechanical loading plays an important role in skeletal muscle tissue *in-vivo* and can regulate muscle size, muscle cell differentiation as well as matrix remodelling. Indeed, continuous passive tension to skeletal muscle by bone growth of approximately 2 mm/wk during neonatal development influences muscle weight, length and myofibrillar organisation (Olwin et al., 1994; Stewart, 1972). Following the first work by Goldberg that demonstrated a rapid hypertrophic response after only 24 hr post tenotomy of a synergistic muscle (synergistic ablation) (Goldberg, 1967), several groups have used this model of overload induced hypertrophy (Armstrong et al., 1979; Esser and White, 1995; Kandarian et al., 1992; Linderman et al., 1996). Indeed, using this model, mechanical overload can result in increased amino acid transport (Goldberg, 1967; Goldberg and Goodman, 1969; Henriksen et al., 1993) and satellite cell activation and proliferation (Rosenblatt and Parry, 1992; Schiaffino et al., 1972) and a total increase in protein synthesis (Goldspink, 1977) along with increases in protein sub-

fractions within skeletal muscle (Cuthbertson et al., 2006). Vandenburgh and colleagues (1989) initially laid down the foundations for the investigation of mechanical cyclic stretch in skeletal muscle cells cultures using a collagen matrix and found that stretch elicited an increase in total protein and myotube hypertrophy (Vandenburgh et al., 1989; Vandenburgh and Karlisch, 1989; Vandenburgh et al., 1988). Vandenburgh and Karlisch (1989) produced the first study to mechanically stimulate 3D muscle used collagen coated elastic substratum's seeded with muscle cells isolated from embryonic avian pectoralis muscle. The authors subjected these constructs to mechanical stretch of 300% of starting collagen substratum length over 3 days at a rate of 0.35 mm/h (which is same rate that which stretch stimulates *in-vivo* bone elongation during development) (Vandenburgh and Karlisch, 1989). In these stretched constructs the authors observed increased myoblast proliferation and fusion as well as increases in myotube length (Vandenburgh and Karlisch, 1989). Furthermore, to the authors knowledge the first study to mechanically load primary human skeletal muscle cells (vastus lateralis) in 3D collagen/matrigel solutions cast in silicone moulds was over a decade later (Powell et al., 2002). Once cast, muscle derived cells were grown for a period of 2 days, and induced to differentiate in low serum media for 5 days, before being mechanically loaded after 8 days of myotube maturation (fibre diameter average 6.4 μm). The mechanical load regime included stretch at 5% strain (1 mm) for 2 days, 10% strain (2 mm) for 2 days, and 15% strain (3 mm) for 4 days (total of 16 days; 8 days maturation, 8 days loading). This loading regime induced an increase in myotube diameter by 12% and area by 40% (Powell et al., 2002), suggestive that mechanical load evoked considerable hypertrophy in 3D bioengineered human muscle. In an attempt to look more closely at the molecular mechanisms of loading 3D muscle, Cheema *et al.,* (2005) mechanically stretched 3D matrices seeded with C2C12 cells and polymerised in type I collagen. Following differentiation to promote myotube formation (6 days low serum) they elicited ramp and cyclic stretch regimes with 10% stretch of resting length applied as either 1, 5 or 10 cycles/h for 12 h, and ramp stretch of 10% resting length over 10 min, 1 and 12 hr (10 min and 1 hr ramp load held at 10% stretch for the remaining 12 hrs). The authors observed an increase in gene transcription of IGF-IEa that would code for IGF-I isoform IEa, shown previously to be involved in myoblast differentiation (Yang and Goldspink, 2002) following a single 10% ramp stretch over 1hr (and held at 10% for the remaining 12 hrs) but not after

any of the cyclic stretch regimes. Indeed, IGF-IEa actually decreased in a dose response manner with increasing 10% stretch cycling per hour. In contrast, mechano growth factor (MGF/IGF-IEb in rodents/IGF-IEc in humans), involved in the proliferation and self-renewal of myoblasts, yet inhibition of differentiation (Yang and Goldspink, 2002), was upregulated by both a single 1hr 10% ramp stretch and by 1 cycle/hr cyclic loading. Therefore, this study was suggestive of a suitable anabolic response (IGF-IEa) following ramp stretch that mimics bone growth, whereas cyclic stretch (that would not occur during development) perhaps evoked enhanced proliferation and self-renewal of muscle cells. The authors stated however, that due to only 6 days' maturation, only 5% of all cells were myotubes in the 3D collagen matrices. Therefore, as IGF-IEa and Eb are involved in proliferation, differentiation and self-renewal in myoblasts, this study perhaps represented model of muscle development following bone growth (e.g. ramp stretch) and therefore its results cannot be necessarily extrapolated to the effect of mechanical loading on mature skeletal myotubes. Subsequent studies confirmed that the application of mechanical static load (10% stretch of resting length for 6 hrs per day, separated by 18 hrs of 3% stretch for a total of 6 days- 3 days after initial cell seeding) during C2C12 myoblast differentiation (rather than in mature myotubes) in 3D fibrin cast bioengineered skeletal muscle did lead to increased differentiation, myotube formation (myotube number), size (diameter and length). This corresponded with increases in the gene expression of myogenic regulator factor, myogenin, as well as sarcomeric patterning more similar to native skeletal muscle tissue (Heher et al., 2015). However, loading of constructs following extensive myotube maturation was also not provided in this study. Therefore, studies using smaller collagen gel sponge constructs attempted to differentiate constructs for longer (7 days) and in the presence of exogenous IGF-I to promote myotube formation and hypertrophy and applied both ramp and cyclic loading to the resultant myotubes (Auluck et al., 2005). In these studies, 3D collagen sponges containing human craniofacial muscle derived cells were stretched to 15% of resting length that was continuously held over 6 hrs. While authors did not investigate IGF-I gene expression, the regime evoked higher protein activity of matrix metalloproteinase 2 (MMP-2) versus non-loaded controls and vs. cyclic stretch e.g. 1.5 min stretch, 1.5 min hold followed by 1.5 min release of strain, repeated for 6 hrs (Auluck et al., 2005). MMP-2 is an enzyme produced by muscle cells used to degrade and turnover the matrix

