Electrical stimulation increases hypertrophy and metabolic flux in tissue-engineered human skeletal muscle

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\begin{abstract}
In vitro models of contractile human skeletal muscle hold promise for use in disease modeling and drug development, but exhibit immature properties compared to native adult muscle. To address this limitation, 3D tissue-engineered human muscles (myobundles) were electrically stimulated using intermittent stimulation regimes at 1 Hz and 10 Hz. Dystrophin in myotubes exhibited mature membrane localization suggesting a relatively advanced starting developmental maturation. One-week stimulation significantly increased myobundle size, sarcomeric protein abundance, calcium transient amplitude (\textsim 2-fold), and tetanic force (\textsim 3-fold) resulting in the highest specific force generation (19.3mN/mm\textsuperscript{2}) reported for engineered human muscles to date. Compared to 1 Hz electrical stimulation, the 10 Hz stimulation protocol resulted in greater myotube hypertrophy and upregulated mTORC1 and ERK1/2 activity. Electrically stimulated myobundles also showed a decrease in fatigue resistance compared to control myobundles without changes in glycolytic or mitochondrial protein levels. Greater glucose consumption and decreased abundance of acetylcarnitine in stimulated myobundles indicated increased glycolytic and fatty acid metabolic flux. Moreover, electrical stimulation of myobundles resulted in a metabolic shift towards longer-chain fatty acid oxidation as evident from increased abundances of medium- and long-chain acylcarnitines. Taken together, our study provides an advanced \textit{in vitro} model of human skeletal muscle with improved structure, function, maturation, and metabolic flux.
\end{abstract}

1. Introduction

Healthy skeletal muscle function is essential for respiration, locomotion and metabolic homeostasis but can be compromised due to aging and genetic, neuro muscular, and metabolic diseases [1–5]. Despite decades of progress, our ability to treat devastating muscle diseases has been relatively limited, in part due to the failure of small animal models to accurately replicate human disease and predict clinically relevant drug responses [6,7]. The ability to engineer high-fidelity \textit{in vitro} models of human skeletal muscle would significantly advance studies of muscle biology and diseases and allow high-throughput drug development applications in a patient-specific manner. The physiological relevance and predictive power of such assays would be critically dependent on the ability to engineer muscle tissues with mature structural and functional properties [8–15].

We have previously shown that contractile function and contractile properties of rodent engineered skeletal muscle tissues can be improved via mesoscopic hydrogel molding [16], optimization of starting extracellular matrix protein composition [17], biochemical signaling [18], and dynamic culture [8]. Recently, we further refined these methodologies to engineer the first functional human skeletal muscle tissues made from primary myoblasts [19] or pluripotent stem cells [20], termed myobundles. Myobundles recapitulate many of the functional and morphological properties of native muscle and show clinically relevant responses to various drugs that promote or decrease muscle function [19]. However, the resulting myofiber size and isometric contractile properties of myobundles are still inferior to those of native adult muscle indicative of incomplete muscle maturation [19,21].

Complete skeletal muscle development and maintenance of adult muscle function and mass requires functional innervation. Absence of electrical activity in \textit{utero}, via denervation or paralysis, prevents full maturation and growth of skeletal muscle [22–25]. In adult muscle, denervation results in progressive muscle atrophy which ultimately yields myofibers of similar sizes to those reported in engineered tissues [26–28]. In addition to regulating muscle mass and size, the frequency and number of electrical stimuli regulates the contractile and metabolic...
phenotype of skeletal muscles [29–34]. Electrical stimulation of 2D human myotubes has been shown to increase myogenic differentiation and alter metabolic substrate utilization [35,36]. However, performing long-term electrical stimulation studies in 2D cell culture is hindered by the rapid myotube detachment from the underlying substrate [37]. In rodent myogenic cell lines and primary cells, electrical stimulation has been shown to increase engineered muscle force, protein expression, and differentiation markers [37–42]. However, the effects of electrical stimulation on tissue structure and contractile and metabolic function are yet to be studied in functional human-tissue-engineered muscles.

