Effects of topological constraints on the alignment and maturation of multinucleated myotubes

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Funding information
Vetenskapsrådet, Grant/Award Number: 2015-03520 and 2016-00785; Human Frontier Science Program, Grant/Award Number: RGY0082-2014

Abstract
Microfluidic-based technologies enable the development of cell culture systems that provide tailored microenvironmental inputs to mammalian cells. Primary myoblasts can be induced to differentiate into multinucleated skeletal muscle cells, myotubes, which are a relevant model system for investigating skeletal muscle metabolism and physiology in vitro. However, it remains challenging to differentiate primary myoblasts into mature myotubes in microfluidics devices. Here we investigated the effects of integrating continuous (solid) and intermittent (dashed) walls in microfluidic channels as topological constraints in devices designed to promote the alignment and maturation of primary myoblast-derived myotubes. The topological constraints caused alignment of the differentiated myotubes, mimicking the native anisotropic organization of skeletal muscle cells. Interestingly, dashed walls facilitated the maturation of skeletal muscle cells, as measured by quantifying myotube cell area and the number of nuclei per myotube. Together, our results suggest that integrating dashed walls as topographic constraints in microfluidic devices supports the alignment and maturation of primary myoblast-derived myotubes.

Keywords
alignment, maturation, multinucleated myotubes, primary myoblasts, topological constraints

1 | INTRODUCTION

The generation of contraction forces by skeletal muscle is dependent on the highly anisotropic organization of individual skeletal muscle cells (Flaibani et al., 2009; Shimizu et al., 2009; Zhao et al., 2009). Myoblasts fuse to form long and thin multinucleated cells called myotubes that are aligned relative to the long axis of the muscle. When skeletal muscle tissues are damaged by injury or disease, satellite cells, which remain quiescent under normal physiological conditions, convert into myoblasts and initiate skeletal muscle regeneration (Bettadapur et al., 2016; Schultz et al., 1978), differentiating into skeletal muscle cells (Bentzinger et al., 2010; Braun & Gautel, 2011; Kuang et al., 2007; Pélault et al., 2007).

Bioengineered in vitro models of skeletal muscle development and regeneration typically aim to recreate in vitro the anisotropic organization of myotubes in vivo (Uzel et al., 2014). This has been achieved primarily by providing anisotropic topographic or chemical cues to primary myoblasts (Choi et al., 2008), induced pluripotent stem cells (iPSCs) (Osaki et al., 2018) or C2C12 cells, an immortalized myoblast cell line (Anene-Nzelu et al., 2013), during their differentiation into myotubes. Microfabrication methods such as electrospinning (Choi et al., 2008; Şenel Ayaz et al., 2014), microgrooves (Anene-Nzelu et al., 2013; Aubin et al., 2010; Bettadapur...
Microfluidics-based approaches offer the possibility to manipulate fluid flow, providing a strategy to control communication between cells that is mediated by factors present in the fluid phase (Maimon et al., 2018; Mills et al., 2018; Osaki et al., 2018; Southam et al., 2013; Uzel et al., 2016). C2C12 cells are widely used in microfluidic devices as they can be easily differentiated into myotubes. However, C2C12-derived myotubes do not adequately mimic the physiology (Osaki et al., 2018) and metabolic properties (Abdelmoez et al., 2020) of skeletal muscle. Myotubes derived from iPSCs resemble the properties of skeletal muscle cells more closely than C2C12 myotubes but their integration in microfluidic devices is complex, taking up to several months to implement (Osaki et al., 2018). Primary myoblasts differentiate into myotubes that retain many properties of native skeletal muscle in a relatively fast and consistent manner (Ruas et al., 2012; Taylor-Weiner et al., 2020). However, the use of primary myoblasts in microfluidics devices is hampered by difficulties in achieving successful differentiation on glass substrates and the fact that differentiation is dependent on adequate and uniform initial seeding density of the myoblasts, which can be difficult to achieve within microfluidics channels. In this study, we investigated the effects of topological constraints implemented on microfluidics devices on the alignment and maturity of myotubes derived from primary myoblasts. We aimed to design devices that supported a seeding density of primary myoblasts that was conducive to myotube differentiation and maturation while promoting the alignment of differentiated myotubes. We tested two types of topographic constraints: channels with conventional continuous walls (solid walls) and channels with walls composed of 250 μm long segments separated by 50 μm (dashed walls). The rationale for investigating the effects of dashed walls on myotube alignment and maturation was our observation, using simulations of fluid flow in channels, that dashed walls created pockets of low velocity in the channels, which we hypothesized could lead to improved control of seeding densities of primary myoblasts. Cells are grown on tissue culture polystyrene (TCPS) substrates in established protocols of primary myoblast differentiation into myotubes (Ruas et al., 2012), whereas glass often acts as the cell culture substrate in microfluidic devices. This is because microfluidics devices are usually made of polydimethylsiloxane (PDMS), which can be easily mounted on the glass to form a tight seal whereas PDMS adhesion to TCPS is generally poor. To facilitate the implementation of primary myoblast differentiation on microfluidic devices, we used a method we recently developed that improves the strength of adhesion between TCPS and PDMS, consisting of an oxygen plasma treatment of TCPS and PDMS surfaces followed by temperature annealing of both surfaces (Song et al., 2018). Using this setup, here we show that topological constraints promote myotube alignment, irrespective of whether the topological constraints are formed by solid or dashed walls. However, dashed walls were more effective at promoting myotube maturity. Together, these results suggest that using dashed walls as topographic constraints can be beneficial for the development of microfluidics devices when control of seeding density is a critical variable.