around it in order to enable matrix remodelling. Therefore, this data suggests that ramp loading *in-vitro*, may enable the enhanced matrix remodelling for subsequent myotube formation and hypertrophy if stimuli were to be chronically applied. However, authors were unable to characterise myotube formation as the collagen sponges did not permit immunohistological analysis to be performed, and therefore again these studies were unable to confirm the maturation of the 3D muscle myotubes. In an attempt to somewhat rectify this issue, 3D constructs using type I collagen (similar to that used by Cheema et al., 2005 described above), were matured for the longer period of 14 days (vs. 6 days by Cheema et al., 2005) (Player et al., 2014). This maturation period was also similar to that used previously in fibrin 3D muscle (discussed extensively above) that possessed a more mature muscle phenotype vs. shorter maturation periods. Indeed, immunohistological images presented in the manuscript displayed highly aligned myotubes in parallel; Figure 1E&F (Player et al., 2014). Mechanical load was applied to these more mature constructs via static loading comprising of 10% stretch that was held for 1 hr to mimic synergistic ablation *in vitro* vs. ramp load which was a continuous increase over 1 hr until 10% stretch was attained. IGF-I and matrix metalloproteinase 9 (MMP-9) gene expression was higher with static loading vs. ramp loading, suggesting a positive anabolic response to static loading as well as producing an elevated signal for matrix remodelling that would potentially precede hypertrophy if the signal was chronically maintained. Furthermore, there was no changes observed following loading for negative regulators of muscle mass, myostatin or ubiquitin ligase mRNA (MuRF and Marfbx) involved in protein degradation of muscle specific proteins. Interestingly, there was increased IGFBP2 in static loading conditions and reduced IGFBP5 gene expression in both static and ramp loading conditions. At the protein level, these binding proteins act as soluble clearance molecules for IGF. Therefore, modulating the ability of IGF-I to bind to its receptors depends on whether IGF is bound to the binding proteins or cleaved. Indeed, higher/ lower IGFBP2 results in impaired differentiation and myotube atrophy vs. improved differentiation and myotube hypertrophy in myoblasts (Sharples et al., 2013; Sharples et al., 2010), and excess IGFBP2 expression in mice reduces muscle size (Rehfeldt et al., 2010), suggesting that the potential positive anabolic response of increased IGF-I could be compromised with elevating IGFBP2 in static

load conditions. However, both conditions saw reductions in IGFBP5 that would potentially increase the availability of IGF-I in loaded 3D muscle constructs.

Overall, these studies suggest that mechanical loading experimentation within 3D muscle to investigate lengthening and relaxing of muscle and the subsequent molecular responses to load in adult skeletal muscle phenotypes has been somewhat limited by the lack of experimentation in fully mature myotubes in 3D constructs. Furthermore, there are currently limited investigations into relevant anabolic cell signalling allowing relevant comparisons to acute exercise *in-vivo*. To further compound these issues, contradictory evidence in monolayer studies suggests that cyclic stretch in differentiated rat L6 myoblasts actually impairs protein synthetic associated cell signalling as well as protein synthesis itself, albeit increasing the activity of the potential mechanosensor, focal adhesion kinase (FAK) (Atherton et al., 2009). This suggests that there may be an alternative molecular response to lengthening contractions vs. what is observed following shortening concentric contraction. Alternatively however, stretch in C2C12 myotubes in monolayer has been shown to increase activity of the protein translation initiator P70S6K (Baar et al., 2000). Also, the use of more mature aligned myotubes in 3D collagen constructs or via the use of fibrin/laminin self-assembling 3D muscle (that have been shown to produce mature aligned myotubes, especially following electrical stimulation/motor neuron co-culture), may be important to elucidate the influence of mechanical stretch on the molecular mechanisms (perhaps in combination with electrical stimulation for concentric contraction) to determine the underlying physiological adaptation to exercise mimicking stimuli. Furthermore, studies are currently limited to fairly acute stimulation and therefore restricted with respect to assessing the impact of mechanical load on longer term muscle hypertrophy and the associated mechanisms. Also, there are currently limited studies that combine the use of electrical stimulation and mechanical load that would be more relevant to physiological contraction *in-vivo* and could potentially mimic more closely both shortening concentric and lengthening eccentric contraction. Indeed, the only study to the authors knowledge to combine these two modes of stimulation included 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 C2C12 cells seeded within synthetic aligned