Here, we sought to improve the structural and functional properties of our human engineered muscle tissues via use of chronic intermittent electrical stimulation at two frequencies (1 Hz and 10 Hz). We found that electrical stimulation increases myobundle maturation and force production independent of frequency, but that higher stimulation frequency induces greater myofiber hypertrophy, shortens contractile relaxation time, and promotes shift to a more mature metabolic substrate utilization. With improved structural and functional properties, electrically stimulated human myobundles represent a promising in vitro platform for human disease modeling and drug development applications.

2. Methods

2.1. Human myoblast isolation

Nine human skeletal muscle samples (aged 8 to 34, 5 male and 4 female donors) were obtained through standard needle biopsy or surgical waste from donors under informed consent on Duke University IRB protocols (Pro00048509 and Pro00012628). Muscle samples were minced and digested with 0.05% trypsin for 30 min at 37 °C. Isolated cells were centrifuged and resuspended in growth media (GM, consisting of low-glucose DMEM, 10% fetal bovine serum, supplemented with rat SkGM bulletkit minus gentamycin and insulin (Lonza) and then preplated for 2 h to decrease fibroblast numbers. After pre-plating, cells were seeded onto to Matrigel (BD Biosciences) coated flasks and expanded bypassaging upon reaching 70% confluence. At passage 4, cells were detached and used to fabricate myobundles.

2.2. Engineered myobundle formation

Three-dimensional engineered muscle tissues (myobundles) were formed within polydimethylsiloxane (PDMS) molds containing two semi-cylindrical wells (7 mm long, 2 mm diameter), cast from 3D-machined Teflon masters, as described previously [19,20,43]. PDMS molds were coated with 0.2% (w/v) pluronic F-127 (Invitrogen) for 1 h at room temperature to prevent hydrogel adhesion. Laser-cut CereX® frames (9 × 9 mm², 1 mm wide rim) positioned around the 2 wells served toanchor bundle ends and facilitate handling and implantation. Specifically, a cell solution (7.5 × 10⁵ cells in 17.2 µl media per bundle + 0.5 µl 80 µg/ml aprotinin in water + 2 µl of 50 U/ml thrombin in 0.1% BSA in PBS) and a gelling solution (11 µl media + 10 µl Matrigel + 10 µl of 20 mg/ml Fibrinogen in DMEM) were prepared in separate vials on ice for up to six myobundles per vial. Aprotinin was included in the cell solution to reduce excessive fibrinolysis [44]. Gelling solution was added to the cell solution and mixed thoroughly, then each bundle was individual pipetted within the PDMS mold and onto the frame. Cell/hydrogel mixture was injected into the PDMS wells and polymerized at 37 °C for 30 min. Formed iSKM bundles were dynamically cultured on a rocker and fed with GM supplemented with 1.5 mg/ml 6-aminopenicaproic acid (ACA, Sigma) to reduce fibrinolysis for 4 days. Media was then switched to differentiation media consisting of low-glucose DMEM, supplemented with 2% horse serum, 100 µM l-carnitine, 5 µM palmitic acid, 5 µM sodium oleate and 2 mg/ml ACA with media changed daily.

2.3. Electrical stimulation of myobundles

Electrical stimulation was performed between week 1 and 2 of myobundle differentiation (Fig. S1A) using 1-h stimulation bouts separated by 7-h rests. Specifically, myobundles were continuously electrically stimulated at 1 Hz (Fig. S1B) or with a 0.5 s, 10 Hz pulse train every 5 s (Fig. S1C), thus delivering the same total number of stimulation pulses. The 1 Hz and 10 Hz stimulation frequencies were chosen based on a large number of 2D studies in human [35,36] and C2C12 [45,46] myotubes utilizing a similar 1 Hz protocol and 3D studies in primary rat [41] and C2C12 [37–40,47] myotubes using similar 10 Hz protocols. The total numbers of impulses delivered by the two protocols were kept constant to ensure that observed effects can be primarily attributed to changes in stimulation frequency [33]. During electrical stimulation, myobundles were placed between parallel carbon electrodes in a custom-made PDMS chamber (Fig. S1E). Electrical stimulation protocols were programmed using a custom-made pulse-generating Labview program. Electrical impulses delivered to myobundles were bipolar with 70 mA amplitude and 2 ms duration and were delivered using a D330 Multistim system (Digitem Limited).