2 MATERIALS AND METHODS

2.1 Cell preparation

Mouse primary myoblasts were isolated and maintained as described previously (Ruas et al., 2012). Briefly, primary myoblasts were cultured in a growth medium (GM) consisting of a 1:1 mixture of high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) and Ham's F-10 nutrient mixture (Thermo Fisher Scientific), 20% fetal bovine serum (Thermo Fisher Scientific), 1% penicillin-streptomycin (Thermo Fisher Scientific), and 2.5 ng/ml basic fibroblast growth factor (Thermo Fisher Scientific) in sterile 150 mm polystyrene cell culture dishes (Sarstedt), previously coated with 50 μg/ml collagen solution (Rat tail type I; Corning) in 0.02% acetic acid (Sigma-Aldrich) overnight. Polystyrene (PS) dishes were dried before cell seeding. Cells were kept at less than 50% confluency in 20 ml GM in an incubator at 37°C and 5% CO₂, and the GM was changed every 72 h.

2.2 Microfluidic device fabrication

Microfluidic devices with topological constraints were fabricated by standard soft lithography and replica molding processes (Qin et al., 2010; Xia & Whitesides, 1998). For the molding process, glass slides (75 mm × 50 mm, Sigma-Aldrich) were used as a substrate. As SU-8 photoresist adheres poorly to glass, a thin layer of the negative photoresist, SU-8 2 (1-μm in thickness; MicroChem) was first spin-coated as an adhesive layer at 4000 RPM for 30 s (J. Liu, Song, et al., 2014). The photoresist was soft-baked on a hot plate at 65°C for 1 min and 95°C for 2 min before UV light exposure at 10 mW cm⁻² for 60 s without a photomask. The photoresist was then post-baked on a hot plate at 65°C for 1 min and 95°C for 2 min.

On top of the adhesive layer, SU-8 2100 (MicroChem) was spin-coated at 4500 rpm for 30 s, yielding a layer with a thickness of approximately 50 μm. The SU-8 layer was soft-baked on a hot plate at 65°C for 3 min and 100°C for 15 min, exposed to UV-light for patterning at 10 mW cm⁻² for 20 s, and post-baked on a hot plate at 65°C for 5 min and 100°C for 20 min. After being allowed to cool down to room temperature, the SU-8 layer was developed in propane glycol monomethyl ether acetate (Sigma-Aldrich), rinsed with isopropanol alcohol (Sigma-Aldrich), and dried with compressed nitrogen. The surface of the mold was treated with trimethylchlorosilane (Sigma-Aldrich) in a vacuum chamber overnight to silanize the surface for casting. We cast this SU-8 master with a mixture of polydimethylsiloxane (PDMS; Sylgard 184; Dow Corning) and curing agent at an 11:1 weight ratio. This ratio was chosen to promote the adhesion of the PDMS onto a PS petri dish (Song et al., 2016; Clark et al., 2002; Shimizu et al., 2009; Sun et al., 2015; Zhao et al., 2009) and micro-patterns (Atmani & Domian, 2013; H. Liu, Chen, et al., 2014; Nakamoto et al., 2014; Qin et al., 2010) have all been shown to produce aligned myotubes in vitro.
et al., 2018). The mixture was degassed in a vacuum chamber for 30 min and heated at 65°C for 2 h in an oven. After curing, the PDMS microchannel device was peeled off from the master, and biopsy punches (Kai Medical) were used to create 8 mm medium reservoirs as well as 2 mm inlet ports for cell seeding.