electro-spun polyurethane (PU) fibers (Liao et al., 2008). This regime increased the percentage of striated myotubes from 70 to 85%, which was accompanied by an increase in contractile proteins such as α-actinin and myosin heavy chain. Despite this, the combination of mechanical load and electrical stimulation was unable to elicit larger increases versus the individual modes of stimulation alone. However, it is worth mentioning that this was conducted using non-biological scaffolds that perhaps do not mimic the response that may be observed using biological matrices. Also, the stimulation was low frequency and continuous mimicking aerobic exercise where even greater increases in myotube hypertrophy may be observed after higher frequency intermittent contractions with adequate rest cycles repeated over time. Furthermore, given the discrepancy within the literature over the lack of cell signaling response to acute lengthening (Atherton et al., 2009) versus acute electrically stimulated contractions (Baar et al., 2000) and the limited work conducted into the investigation of the underlying mechano-signal transduction thought to lead to hypertrophy in response to high frequency intermittent regimes in 3D muscle, for example; the potential role of focal adhesion kinase (FAK) (Crossland et al., 2013; Fluck et al., 1999), phospholipase D/ phosphatidic acid (Hornberger et al., 2006), mTOR (Baar and Esser, 1999), mitogen activated protein kinases e.g. ERK (Miyazaki et al., 2011), the investigation of these pathways following the combination of electrical stimulation and mechanical stimulation in-vitro muscle requires future investigation. More recently the latest technology bioreactors are becoming more commercially available that allow both mechanical stretch and electrical stimulation of 3D cultures and are fully aseptic with perfused media control (depicted in Figure 3), potentially allowing more chronic exercise mimicking regimes to be applied, especially if using self-assembling myooids using biological scaffolds and primary derived muscle cells that incorporate matrix maintaining fibroblasts that have been shown to be viable for 50 days (Dennis and Kosnik, 2000; Dennis et al., 2001). To the authors knowledge however, there have been no studies undertaken in 3D skeletal muscle that have combined both chronic mechanical loading and electrical stimulation, therefore this also warrants future investigation.

Future directions

There are currently some exciting advances into optimising the maturation of the most adult like skeletal muscle tissue in 3D bioengineered constructs using electrical stimulation, motor neuron co-culture and mechanical loading. However, the molecular mechanisms underpinning the adaptation of 3D bioengineered muscle with a mature adult phenotype following acute continuous stimuli, mimicking aerobic exercise, are only just beginning to emerge with a distinct lack of investigations focusing on higher frequency, intermittent stimulation regimes mimicking resistance type exercise. Therefore, it is envisaged that technology that allows both concentric shortening and eccentric lengthening via the use of the latest bioreactor technology that permits simultaneous mechanical and electrical stimuli, will enable experimentation into this field to progress in the future (depicted in Figure 3). Importantly, while genetic modification in 3D cultures have been performed previously (examples include (Du et al., 2014; Evans et al., 2017; Romero et al., 2016), the use of this molecular tool has only emerged recently in bioengineered skeletal muscle (Cheng et al., 2016). Therefore, we envisage, in the near future, that these 3D *in-vitro* skeletal muscle systems may more easily allow for genetic modification (gene silencing/overexpression) (Cheng *et al.,* 2016), pharmacological inhibition (Khodabukus and Baar, 2012), alterations in the hormonal background (Huang et al., 2005) and/ or pre-clinical therapeutics being applied to more biologically relevant human muscle testing systems that cannot be readily undertaken within *in-vivo* human experimentation due to ethical and safety concerns. Additionally, studies have also attempted to mimic aged-related muscle loss in 3D bioengineered skeletal muscle (Sharples et al., 2012) opening up the possibility to investigate the associated mechanisms of anabolic resistance to load and nutrients observed in ageing muscle *in-vivo* following the mimicking of exercise and/or nutrient manipulation in 3D bioengineered skeletal muscle. Finally, because isolated muscle cells from different environmental niches *in-vivo* (e.g. physically active, diabetic, cancer cachectic, obese and sarcopenic) remember their prior environment once isolated *in-vitro*, and take on characteristics of their disease state, reviewed in detail by our group recently (Sharples et al., 2016), bioengineering of skeletal muscle may enable the application of potential therapies in models of human age-related muscle loss and muscle loss disease using cells isolated from the skeletal muscle from patients (e.g. sarcopenia, sarcopenic obese, type II diabetic, cancer cachectic).

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References

Armstrong RB, Marum P, Tullson P, Saubert CWt. 1979. Acute hypertrophic response of skeletal muscle to removal of synergists. Journal of applied physiology: respiratory, environmental and exercise physiology 46(4):835-842.

Atherton PJ, Szewczyk NJ, Selby A, Rankin D, Hillier K, Smith K, Rennie MJ, Loughna PT. 2009. Cyclic stretch reduces myofibrillar protein synthesis despite increases in FAK and anabolic signalling in L6 cells. The Journal of physiology 587(Pt 14):3719-3727.

Auluck A, Mudera V, Hunt NP, Lewis MP. 2005. A three-dimensional in vitro model system to study the adaptation of craniofacial skeletal muscle following mechanostimulation. European journal of oral sciences 113(3):218-224.

Baar K, Esser K. 1999. Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. Am J Physiol 276(1 Pt 1):C120-127.

Baar K, Torgan CE, Kraus WE, Esser K. 2000. Autocrine phosphorylation of p70(S6k) in response to acute stretch in myotubes. Molecular cell biology research communications : MCBRC 4(2):76-80.

Baker EL, Dennis RG, Larkin LM. 2003. Glucose transporter content and glucose uptake in skeletal muscle constructs engineered in vitro. In vitro cellular & developmental biology Animal 39(10):434-439.

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3(11):1014-1019.

Breslin S, O'Driscoll L. 2013. Three-dimensional cell culture: the missing link in drug discovery. Drug discovery today 18(5-6):240-249.

Brevet A, Pinto E, Peacock J, Stockdale FE. 1976. Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. Science (New York, NY) 193(4258):1152-1154.

Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. 1999. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. The American journal of physiology 277(2 Pt 2):H433-444.

Cairns SP, Chin ER, Renaud JM. 2007. Stimulation pulse characteristics and electrode configuration determine site of excitation in isolated mammalian skeletal muscle: implications for fatigue. Journal of applied physiology (Bethesda, Md : 1985) 103(1):359-368.