2.4. Imaging of calcium transients

Myobundles expressing the MHCK7-gCaMP6 reporter were non-destructively monitored for calcium transients prior to isometric contractile testing as described previously [9,19]. A live imaging chamber with heated enclosure was used to maintain cells in physiological conditions during recording. Myobundles were placed in sterile Tyrode’s solution in a custom-designed glass-bottom bath containing carbon electrodes for stimulation. Fluorescent images were acquired through a FITC filter set at 50 fps rate under 4 × magnification using an Andor iXon camera affixed to a Nikon microscope. Video analysis was performed using Andor Solis software and relative changes in fluorescence signal were calculated by ΔF/F = (Peak-Trough)/(Trough-Baseline).

2.5. Measurement of isometric contractile properties

Electrically or chemically stimulated contractile force generation in myobundles was measured using a custom force measurement set-up as previously described [8,9,17,19,20]. Briefly, single myobundles were transferred to the bath of the force measurement set-up, maintained at 37 °C. One end of the bundle was secured by a pin to an immobile PDMS block and the other end was attached to a PDMS float connected to the force transducer mounted on a computer-controlled motorized linear actuator (Thor Labs). The sides of the frame were cut to allow myobundle stretch by the actuator and isometric measurement of contractile force. Initially, the myobundle was set to its baseline length using the motorized linear actuator. To assess the force-length relationship, myobundle was stretched by 2% of its culture length then stimulated by a 40 V/cm, 10 ms electrical pulse using a pair of platinum electrodes and the twitch force was recorded. At 12% stretch, 1 s long stimulations at 1, 5, 10, and 20 Hz were applied and the subsequent contractile force was recorded to assess the force-frequency relationship. Contractile force traces were analyzed for peak twitch or tetanus force, time to peak twitch, and half relaxation time using a custom MATLAB program.

2.6. Immunohistochemistry

Cell monolayers were fixed in 4% paraformaldehyde in PBS for 10 min and myobundles were fixed in 2% paraformaldehyde in PBS overnight at 4 °C. Following fixation, samples were washed in PBS then blocked in 5% chick serum with 0.2% Triton-X 100. Primary antibodies were applied at optimized concentrations for 45 min prior to application of fluorescently labeled secondary antibodies for 45 min. Images
were acquired using a Leica SP5 inverted confocal microscope and analyzed using ImageJ. Primary and secondary antibody information can be found in Supplementary Table 1.

2.7. Western blotting

Western blotting was performed as described previously [19,43,48]. Briefly, protein was isolated in RIPA lysis and extraction buffer with protease inhibitor (Sigma) phosphatase inhibitor cocktail (Roche) and protein concentration determined by BCA assay (Thermofisher). Western blot was performed using Bio-Rad Mini-PROTEAN gels and transferred using a Pierce 2000 powerblot semi-dry transfer system (Thermofisher). Primary antibodies were applied overnight 4°C before application of HRP-conjugated anti-mouse (1:20,000) and HRP conjugated anti-rabbit was purchased from SCBT (1:5000). Chemiluminescence was performed using Clarity Western ECL substrate (Bio-Rad). Images were acquired using a Bio-Rad Chemidoc and analyzed using ImageJ.

2.7.1. Quantitative RT-qPCR

RNA was isolated from myobundles using the Aurum Total RNA Mini Kit (Bio-Rad) and then reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR for muscle related genes was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) and was performed with iTaq Universal SYBR Green Supermix (Bio-Rad). Primary and secondary antibody information were acquired using a Leica SP5 inverted confocal microscope and analyzed using ImageJ.

2.8. Metabolomics

Two-week myobundles were snap frozen in liquid nitrogen immediately following the last bout of electrical stimulation. Acylcarnitines and amino acids were analyzed by flow injection tandem mass spectrometry using sample preparation methods described previously [49,50]. The data were acquired using a Waters AcquityTM UPLC system equipped with a TQ (triple quadrupole) detector and a data system controlled by MassLynx 4.1 operating system (Waters, Milford, MA). (MS/MS)

2.9. Statistics

Data are expressed as mean ± SEM. Statistically significant differences were determined by one-way ANOVA with Tukey’s post hoc test and p < 0.05 was considered significantly different.