After the cleaning process, the surface of the device was treated with oxygen plasma at 300 mTorr (Harrick Plasma) for 1 min and coated with polyethylene glycol (PEG) solution (PEG; acetone: 1:1 in volume) on parafilm for 1 h at room temperature to maintain the hydrophilicity of the device over time (Kovach et al., 2014). We observed that the devices remained hydrophilic for ~48 h after PEG coating. Then, the device was rinsed with DI water, dried with compressed nitrogen, and attached to a PS cell culture dish. Finally, the device mounted on the dish was incubated at 65°C for 1 h in an oven, which resulted in reversible bonding with a tight seal and good adhesion. This method allows us to keep the device in a dry state for further processing (e.g., UV sterilization) and to use the standard extracellular matrix coating for primary myoblasts culture, avoiding the need for further physical and/or chemical surface modifications (Lee & Ram, 2009; Song et al., 2018; Sunkara et al., 2011; Tang & Lee, 2010).

2.3 | Cell culture in the microfluidic device

For sterilization, the device was exposed to UV light in a laminar flow cell culture hood for 20 min. Then, the device was filled with 50 µg/ml collagen solution overnight. After aspirating the collagen solution, we added 100 µl GM in each reservoir and kept the device in an incubator at 37°C until the flow was stabilized in the device. In the meantime, primary myoblasts were trypsinized and prepared in Eppendorf tubes.

Primary myoblasts were suspended in 3.5 µl GM and carefully injected into the device prefilled with GM through the inlet port, which promoted uniform cell seeding. The hydrostatic pressure formed by the cell suspension allowed the cells to flow into the device. We kept the device in an incubator at 37°C and 5% CO₂ overnight to allow primary myoblasts to adhere to the PS substrate on the device. The following day (D1), we exchanged GM with differentiation medium (DM) consisting of high-glucose DMEM, 5% horse serum (HS; Thermo Fisher Scientific; cat #16050-122), and 1% penicillin-streptomycin. DM was changed daily, and the device was kept in the incubator at 37°C and 5% CO₂ for 4 days (D1–D4). Three samples of each device were used for each batch of cells (technical replicates), and the experiment was repeated three times with different batches of cells (biological replicates) from new vials. For each device design, we employed three different densities of seeded primary myoblasts: 5 × 10³, 10 × 10³, and 20 × 10³ cells/mm².

2.4 | Immunostaining of cells on-chip

On D5, we fixed the cells using 10% formalin for 20 min at room temperature and permeabilized them with 0.1% Triton X-100 for 10 min at room temperature. Then, the nuclei and the cytoskeleton were labeled with 4,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) and Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific), respectively, for 40 min at room temperature.

2.5 | Imaging and image analysis

For each device, 12 consecutive locations along the topological constraints in the culturing area were imaged using a ×10 objective on a ZEISS inverted fluorescence microscope. Three types of image analysis were carried out with ImageJ: angle of myotube orientation, number of nuclei per myotube, and myotube area. To facilitate image analysis, the 12 consecutive images were aligned and assembled to generate a single image, covering half of the culturing area with constraints, thereby avoiding the areas of the input port and areas without constraints. Imaged cells that were found to overlap with other cells were manually separated by adding black lines at the intersections using Adobe Photoshop. The assembled ×10 images were used for analysis of cell orientation and maturity. The angles of myotube orientation were measured by using the Directionality plugin of ImageJ. Myotubes were identified by Alexa Fluor 488 Phalloidin staining. The number of nuclei in a myotube was determined by counting the number of DAPI positive nuclei contained in an Alexa Fluor 488 Phalloidin labeled cell. The area of a myotube was measured by counting the number of pixels per myotube. The image processing for counting nuclei and sizes was also performed by ImageJ. The processed data were collected and sorted by wall types, wall spacings, and seeding densities. Subsequently, the data for each condition were integrated and analyzed without normalization.

3 | RESULTS

3.1 | Microfluidic devices with topological constraints

We designed two types of microfluidic devices with integrated topological constraints formed by solid walls or dashed walls (Figure 1).
For each design, several spacings between the constraints were tested. The narrowest spacing, 25 µm, corresponds to about a single myotube width, and we further included spacings of 50, 100, and 200 µm (Table 1). The spacings were chosen to create a range of fluid flow conditions upon cell seeding, which we hypothesized would impact the density and uniformity of cell seeding within the channels.