Chang YJ, Chen YJ, Huang CW, Fan SC, Huang BM, Chang WT, Tsai YS, Su FC, Wu CC. 2016. Cyclic Stretch Facilitates Myogenesis in C2C12 Myoblasts and Rescues Thiazolidinedione-Inhibited Myotube Formation. Frontiers in bioengineering and biotechnology 4:27.

Chen R, Feng L, Ruan M, Liu X, Adriouch S, Liao H. 2013. Mechanical-stretch of C2C12 myoblasts inhibits expression of Toll-like receptor 3 (TLR3) and of autoantigens associated with inflammatory myopathies. PloS one 8(11):e79930.

Cheng CS, Ran L, Bursac N, Kraus WE, Truskey GA. 2016. Cell Density and Joint microRNA-133a and microRNA-696 Inhibition Enhance Differentiation and Contractile Function of Engineered Human Skeletal Muscle Tissues. Tissue engineering Part A 22(7-8):573-583.

Close R. 1964. Dynamic properties of fast and slow skeletal muscles of the rat during development. The Journal of physiology 173:74-95.

Coffey VG, Hawley JA. 2007. The molecular bases of training adaptation. Sports medicine (Auckland, NZ) 37(9):737-763.

Crossland H, Kazi AA, Lang CH, Timmons JA, Pierre P, Wilkinson DJ, Smith K, Szewczyk NJ, Atherton PJ. 2013. Focal adhesion kinase is required for IGF-I-mediated growth of skeletal muscle cells via a TSC2/mTOR/S6K1-associated pathway. American journal of physiology Endocrinology and metabolism 305(23695213):183-193.

Cuthbertson DJ, Babraj J, Smith K, Wilkes E, Fedele MJ, Esser K, Rennie M. 2006. Anabolic signaling and protein synthesis in human skeletal muscle after dynamic shortening or lengthening exercise. American journal of physiology Endocrinology and metabolism 290(4):E731-738.

Danoviz ME, Yablonka-Reuveni Z. 2012. Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. Methods in molecular biology (Clifton, NJ) 798:21-52.

Dennis RG, Dow DE. 2007. Excitability of skeletal muscle during development, denervation, and tissue culture. Tissue engineering 13(10):2395-2404.

Dennis RG, Kosnik PE, 2nd. 2000. Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. In Vitro Cell Dev Biol Anim 36(5):327-335.

Dennis RG, Kosnik PE, 2nd, Gilbert ME, Faulkner JA. 2001. Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines. Am J Physiol Cell Physiol 280(2):C288-295.

Donnelly K, Khodabukus A, Philp A, Deldicque L, Dennis RG, Baar K. 2010. A novel bioreactor for stimulating skeletal muscle in vitro. Tissue engineering Part C, Methods 16(4):711-718.

Drummond MJ, Fujita S, Takashi A, Dreyer HC, Volpi E, Rasmussen BB. 2008. Human muscle gene expression following resistance exercise and blood flow restriction. Medicine and science in sports and exercise 40(4):691.

Du X, Wang J, Diao W, Wang L, Long J, Zhou H. 2014. A genetically modified protein-based hydrogel for 3D culture of AD293 cells. PloS one 9(9):e107949.

Esser KA, White TP. 1995. Mechanical load affects growth and maturation of skeletal muscle grafts. Journal of applied physiology (Bethesda, Md : 1985) 78(1):30-37.

Evans JC, Malhotra M, Fitzgerald KA, Guo J, Cronin MF, Curtin CM, O'Brien FJ, Darcy R, O'Driscoll CM. 2017. Formulation and Evaluation of Anisamide-Targeted Amphiphilic Cyclodextrin Nanoparticles To Promote Therapeutic Gene Silencing in a 3D Prostate Cancer Bone Metastases Model. Molecular pharmaceutics 14(1):42-52.

Fluck M, Carson JA, Gordon SE, Ziemiecki A, Booth FW. 1999. Focal adhesion proteins FAK and paxillin increase in hypertrophied skeletal muscle. Am J Physiol 277(10409118):152-162.

Fredette BJ, Landmesser LT. 1991. A reevaluation of the role of innervation in primary and secondary myogenesis in developing chick muscle. Developmental biology 143(1):19-35.

Goldberg AL. 1967. Work-induced growth of skeletal muscle in normal and hypophysectomized rats. The American journal of physiology 213(5):1193-1198.

Goldberg AL, Goodman HM. 1969. Amino acid transport during work-induced growth of skeletal muscle. The American journal of physiology 216(5):1111-1115.

Goldspink DF. 1977. The influence of immobilization and stretch on protein turnover of rat skeletal muscle. The Journal of physiology 264(1):267-282.

Grifone R, Laclef C, Spitz F, Lopez S, Demignon J, Guidotti JE, Kawakami K, Xu PX, Kelly R, Petrof BJ, Daegelen D, Concordet JP, Maire P. 2004. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. Molecular and cellular biology 24(14):6253-6267.

Harris AJ, Fitzsimons RB, McEWAN JC. 1989. Neural control of the sequence of expression of myosin heavy chain isoforms in foetal mammalian muscles. Development 107(4):751-769.

Heher P, Maleiner B, Prüller J, Teuschl AH, Kollmitzer J, Monforte X, Wolbank S, Redl H, Rünzler D, Fuchs C. 2015. A novel bioreactor for the generation of highly aligned 3D skeletal muscle-like constructs through orientation of fibrin via application of static strain. Acta biomaterialia 24:251-265.

Henneman E, Somjen G, Carpenter DO. 1965. Excitability and inhibitability of motoneurons of different sizes. Journal of neurophysiology 28(3):599-620.