3. Results

3.1. Electrical stimulation promotes growth and structural maturation of myobundles

To investigate the effects of simulated exercise on engineered human muscle structure and function, we applied 1-week electrical field stimulation to myobundles that had been differentiated for 7 days (Sup. Fig. 1A). Electrical stimulation was applied in 1-h bouts separated by 7-h pauses using a continuous 1 Hz train or a 0.5 s 10 Hz train applied every 5 s to ensure that the same number of electrical impulses were delivered by both protocols (Sup. Fig. 1B and C). Following 7-day stimulation, myobundles were collected and analyzed by immunohistology and contractile force testing.

From immunostaining analyses, we found that the number of nuclei in transverse cross-sections increased 1.5-fold compared to non-stimulated controls (CTL: 464 ± 24, 1 Hz: 689 ± 52, 10 Hz: 699 ± 42, Fig. 1A and C). Electrical stimulation also increased the size of the entire muscle bundle as evident from a 1.8-fold increase in myobundle cross-sectional area (CTL: 0.10 ± 0.004 mm², 1 Hz: 0.17 ± 0.013 mm², 10 Hz: 0.18 ± 0.012 mm², Fig. 1B and D) and resulted in a 1.6-fold increase in the total F-actin area (primarily labeling muscle cells [8,19,20]) compared to non-stimulated controls (CTL: 0.08 ± 0.003 mm², 1 Hz: 0.14 ± 0.010 mm², 10 Hz: 0.15 ± 0.011 mm²).

In contrast to developmentally immature cytoplasmic localization of dystrophin in 2D cultures (Fig. S2A), also reported in previous studies [51,52], all cross-sectional (Fig. 2A) and longitudinal (Fig. S2B) staining of 3D myobundles demonstrated mature localization of dystrophin in myotube membranes, which allowed precise quantification of myotube cross-sectional area and diameter. We found that myotube cross-sectional area (CTL: 49.0 ± 0.97 μm²; 1 Hz: 92.7 ± 3.05 μm²; 10 Hz: 110.2 ± 3.32 μm², Fig. 2B) and diameter (CTL: 9.9 ± 0.10 μm; 1 Hz: 13.1 ± 0.20 μm; 10 Hz: 14.4 ± 0.2 μm, Fig. 2C) significantly increased with electrical stimulation, which was further evident from a rightward shift in myotube diameter distribution (Fig. 2D). The 10 Hz stimulation showed the largest increase (P < 0.001) resulting from reduced proportion of myotubes with small diameters (4–8 μm) and increased proportion of the largest myotubes (> 20 μm, Fig. 2D).

Electrical stimulation also promoted myobundle sarcromerogization and maturity as evidenced from increased proportion of myotubes with sarcomeric α-actinin® cross-striations (Fig. 3A and B) and 20% increased myotube length (CTL: 468 ± 14.7 μm; 1 Hz: 577 ± 20.6 μm; 10 Hz: 549 ± 21.8 μm, Fig. 3C). Consistent with immunostaining results, western blot analysis showed that electrical stimulation upregulated expression of several sarcomeric proteins including dystrophin, myosin heavy chain, and sarcomeric α-actinin (Fig. 3E and F).

Protein synthesis in skeletal muscle is regulated by the protein kinase mTOR which interacts with several proteins to form two complexes: mTORC1 complex 1 (mTORC1) containing raptor and mTORC2 complex 2 (mTORC2) containing rictor [53,54]. Specifically, mTORC1 activity is required for and correlates with the degree of muscle hypertrophy in response to exercise in rodents and humans [55-58]. Therefore, we analyzed the phosphorylation of mTOR-related kinases immediately following the last bout of electrical stimulation in 2-week myobundles (Fig. 4A and B). We found that electrical stimulation significantly increased mTORC1 activity, measured by S6 phosphorylation, in a frequency-dependent manner. Frequency dependent upregulation was also found for mTOR phosphorylation at serine 2448, a marker of both mTORC1 and mTORC2 activation, as well as phosphorylation of ERK1/2, a protein kinase that can regulate mTORC1 activity [59]. Phosphorylation of Akt at serine 473, a positive regulator of mTORC1 activity and a marker of both mTORC1 and mTORC2 activation [53,60,61], was increased similarly by both electrical stimulation regimens, while the phosphorylation of AMPK, a negative regulator of mTORC1 that is activated during energy stress [62], was unchanged by stimulation. Overall, this protein kinase signaling analysis revealed upregulated activation of the anabolic mTOR, S6k, Akt and ERK1/2 pathways.

