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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 accelerating bioreactor technology, providing simultaneous electrical and mechanical loading and flow perfusion *in-vitro*, may provide the basis for advancing knowledge into the future. We also envisage, that more studies using genetic, pharmacological and hormonal modifications applied in human 3D bioengineered skeletal muscle may allow for the discovery in-depth mechanisms underlying the response to exercise in relevant human testing systems. Finally, that 3D bioengineered skeletal muscle could 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 the 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 in an 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 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 in isolation (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 limited comparisons of monolayer vs. 3D bioengineered skeletal muscle with the most notable 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). However, electrical stimulation regimes in monolayer have broadly

mimicked a cell signalling exercise response, such as increased AMPK (Nedachi et al., 2008), enhanced glucose metabolism (uptake and oxidation) and complete fatty acid oxidation (Nikolic et al., 2012) in response to continuous low-frequency stimulation, where high-frequency stimulation to C2C12 myotubes resulted 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 and resistance exercise, 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 monolayer and 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. 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 aligned 3D mature muscle fibres *in-vitro* over a 2-3 week period (Martin et al., 2013). The use of bioengineered systems also permits 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 (Khodabukus and Baar, 2016) and therefore not discussed extensively in the current review. The majority of literature within this area looking to investigate mechanisms 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 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 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 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 (via SDS-PAGE) and protein synthesis (via incorporation of [^{3}H] Leucine into myosin extracts) by an average of 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) 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 being advantageous for evoking a 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-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 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 Y)-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*.

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 more 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 a 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 myogenically enriched cell populations myoids 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/m² that has been observed in adult skeletal muscle (Urbanchek et al., 2001). However, the specific force of 36.3 kN/m² reported by Huang et al., (2005) using myogenically enriched fibrin constructs was 12.5-fold higher than the 2.9 kN/m² 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 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 more 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 can 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 an increased 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), maybe not as 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 p70s6K phosphorylation, 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 mimicking 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 than 14 days, and/or that mimic resistance exercise type stimulation regimes via higher frequency contractions, and the 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 resistance exercise to evoke increases in muscle size.

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), 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 organisation 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 and indicative of a 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. an 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 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). The first study to mechanically stimulate 3D muscle used collagen coated elastic substratum's seeded with muscle cells isolated from embryonic avian pectoralis muscle was Vandenburgh and Karlisch (1989). 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 of mature myotubes) within 3D fibrin cast bioengineered skeletal muscle did lead to increased differentiation, myotube formation (myotube number), size (diameter and length), and increases in myogenic regulator factors gene expression, 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 again both ramp and cyclic loading (Auluck et al., 2005) to the resultant myotubes. 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 the 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 was 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 preceding hypertrophy if the signal was chronically maintained. Furthermore, there was no changes observed following loading for negative regulators of muscle mass, myostatin mRNA or ubiquitin ligases (MuRF and Marfbx) involved in protein degradation of muscle specific proteins gene expression. 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 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 P70S6K activation (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 has been shown to produce mature aligned myotubes (specially following electrical stimulation/motor neuron co-culture) may be important to elucidate the influence of mechanical stretch on the molecular mechanisms (and perhaps in combination with electrical stimulation for concentric contraction) to determine the underlying physiological adaptation to exercise mimicking stimuli. Furthermore, studies are limited to fairly acute stimulation and therefore limited 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 maybe 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 and the limited work conducted into the investigation of the underlying mechano-signal transduction thought to lead to hypertrophy, 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) in 3D muscle in response to high frequency intermittent regimes, the investigation of these pathways following the combination of electrical stimulation and mechanical stimulation in 3D bioengineered skeletal 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 (depicted in Figure 3), and are fully aseptic with perfused media control, potentially also 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.

Summary and 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 investigating 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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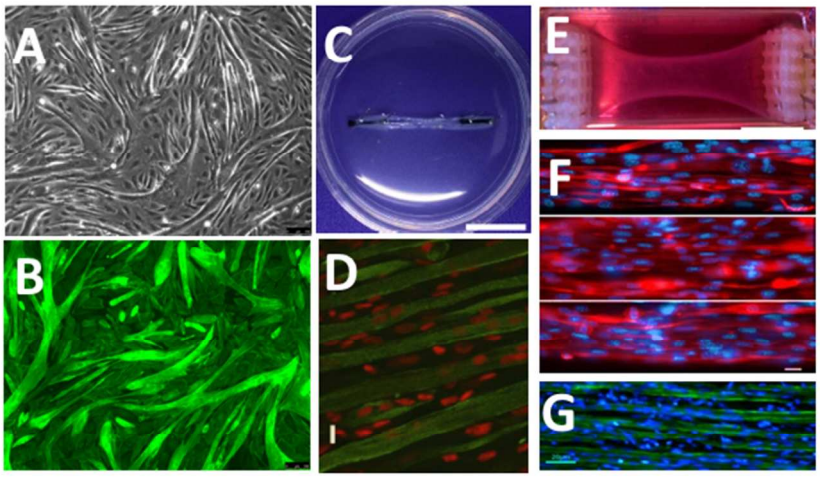

Figure 1. A) Typical Light and, B) fluorescent microscope images (10X, actin- green) of skeletal muscle cells (C2C12) in monolayer cultures and the resulting swirling myotube formations that occur when no tension is applied to cultures (scale bar 100 um) (images from unpublished images taken by Sharples 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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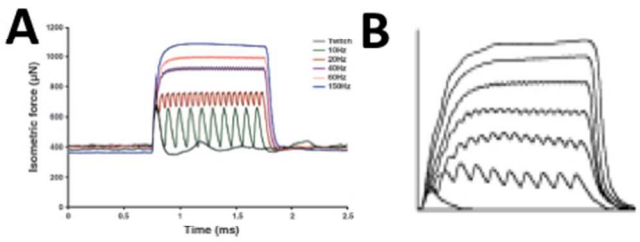

Figure 2. Data suggest that force-frequency traces (A, left) produced by 3D bioengineered skeletal muscle at different frequencies of electrical stimulation with summation beginning at 20 Hz and the constructs reaching a fused tetanus above 80 Hz are similar with (B) force frequency relationship of in-vivo 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)

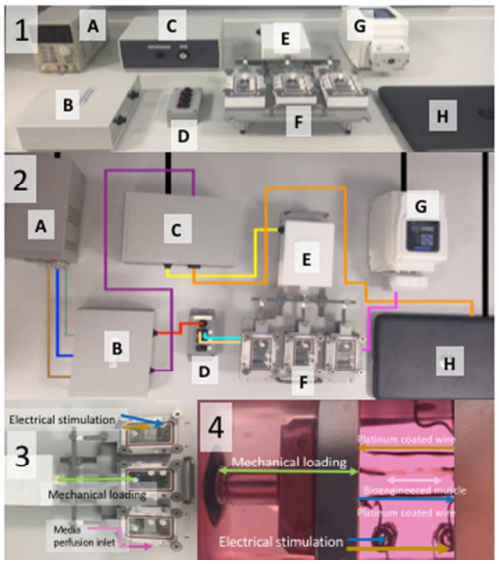

Figure 3. 1)An example of a novel bioreactor system allowing mechanical loading, electrical stimulation and perfused 3D cell culture chamber that can all be housed into an humidified Co2 incubator. A) Electrical stimulation module, B) Electrical stimulation output box, C) Control Module, D) Electrode 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)