Henriksen EJ, Schneider MC, Ritter LS. 1993. Regulation of contraction-stimulated system A amino acid uptake in skeletal muscle: role of vicinal sulfhydryls. Metabolism: clinical and experimental 42(4):440-445.

Hinds S, Bian W, Dennis RG, Bursac N. 2011. The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. Biomaterials 32(14):3575-3583.

Holloszy JO. 1973. Biochemical Adaptations to Exercise; Aerobic Metabolism1. Exercise and sport sciences reviews 1(1):45-72.

Hood DA. 2009. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle This paper is one of a selection of papers published in this Special Issue, entitled 14th International Biochemistry of Exercise Conference–Muscles as Molecular and Metabolic Machines, and has undergone the Journal's usual peer review process. Applied Physiology, Nutrition, and Metabolism 34(3):465-472.

Hornberger TA, Chu WK, Mak YW, Hsiung JW, Huang SA, Chien S. 2006. The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc Natl Acad Sci U S A 103(12):4741-4746.

Huang Y-C, Dennis RG, Baar K. 2006. Cultured slow vs. fast skeletal muscle cells differ in physiology and responsiveness to stimulation. American Journal of Physiology-Cell Physiology 291(1):C11-C17.

Huang Y-C, Dennis RG, Larkin L, Baar K. 2005. Rapid formation of functional muscle in vitro using fibrin gels. Journal of Applied Physiology 98(2):706-713.

Jørgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA. 2005. Effects of α-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. The FASEB journal 19(9):1146-1148.

Joseph A-M, Pilegaard H, Litvintsev A, Leick L, Hood DA. 2006. Control of gene expression and mitochondrial biogenesis in the muscular adaptation to endurance exercise. Essays in Biochemistry 42:13-29.

Juhas M, Bursac N. 2014. Roles of adherent myogenic cells and dynamic culture in engineered muscle function and maintenance of satellite cells. Biomaterials 35(35):9438-9446.

Juhas M, Engelmayr GC, Fontanella AN, Palmer GM, Bursac N. 2014. Biomimetic engineered muscle with capacity for vascular integration and functional maturation in vivo. Proceedings of the National Academy of Sciences 111(15):5508-5513.

Kandarian SC, Schulte LM, Esser KA. 1992. Age effects on myosin subunit and biochemical alterations with skeletal muscle hypertrophy. Journal of applied physiology (Bethesda, Md : 1985) 72(5):1934-1939.

Kawahara Y, Yamaoka K, Iwata M, Fujimura M, Kajiume T, Magaki T, Takeda M, Ide T, Kataoka K, Asashima M. 2007. Novel electrical stimulation sets the cultured myoblast contractile function to 'on'. Pathobiology 73(6):288-294.

Khodabukus A, Baar K. 2009. Regulating fibrinolysis to engineer skeletal muscle from the C2C12 cell line. Tissue engineering Part C, Methods 15(3):501-511.

Khodabukus A, Baar K. 2012. Defined electrical stimulation emphasizing excitability for the development and testing of engineered skeletal muscle. Tissue engineering Part C, Methods 18(5):349-357.

Khodabukus A, Baar K. 2015. Contractile and metabolic properties of engineered skeletal muscle derived from slow and fast phenotype mouse muscle. Journal of cellular physiology 230(8):1750-1757.

Khodabukus A, Baar K. 2016. Factors That Affect Tissue-Engineered Skeletal Muscle Function and Physiology. Cells, tissues, organs 202(3-4):159-168.

Khodabukus A, Baehr LM, Bodine SC, Baar K. 2015. Role of contraction duration in inducing fast-to-slow contractile and metabolic protein and functional changes in engineered muscle. Journal of cellular physiology 230(10):2489-2497.

Khodabukus A, Paxton JZ, Donnelly K, Baar K. 2007. Engineered muscle: a tool for studying muscle physiology and function. Exercise and sport sciences reviews 35(4):186-191.

Kook S-H, Lee H-J, Chung W-T, Hwang I-H, Lee S-A, Kim B-S, Lee J-C. 2008. Cyclic mechanical stretch stimulates the proliferation of C2C12 myoblasts and inhibits their differentiation via prolonged activation of p38 MAPK. Molecules & Cells (Springer Science & Business Media BV) 25(4).

Kumar A, Murphy R, Robinson P, Wei L, Boriek AM. 2004. Cyclic mechanical strain inhibits skeletal myogenesis through activation of focal adhesion kinase, Rac-1 GTPase, and NF-κB transcription factor. The FASEB Journal 18(13):1524-1535.

Langelaan ML, Boonen KJ, Rosaria-Chak KY, van der Schaft DW, Post MJ, Baaijens F. 2011. Advanced maturation by electrical stimulation: Differences in response between C2C12 and primary muscle progenitor cells. Journal of tissue engineering and regenerative medicine 5(7):529-539.

Larkin LM, Van der Meulen JH, Dennis RG, Kennedy JB. 2006. Functional evaluation of nerve-skeletal muscle constructs engineered in vitro. In vitro cellular & developmental biology Animal 42(3-4):75-82.

Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JF, Hidalgo J, Pilegaard H. 2009. PGC-1α mediates exercise-induced skeletal muscle VEGF expression in mice. American Journal of Physiology-Endocrinology and Metabolism 297(1):E92-E103.

Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C. 2005. PGC-1α deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol 3(4):e101.

Liao IC, Liu JB, Bursac N, Leong KW. 2008. Effect of Electromechanical Stimulation on the Maturation of Myotubes on Aligned Electrospun Fibers. Cellular and molecular bioengineering 1(2-3):133-145.