3.2. Electrical stimulation increases force generation in myobundles

To assess if the stimulation-induced changes in myobundle and myotube structure had an effect on muscle function, we measured twitch and tetanic forces of contraction (Fig. 5A). For each of the nine donors tested, electrical stimulation improved myobundle contractile force (Fig. 5B) yielding a 3-fold higher average twitch (CTL: 0.5 ± 0.03 mN; 1 Hz: 1.7 ± 0.13 mN; 10 Hz: 1.5 ± 0.16 mN, Fig. 5C) and tetanic (CTL: 0.9 ± 0.07 mN, 1 Hz: 3.4 ± 0.18 mN, 10 Hz: 3.2 ± 0.19 mN, Fig. 5D) forces compared to unstimulated control. Specific force (peak tetanic force divided by myobundle cross-sectional area), a marker of muscle maturity, was increased in similarly fashion (CTL: 9.1 ± 0.38 mN/mm²; 1 Hz: 19.3 ± 0.63 mN/mm²; 10 Hz: 18.9 ± 0.69 mN/mm², Fig. 5E). The higher specific force indicated that the increased absolute force production was a result of not only increased myobundle and F-actin cross-sectional areas, but also possible changes in calcium handling.
Fig. 1. Effects of electrical stimulation on myobundle structure. (A, B) Representative myobundle cross-sectional images stained for (A) nuclei (blue) and (B) filamentous actin (F-act, green) and nuclei in myobundles that were not stimulated (CTL) or electrically stimulated at 1 or 10 Hz. (C-E) Quantification of number of (C) nuclei per cross-section, (D) myobundle cross-sectional area and (E) F-actin area (n = 16 myobundles from N = 4 donors per group). *P < 0.01 vs. CTL. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Effects of electrical stimulation on myotube size. (A) Representative myobundle cross-sectional images stained for dystrophin (red). (B, C) Quantification of (B) myotube cross-sectional area and (C) myotube diameter. (D) Frequency histogram of myotube diameters (n = 500 myotubes from N = 4 donors per group). *P < 0.001 vs. CTL; #P < 0.001 vs. 1 Hz. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.3. Electrical stimulation increases calcium transient amplitude and alters contractile kinetics of myobundles

To determine if the increase in muscle force generation was related to increases in calcium transient amplitude, we transduced myoblasts with the genetic indicator of intracellular Ca\(^{2+}\) concentration, gCaMP6, driven by a muscle-specific promoter, MHCK7, as previously described [9,19,20]. From normalized changes in gCaMP6 fluorescence intensity (ΔF/F), we found that electrical stimulation of myobundles resulted in a 2-fold increase in Ca\(^{2+}\) transient amplitude (Fig. 6A and B). We next determined if this increase was accompanied by changes in twitch kinetics (Fig. 6C), a classical marker of muscle fiber type and developmental maturity. While electrical stimulation had no effect on time-to-peak tension (TPT, Fig. 6D), half-relaxation time (1/2RT, CTL: 236 ± 13.3 msec, 1 Hz: 244 ± 6.0 msec, 10 Hz: 192 ± 10.5 msec, Fig. 6E) was significantly shortened by 10 Hz stimulation. Additionally, 90% decay time (CTL: 534 ± 29.6 msec, 1 Hz: 414 ± 14.0 msec, 10 Hz: 351 ± 7.8 msec, Fig. 6F) was significantly shortened in frequency-dependent manner. Relaxation rates of cytoplasmic calcium transient are regulated, in part, by Ca\(^{2+}\) sequestration by calsequestrin, re-uptake to the sarcoplasmic reticulum by SERCA pump, and outflow through sarcolemma via PMCA. To determine if electrical stimulation induced changes in the expression of related Ca\(^{2+}\) handling genes, we performed qRT-PCR. In agreement with the faster relaxation kinetics,
Fig. 5. Effects of electrical stimulation on myobundle force generation. (A) Representative twitch and peak tetanic (20 Hz) force traces in 2-week myobundles that were either unstimulated (CTL) or electrically stimulated using the 1 Hz or 10 Hz protocols. (B) Normalized peak tetanic force generation from 9 independent donors (D1 - 9) (n = 4–22 myobundles per donor per group). (C-D) Average peak (C) twitch and (D) tetanic forces from all donors (n = 66 – 98 myobundles from N = 9 myobundles per group). (E) Specific force generation (n = 16–32 myobundles from N = 4 donors per group). *P < 0.01 vs. CTL.