We analyzed the fluid flow in the microfluidic devices using a commercial simulation software package (COMSOL Multiphysics 5.2) (Figure 2). We used boundary conditions corresponding to laminar flow in two dimensions, assuming no-slip conditions at the walls. The properties of the fluid (cell culture medium) were set to those of water and the entrance pressure of the fluid was set at a constant hydrostatic pressure of 39.2 Pa, with 4 mm height of the reservoir in the device. The entrance length was set as 0 m, and the outlet pressure was set as 0 Pa. The simulation shows that the velocity increases as the wall spacing \( w \) between constraints increases, as expected in conditions where the inlet and outlet pressure are kept constant. The dashed walls created low-velocity zones between the walls along the flow, which rendered the flow velocity nonuniform along the channel. The nonuniformity of the velocity along the channel decreased as \( w \) increased.

During cell seeding into the devices, the pressure drop decreased continuously until it reached zero, when the height of fluid in the inlet port and the reservoirs became the same, unlike the simulations, where the pressure drop was kept constant. Nonetheless, the observation of low-velocity pockets in the simulations of fluid flow in channels with dashed walls prompted us to investigate the effects of dashed and solid walls on the seeding patterns of primary myoblasts in channels and their maturation into myotubes. In the channels with solid walls, most of the cells moved downstream continuously until the flow stopped when the pressure drop reached zero. However, in the channels with dashed walls, cells were able to stay at and near the low-velocity zones, especially in the channels with wall spacings of 25 and 50 µm, resulting in more uniform cell seed in channels with dashed walls compared to solid walls (Figure 3). Cell spreading could be observed at 2 h after seeding, primarily in the wider wall spacings (100 and 200 µm).

### 3.2 Primary myoblasts differentiate into myotubes on the microfluidics devices

To monitor the differentiation of primary myoblasts into myotubes, primary myoblasts were seeded at a density of \( 10^4 \) cells/mm² on a device with solid walls with 200 µm wide spacings (W200S) and incubated for 3 h to allow the cells to adhere to the underlying tissue culture polystyrene substrate on the device. Then, the microfluidic device was mounted on a cell monitoring device (Cytomate Technologies B.V.), and microscopy images of primary myoblasts were acquired every 15 min. The medium was changed to a DM at 20 h (D1), and fresh DM was supplied every day until Day 4 (D4). Primary myoblasts in DM started to differentiate and fuse, forming long

### TABLE 1 Design parameters of the microfluidic devices

| Device | Type of constraint | Wall spacing (w) (µm) | Culturing area (mm²) | # of constraints |
|--------|-------------------|-----------------------|---------------------|-----------------|
| W25S   | Solid wall        | 25                    | ~7                  | 6               |
| W25D   | Dashed wall       |                       |                     |                 |
| W50S   | Solid wall        | 50                    | ~8                  | 4               |
| W50D   | Dashed wall       |                       |                     |                 |
| W100S  | Solid wall        | 100                   | ~10                 | 3               |
| W100D  | Dashed wall       |                       |                     |                 |
| W200S  | Solid wall        | 200                   | ~10                 | 1               |
| W200D  | Dashed wall       |                       |                     |                 |

During cell seeding into the devices, the pressure drop decreased continuously until it reached zero, when the height of fluid in the inlet port and the reservoirs became the same, unlike the simulations, where the pressure drop was kept constant. Nonetheless, the observation of low-velocity pockets in the simulations of fluid flow in channels with dashed walls prompted us to investigate the effects of dashed and solid walls on the seeding patterns of primary myoblasts in channels and their maturation into myotubes. In the channels with solid walls, most of the cells moved downstream continuously until the flow stopped when the pressure drop reached zero. However, in the channels with dashed walls, cells were able to stay at and near the low-velocity zones, especially in the channels with wall spacings of 25 and 50 µm, resulting in more uniform cell seeding in channels with dashed walls compared to solid walls (Figure 3). Cell spreading could be observed at 2 h after seeding, primarily in the wider wall spacings (100 and 200 µm).

#### FIGURE 2 Simulated velocity profiles of the flow in the microfluidic devices. The unit of the velocity on the color legend is m/s. The fluid flows from right to left [Color figure can be viewed at wileyonlinelibrary.com]
multinucleated myotubes, which could be observed within a day after changing the medium (Figure 4). On Day 2 (D2), longer myotubes started to be observed, and on Day 3 (D3), thick and long myotubes were observed. Single primary myoblasts that did not differentiate into myotubes were also observed throughout the experiment.