Linderman JK, Talmadge RJ, Gosselink KL, Tri PN, Roy RR, Grindeland RE. 1996. Synergistic ablation does not affect atrophy or altered myosin heavy chain expression in the non-weight bearing soleus muscle. Life sciences 59(10):789-795.

Martin NR, Passey SL, Player DJ, Khodabukus A, Ferguson RA, Sharples AP, Mudera V, Baar K, Lewis MP. 2013. Factors affecting the structure and maturation of human tissue engineered skeletal muscle. Biomaterials 34(23):5759-5765.

Martin NR, Passey SL, Player DJ, Mudera V, Baar K, Greensmith L, Lewis MP. 2015. Neuromuscular Junction Formation in Tissue-Engineered Skeletal Muscle Augments

Contractile Function and Improves Cytoskeletal Organization. Tissue engineering Part A 21(19-20):2595-2604.

Mertens JP, Sugg KB, Lee JD, Larkin LM. 2014. Engineering muscle constructs for the creation of functional engineered musculoskeletal tissue. Regenerative medicine 9(1):89-100.

Miyazaki M, McCarthy JJ, Fedele MJ, Esser KA. 2011. Early activation of mTORC1 signalling in response to mechanical overload is independent of phosphoinositide 3-kinase/Akt signalling. The Journal of Physiology 589(7):1831-1846.

Morimoto Y, Kato-Negishi M, Onoe H, Takeuchi S. 2013. Three-dimensional neuron-muscle constructs with neuromuscular junctions. Biomaterials 34(37):9413-9419.

Mudera V, Smith A, Brady M, Lewis M. 2010. The effect of cell density on the maturation and contractile ability of muscle derived cells in a 3D tissue-engineered skeletal muscle model and determination of the cellular and mechanical stimuli required for the synthesis of a postural phenotype. Journal of cellular physiology 225(3):646-653.

Naumann K, Pette D. 1994. Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation 55(3):203-211.

Nedachi T, Fujita H, Kanzaki M. 2008. Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. American journal of physiology Endocrinology and metabolism 295(5):E1191-1204.

Nikolic N, Bakke SS, Kase ET, Rudberg I, Flo Halle I, Rustan AC, Thoresen GH, Aas V. 2012. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. PloS one 7(3):e33203.

Niro C, Demignon J, Vincent S, Liu Y, Giordani J, Sgarioto N, Favier M, Guillet-Deniau I, Blais A, Maire P. 2010. Six1 and Six4 gene expression is necessary to activate the fast-type muscle gene program in the mouse primary myotome. Dev Biol 338(2):168-182.

O'Connor MS, Carlson ME, Conboy IM. 2009. Differentiation rather than aging of muscle stem cells abolishes their telomerase activity. Biotechnol Prog 25(4):1130-1137.

Olwin BB, Arthur K, Hannon K, Hein P, McFall A, Riley B, Szebenyi G, Zhou Z, Zuber ME, Rapraeger AC, et al. 1994. Role of FGFs in skeletal muscle and limb development. Molecular reproduction and development 39(1):90-100; discussion 100-101.

Owens DJ, Sharples AP, Polydorou I, Alwan N, Donovan T, Tang J, Fraser WD, Cooper RG, Morton JP, Stewart C, Close GL. 2015. A systems-based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy. American journal of physiology Endocrinology and metabolism 309(12):E1019-1031.

Pardo PS, Mohamed JS, Lopez MA, Boriek AM. 2011. Induction of Sirt1 by mechanical stretch of skeletal muscle through the early response factor EGR1 triggers an antioxidative response. Journal of Biological Chemistry 286(4):2559-2566.

Park H, Bhalla R, Saigal R, Radisic M, Watson N, Langer R, Vunjak-Novakovic G. 2008. Effects of electrical stimulation in C2C12 muscle constructs. Journal of tissue engineering and regenerative medicine 2(5):279-287.

Player D, Martin N, Castle P, Passey S, Sharples A, Mudera V, Lewis M. Mechanical stimulation of 3D bio-engineered skeletal muscle; 2011.

Player D, Martin N, Passey S, Sharples A, Mudera V, Lewis M. 2014. Acute mechanical overload increases IGF-I and MMP-9 mRNA in 3D tissue-engineered skeletal muscle. Biotechnology letters 36(5):1113-1124.

Powell CA, Smiley BL, Mills J, Vandenburgh HH. 2002. Mechanical stimulation improves tissue-engineered human skeletal muscle. American Journal of Physiology-Cell Physiology 283(5):C1557-C1565.

Rangarajan S, Madden L, Bursac N. 2014. Use of flow, electrical, and mechanical stimulation to promote engineering of striated muscles. Annals of biomedical engineering 42(7):1391-1405.

Rehfeldt C, Renne U, Sawitzky M, Binder G, Hoeflich A. 2010. Increased fat mass, decreased myofiber size, and a shift to glycolytic muscle metabolism in adolescent male transgenic mice overexpressing IGFBP-2. American journal of physiology Endocrinology and metabolism 299(2):E287-298.

Richard AF, Demignon J, Sakakibara I, Pujol J, Favier M, Strochlic L, Le Grand F, Sgarioto N, Guernec A, Schmitt A, Cagnard N, Huang R, Legay C, Guillet-Deniau I, Maire P. 2011. Genesis of muscle fiber-type diversity during mouse embryogenesis relies on Six1 and Six4 gene expression. Dev Biol 359(2):303-320.

Romero D, Al-Shareef Z, Gorrono-Etxebarria I, Atkins S, Turrell F, Chhetri J, Bengoa-Vergniory N, Zenzmaier C, Berger P, Waxman J, Kypta R. 2016. Dickkopf-3 regulates prostate epithelial cell acinar morphogenesis and prostate cancer cell invasion by limiting TGF-beta-dependent activation of matrix metalloproteases. Carcinogenesis 37(1):18-29.