Fig. 6. Effects of electrical stimulation on calcium handling in myobundles. (A) Representative traces of gCaMP6-reported Ca\[^{2+}\] transient signal from myobundles stimulated at 1 Hz (twitch) and 20 Hz (tetanus). (B) Quantified amplitudes of Ca\[^{2+}\] transients (n = 12 myobundles from N = 3 donors per group). (C) Representative twitch traces in non-stimulated (CTL) and 1 and 10 Hz stimulated myobundles. (D) Time-to-peak tension (TPT), (E) Half-relaxation time (1/2RT) and (F) 90% decay time (n = 66–98 myobundles from N = 9 donors per group). (G) Quantified mRNA expression of calcium-handling related genes (n = 9–12 myobundles from N = 3 donors per group). *P < 0.05 vs. CTL; #P < 0.05 vs. 1 Hz.
expressions of PMCA and the fast calsequestrin (CSQ1) and SERCA (SERCA1) isoforms were increased in the 10 Hz stimulation group (Fig. 6G).

3.4. Electrical stimulation decreases fatigue resistance in myobundles

We next determined if electrical stimulation induced changes in fatigue resistance, along with changes in force generation and calcium handling. Fatigue was measured as the loss of force generation during a continuous 30-s tetanic contraction (Fig. 7A). Electrical stimulation increased fatigability of myobundles as evidenced by a larger loss of force at the end of a 30-s contraction (CTL: $-55.8 \pm 3.03\%$, 1 Hz: $-77.7 \pm 1.59$, 10 Hz: $-81.3 \pm 2.31\%$, Fig. 7B). Typically, decreased fatigue resistance is associated with increased glycolytic metabolism or decreased oxidative metabolism. To determine if electrical stimulation increased glucose consumption, we measured media concentrations of glucose and lactate, a byproduct of anaerobic glycolysis, following 24-hr culture with and without stimulation. Glucose metabolism of electrically stimulated myobundles was increased as shown by decreased media concentration of glucose (Fig. 7C) and increased concentration of lactate (Fig. 7D). To determine if this increase in glucose consumption was associated with increased expression of glucose transporter proteins, we measured levels of the 2 major glucose transporters in skeletal muscle, GLUT1 and GLUT4. Furthermore, to determine if changes in fatigability resulted from changes in mitochondrial content, we assessed the expression of mitochondrial enzymes Complex III and V (Fig. 7E). We found no significant effects of electrical stimulation on the expression of GLUT1 and GLUT4 proteins or mitochondrial enzymes Complex III and V (Fig. 7F).

3.5. Electrical stimulation alters intracellular acylcarnitine species profile and amino acid levels

We next performed intracellular metabolomic analyses to determine if electrical stimulation of myobundles caused changes in their metabolic flux and substrate utilization. Carnitine regulates mitochondrial fatty acid import and oxidation and thus metabolic substrate utilization can be assessed by changes in carbon length and abundance of acylcarnitine species [50]. We thus profiled acylcarnitine species immediately following the last bout of electrical stimulation (Fig. 8 and Supplementary Table 3) and found that, consistent with upregulated fatty acid metabolism, stimulation decreased levels of acetylcarnitine (C2, Fig. 8A) and small chain acylcarnitine (C4/Ci4 and C6-DC/C8-OH, Fig. 8B–C) species, while the levels of medium-chain acylcarnitine species (C10, C14.1, and C18.3) were increased (Fig. 8D–F). For the 10 Hz stimulation only, we found an increase in the long-chain acylcarnitine species (C22, C22.1 and C22.2) (Fig. 8G–I), indicating increased longer-chain fatty acid metabolism. In addition to upregulated fatty acid oxidation, increase in amino acid catabolism is linked to increase in C3, C4/C5 and C5 acylcarnitine species. Therefore, we next determined if changes in intracellular amino acids concentrations occurred with electrical stimulation and found significant increases in 6 amino acids, namely the branch chain amino acids valine and leucine/isoleucine, methionine, proline, ornithine and citrulline (Fig. S3, Supplementary Table 4). Together, these results demonstrated that electrical stimulation of myobundles yields a shift toward fatty acid oxidation and increased production of several intracellular amino acids.