Rosenblatt JD, Parry DJ. 1992. Gamma irradiation prevents compensatory hypertrophy of overloaded mouse extensor digitorum longus muscle. J Appl Physiol (1985) 73(6):2538-2543.

Schiaffino S, Bormioli SP, Aloisi M. 1972. Cell proliferation in rat skeletal muscle during early stages of compensatory hypertrophy. Virchows Archiv B, Cell pathology 11(3):268-273.

Seale P, Rudnicki MA. 2000. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Developmental biology 218(2):115-124.

Sharples AP, Al-Shanti N, Hughes DC, Lewis MP, Stewart CE. 2013. The role of insulin-like-growth factor binding protein 2 (IGFBP2) and phosphatase and tensin homologue (PTEN) in the regulation of myoblast differentiation and hypertrophy. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 23(3):53-61.

Sharples AP, Al-Shanti N, Stewart CE. 2010. C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: relevance to disease and ageing? Journal of cellular physiology 225(1):240-250.

Sharples AP, Player DJ, Martin NR, Mudera V, Stewart CE, Lewis MP. 2012. Modelling in vivo skeletal muscle ageing in vitro using three-dimensional bioengineered constructs. Aging cell 11(6):986-995.

Sharples AP, Stewart CE. 2011. Myoblast models of skeletal muscle hypertrophy and atrophy. Current Opinion in Clinical Nutrition & Metabolic Care 14(3):230-236.

Sharples AP, Stewart CE, Seaborne RA. 2016. Does skeletal muscle have an 'epi'-memory? The role of epigenetics in nutritional programming, metabolic disease, aging and exercise. Aging cell 15(4):603-616.

Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. 1998. Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase. The EMBO journal 17(22):6649-6659.

Smith A, Passey S, Greensmith L, Mudera V, Lewis M. 2012. Characterization and optimization of a simple, repeatable system for the long term in vitro culture of aligned myotubes in 3D. Journal of cellular biochemistry 113(3):1044-1053.

Smith AS, Passey SL, Martin NR, Player DJ, Mudera V, Greensmith L, Lewis MP. 2016. Creating Interactions between Tissue-Engineered Skeletal Muscle and the Peripheral Nervous System. Cells, tissues, organs 202(3-4):143-158.

Stern-Straeter J, Bach A, Stangenberg L, Foerster V, Horch R, Stark G, Beier J. 2005. Impact of electrical stimulation on three-dimensional myoblast cultures-a real-time RT-PCR study. Journal of Cellular and Molecular Medicine 9(4):883-892.

Stewart DM. 1972. Chapter 5 - The role of tension in musle growth A2 - Goss, Richard J. Regulation of Organ and Tissue Growth: Academic Press. p 77-100.

Thelen MH, Simonides WS, van Hardeveld C. 1997. Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene. Biochemical Journal 321(3):845-848.

Urbanchek MG, Picken EB, Kalliainen LK, Kuzon WM, Jr. 2001. Specific force deficit in skeletal muscles of old rats is partially explained by the existence of denervated muscle fibers. The journals of gerontology Series A, Biological sciences and medical sciences 56(5):B191-197.

van der Schaft DW, van Spreeuwel AC, van Assen HC, Baaijens FP. 2011. Mechanoregulation of vascularization in aligned tissue-engineered muscle: a role for vascular endothelial growth factor. Tissue Engineering Part A 17(21-22):2857-2865.

Vandenburgh H, Kaufman S. 1979. In vitro model for stretch-induced hypertrophy of skeletal muscle. Science (New York, NY) 203(4377):265-268.

Vandenburgh HH, Hatfaludy S, Karlisch P, Shansky J. 1989. Skeletal muscle growth is stimulated by intermittent stretch-relaxation in tissue culture. American Journal of Physiology-Cell Physiology 256(3):C674-C682.

Vandenburgh HH, Karlisch P. 1989. Longitudinal growth of skeletal myotubes in vitro in a new horizontal mechanical cell stimulator. In vitro cellular & developmental biology 25(7):607-616.

Vandenburgh HH, Karlisch P, Farr L. 1988. Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel. In vitro cellular & developmental biology 24(3):166-174.

Wehrle U, Düsterhöft S, Pette D. 1994. Effects of chronic electrical stimulation on myosin heavy chain expression in satellite cell cultures derived from rat muscles of different fiber-type composition. Differentiation 58(1):37-46.

Wilson SJ, Harris AJ. 1993. Formation of myotubes in aneural rat muscles. Dev Biol 156(2):509-518.

Wilson SJ, Ross JJ, Harris AJ. 1988. A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats. Development 102(4):815-821.

Yang SY, Goldspink G. 2002. Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett 522(1-3):156-160.