4. Discussion

In this study, we investigated the effects of chronic intermittent
electrical stimulation at 2 different frequencies on human engineered muscle structure, function and metabolism. Consistent with previous studies in engineered rodent muscles from primary and immortalized cells [37–41,63], we found that electrical stimulation of human myobundles increased their absolute force production by 3-fold. The reproducibility and robustness of this result was confirmed in myobundles generated from multiple donors of different age and sex. In addition to contractile force, electrical stimulation increased myobundle size, myotube diameter and length, as well as glucose and fatty acid metabolic flux, independent of stimulation frequency. We also found frequency-dependent (10 Hz vs. 1 Hz) increase in myotube hypertrophy, speed of twitch relaxation and metabolism of longer chain fatty acids.

The role of functional innervation in inducing and maintaining adult myofiber size and function is well established with denervated human myofibers showing diameters and specific forces more than 50-fold smaller than innervated myofibers [21,28,64–70]. In our study, electrical stimulation increased specific force of human myobundles by 2-fold (Fig. 5E) yielding the highest specific force reported for human engineered muscle (19.3 ± 0.63mN/mm²), but still significantly lower than those of native human skeletal muscles (150–250mN/mm²) [21,68]. Similarly, despite a 30–40% increase, myotube diameter (to 14.4 ± 0.2μm) remained small, in the size range of long-term denervated myofibers [28,64,66]. This result after only 7-day stimulation is expected, given that muscle development takes several months and that recovery of long-term denervated myofiber size to normal values by electrical stimulation requires 6–24 months [28,64,66]. On the other hand, long-term in vitro electrical stimulation could result in cell damage due to induction of electrochemical imbalance [71] and reactive oxygen species [72,73], which could be minimized by use of appropriate carbon electrodes [73,74] to apply bipolar, constant current stimuli [71]. Even if non-damaging to cells, long-term stimulation of avascular myobundles would likely increase muscle size and metabolic demand to yield hypoxic stress, cell death, and formation of necrotic core [8,13], which could be countered by applying oxygen carriers [75], perfusion bioreactors [76–78], or altered tissue geometry [13,79,80]. Nevertheless, long-term electrical stimulation alone may not permit complete engineered muscle maturation and additional neurotrophic factors [18], hormones [81], cells [82], and mechanical loading regimes [83] may be required to achieve adult myofiber size and force generating capacity in vitro.

![Fig. 8. Effects of electrical stimulation on the concentrations of intracellular acylcarnitine species in myobundles. 2-week myobundles were collected immediately following the last bout of stimulation for. (A-I) Intracellular concentration of (A) acetylcarnitine, (B-C) short-chain, (D-E) medium-chain, and (F-I) long-chain acylcarnitines. (N = 1 donor, n = 6 myobundles per group). *P < 0.05 vs. CTL; #P < 0.05 vs. 1 Hz.](image-url)
Skeletal muscle hypertrophy occurs through two mechanisms: 1) increased satellite cell activation, proliferation and fusion into pre-existing or new myofibers and; 2) increased net protein synthesis in muscle myofibers [11]. We found that electrical stimulation increased both nuclei number (Fig. 1C) and myofiber size suggesting increased satellite cell or myoblast proliferation, which we [9] and others [38, 84] have previously reported in rodent engineered muscles. Furthermore, a single bout of exercise in native rodent and human skeletal muscle has been shown to increase mTORC1 activity that correlates with increased muscle mass [55, 57], while inhibition of mTORC1 prevented muscle hypertrophy [58]. Activation of mTORC1 increases protein synthesis through 4E-BP1 and S6K phosphorylation-dependent increase in translation initiation, elongation and ribosome biogenesis [85, 86]. Additionally, Akt and ERK1/2 activation are required for maximal mTORC1 activity through phosphorylation of TSC2 and consequent increase in branch chain amino acid (BCAA) concentration [87, 90–94]. Despite greater increase in anabolic signaling (Fig. 4) and myotube diameter (Fig. 2B) with 10 Hz vs. 1 Hz stimulation, both stimulation protocols induced similar increase in myobundle and F-actin areas (Fig. 1D and E) and force generation (Fig. 5). Recent studies have shown that human muscle hypertrophy and strength are similarly increased during low-load, high-repetition and high-load, low-repetition exercises until their total works are matched [95, 96] and/or exercise is performed to failure [97, 98]. While each myobundle contraction at 10 Hz stimulation produced higher force, the total number of contractions was higher at 1 Hz stimulation (Fig. S1E), which resulted in similar total muscle activities likely yielding similar muscle growths. Additionally, exercise-induced muscle hypertrophy in vivo is often supported with increased capillary numbers and nutrient supply [99, 100], the absence of which in avascular myobundles may eventually limit the resulting tissue growth.

The electrical stimulation protocols used in our study did not induce mitochondrial biogenesis or glucose transporter upregulation (Fig. 7E and F), unlike other studies utilizing 2 week chronic (24 h/day) chronic electrical stimulation regimes [38, 39]. A likely difference is that our protocol did not induce metabolic stress as evidenced by the lack of AMPK phosphorylation (Fig. 5A and B) [62]. The increase in sarcomeric but not metabolic proteins in stimulated myobundles was a likely reason for their increased fatigability, although the potentially role of oxygen diffusion limit due to increased myobundle size cannot be ruled out. The intermittent electrical stimulation used here mimics neural firing pattern of fast fibers and the increased fatigability could also signal a shift toward a fast-fiber phenotype [33, 34]. Only with higher frequency electrical stimulation did we find shorter half-relaxation times (Fig. 6E) and increased expression of fast SERCA and CSQ isoforms, indicative of a partial fast-fiber type shift (Fig. 6G). A more complete shift to a fast fiber phenotype may require increases in media glucose concentration [101] and longer culture times and higher stimulation frequencies (> 40 Hz) as required for full fiber-type shifts in vivo [32–34].

A classical feature of increased muscle activity is an increase in metabolic flux and a shift toward fatty acid substrate utilization [102]. Here, we found that electrical stimulation increased both glycolytic flux (Fig. 7C and D) and fatty acid oxidation. Specifically, independent of electrical stimulation frequency, we found a decrease in short-chain acylcarnitine species and an increase in medium acylcarnitines as recently reported in human subjects following exercise [49, 103, 104]. Furthermore, with higher frequency electrical stimulation we found increased abundance of long-chain acylcarnitines, which increase with exercise intensity in humans [105]. The changes in acylcarnitine species profile are indicative of increased fatty acid oxidation and increased longer chain fatty acid metabolism which occurs with developmental maturation and limited glucose availability [102, 106, 107].

Importantly, the increased myotube size, abundance of cross-stra-tions, and muscle function all indicate a shift toward a more develop-mentally mature model of skeletal muscle. For increased physiologi-cal relevance, the human myobundles should accurately replicate structural and functional features of native adult skeletal muscle. For example, dystrophin is a cytoskeletal protein that functions in skeletal muscle to stabilize the plasma membrane and its mutations can result in the lethal Duchenne and Becker muscular dystrophies [108, 109]. Traditionally, in vitro 2D culture systems on both rigid tissue culture plastic and soft substrates show a primarily cytoplasmic localization of dystrophin [51, 52, 110]. Here we report for the first time correct mem-brane localization of dystrophin in an in vitro primary muscle culture system, which we have previously observed in human iPSC-derived myobundles [20]. These findings suggest that our 3D myobundle system is a superior biomimetic muscle equivalent compared to tradi-tional 2D culture. Furthermore, we show that electrical stimulation can be used to increase dystrophin protein content without affecting mature membrane localization (Fig. 3D and E). As such, we believe that human myobundles could be developed into an attractive in vitro system for studying human myopathies including Duchenne and Becker muscular dystrophy.

In summary, we have shown that electrical stimulation can promote engineered human muscle hypertrophy, structural organization, myo-tube maturation, force generation, and metabolic flux. Higher frequency stimulation yielded larger myotube size suggesting that further in vitro human myogenic maturation may be induced by stimulation patterns mimetic of in vivo muscle activity. The approach described here is a step towards engineering high-fidelity organ-on-a-chip models for studying human muscle biology and disease and discovering new therapeutics.

Data availability

All data supporting the results of these studies are available within the paper, supplemental materials or upon request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2018.08.058.

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