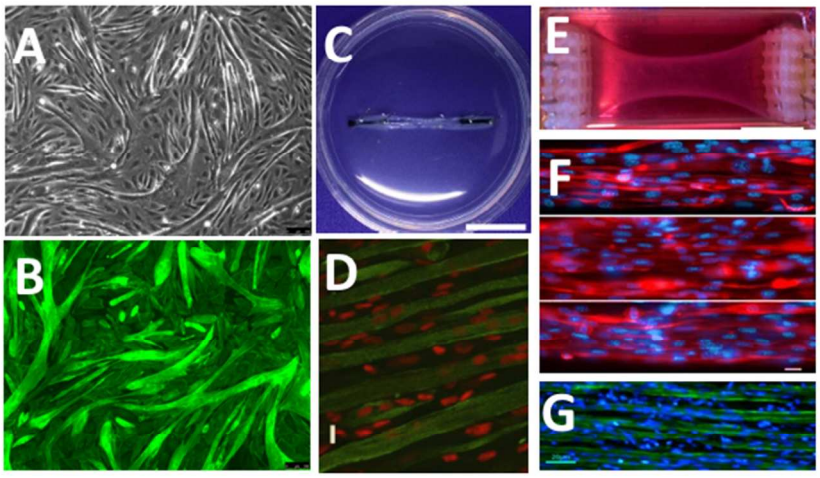

Figure 1. A) Typical Light and, B) fluorescent microscope images (10X, actin- green) of skeletal muscle cells (C2C12) in monolayer cultures and the resulting swirling myotube formations that occur when no tension is applied to cultures (scale bar 100 um) (images from unpublished images taken by Sharples Lab). C) Fibrin 3D bioengineered skeletal muscle using human derived muscle cells (scale bar 1 cm), used with permission from Martin et al. (2013) D) Aligned myotube formation in 3D bioengineered fibrin/human muscle derived cell constructs under tension (scale bar 20 m) demonstrated by fluorescently staining for desmin (green) and nuclei (red), used with permission from Martin et al., (2013). E) Type I collagen 3D bioengineered skeletal muscle using myoblast cell line C2C12s (scale bar 10 mm), used with permission from Player et al. (2014). F) Aligned myotube formation muscle using C2C12 myoblasts in collagen type I bioengineered constructs under unilateral tension (scale bar 20 m), used with permission from Player et al., 2014. G) Fluorescently stained muscle fibres from muscle tissue suggesting that bioengineered muscle morphologically mimics native skeletal muscle tissue (scale bar 20 m), used with permission from Smith et al. (2012).

Figure 1
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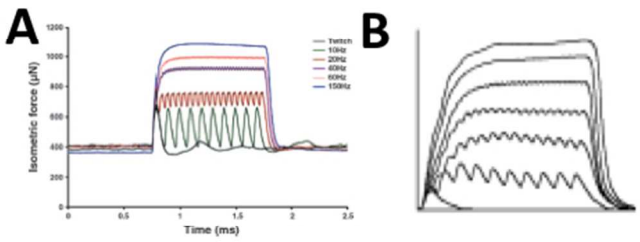

Figure 2. Data suggest that force-frequency traces (A, left) produced by 3D bioengineered skeletal muscle at different frequencies of electrical stimulation with summation beginning at 20 Hz and the constructs reaching a fused tetanus above 80 Hz are similar with (B) force frequency relationship of in-vivo muscle tissue observed in men and women. when stimulating at 1, 10, 15, 20, 30, 50 and 100 Hz (in ascending order). A) Used with permission from Huang et al. (2005) B) Used with permission from Wust, et al. (2003).
Figure 2
338x190mm (54 x 54 DPI)

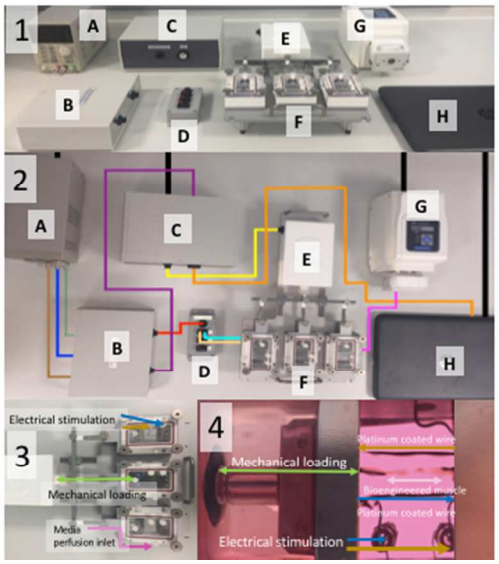

Figure 3. 1)An example of a novel bioreactor system allowing mechanical loading, electrical stimulation and perfused 3D cell culture chamber that can all be housed into an humidified Co2 incubator. A) Electrical stimulation module, B) Electrical stimulation output box, C) Control Module, D) Electrode anode/cathode Splitter, E) Bioreactor mechanical stimulation box, F) Bioreactor 3D cell culture chambers, G) Peristaltic pump/ perfusion Box, H) Laptop and controlling software. 2) Depicts electrical connection setup of the bioreactor. Black - Mains power, Orange – Laptop (G) to Control Module (C), Yellow - Control Module (C) to Bioreactor mechanical stimulation box (E), Brown – Anode (+ve) from Electrical Stimulation Module (A) to electrical stimulation output box (B) Green - Neutral from electrical stimulation module (A) to electrical stimulation output box (B), Blue- Cathode (-ve) from electrical stimulation module to electrical stimulation output box (B), Purple - Control Module (C) to electrical Stimulation Module (B), Red - Electrical Stimulation Output Box (B) to Electrode Anode/Cathode Splitter (D). Peach/Gold - Anode (+ve) from Electrode Anode/Cathode Splitter (D) to Bioreactor 3D cell culture chambers (F), Light blue –Cathode (-ve) from Electrode anode/cathode splitter (D) to Bioreactor 3D cell culture chambers (F), Pink – Perfusion Box (G) to bioreactor 3D cell culture chambers (F). 3) Detailed image of the bioreactor allowing simultaneous mechanical loading, electrical stimulation and media perfusion into the 3D cell culture chambers. 4) Detailed image of the bioreactor 3D cell culture chamber with a C2C12 fibrin self-assembling 3D myooid inserted. Image shows that the 3D muscle can be mounted and mechanically loaded via movement of the mechanical arm as well as electrically stimulated via surrounding platinum coated wires from the incoming electrical stimulation input.

Figure 3
338x190mm (54 x 54 DPI)