3.3 | Topographical constraints induced cell alignment

Immunofluorescence imaging at Day 5 (D5) revealed that topographical constraints induced the alignment of primary myoblasts and myotubes in the devices (Figure 5). The fraction of aligned cells was calculated as the fraction of cells that had a long axis oriented within 10° of the direction of the walls. At all seeding densities tested (5K: 5 × 10³ cells/mm², 10K: 10 × 10³ cells/mm², and 20K: 20 × 10³ cells/mm²) cell alignment showed a trend of decreasing with the increasing spacing of the walls (Figure 6). The fraction of aligned cells tended to be slightly lower in channels with dashed walls than solid walls, and this difference was statistically significant for wall spacings of 200 µm at 10K (p < .05) and 25 µm at 20K (p < .01). Immunofluorescence images showed that in channels with dashed walls, the cells were able to cross the walls, which contributed to the decrease in cell alignment compared to cells in channels with solid walls.

3.4 | Topographical constraints affected myotube maturation

We investigated the effects of the topological constraints on the maturity of primary myoblast-derived myotubes by measuring myotube areas and counting the nuclei numbers per myotube (Figure 7). For this analysis, we considered only multinucleated myotubes that contained two or more nuclei. The myotube area was generally larger in devices with channels containing dashed walls than in channels with solid walls. In particular, at 5K, a statistically significant increase in myotube area in dashed walls compared to solid walls was observed for wall spacings of 25 µm (p < .001). At 10K, statistically significant increases in myotube area for channels dashed walls compared to solid walls were observed for spacings of 100 and 200 µm (p < .05 and p < .001, respectively) and at 20K,
dashed walls with spacings of 200 µm exhibited significantly larger myotube areas than solid walls ($p < .001$).

To further investigate the effect of topological constraints on the maturity of myotubes, we measured the numbers of nuclei per myotube for the different wall types, wall spacings, and cell densities (Figure 8). The results of these analyses were largely consistent with the results for the myotube area, confirming that larger myotubes contain more nuclei. The analysis of effects of wall type showed a tendency for higher numbers of nuclei per myotube for dashed walls compared to solid walls for most conditions, with statistical significance obtained for cells seeded at 5K in devices with 25 µm wall spacings ($p < .01$). At a seeding density of 10K, 100 and 200 µm wall spacings dashed walls supported higher numbers of nuclei per myotube than solid walls ($p < .1$ and $p < .001$, respectively), and for cells that were seeded at 20K, 25 and 200 µm wall spacings showed higher numbers of nuclei per myotube ($p < .1$ and $p < .001$, respectively).

![Figure 5](wileyonlinelibrary.com)

**FIGURE 5** Immunofluorescence images of cells on D5 in the microfluidic devices seeded at densities of (a) 5K, (b) 10K, and (c) 20K. (d) Schematics of the topological constraints in the microfluidic devices. Nuclei are labeled with DAPI (blue), and myotubes are labeled with Alexa Fluor 488 Phalloidin (green). Scale bar = 200 µm. DAPI, 4',6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

![Figure 6](wileyonlinelibrary.com)

**FIGURE 6** Fraction of aligned myotubes in the microfluidic devices at seeding densities of (a) $5 \times 10^3$ cells/mm$^2$ (5K), (b) $10 \times 10^3$ cells/mm$^2$ (10K), and (c) $20 \times 10^3$ cells/mm$^2$ (20K). Both multinucleated myotubes and cells containing a single nucleus were included. The plots show the means and standard deviations of three biological replicates. *$p < .05$ and **$p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]
respectively). Although the cell seeding density did not have clear effects on cell alignment (Figure 6), a cell density of 20K had a negative effect on the indicators of cell maturity studied, cell area, and the number of nuclei per myotube, which was statistically significant for all conditions, except for 200D. Together, these results suggest that there is an interplay between cell seeding density and wall spacing in determining the effects of dashed walls versus solid walls in myotube maturity.

4 | DISCUSSION

Robust and biomimetic in vitro models of myotubes are required to investigate fundamental mechanisms of skeletal muscle physiology. Microfluidics devices allow for control of the microenvironment of skeletal muscle cells in culture, providing insights into skeletal muscle physiology in health and disease that are not achievable using conventional cell culture systems. For example, the study of the interactions between skeletal muscle and peripheral neurons at the neuromuscular junction has greatly benefited from the use of microfluidics technologies (Uzel et al., 2014). However, the cell lines that are widely used to generate myotubes in microfluidics devices, primarily C2C12 cells, do not recapitulate many key aspects of skeletal muscle physiology (Osaki et al., 2018). Therefore, there is a need to improve the compatibility of microfluidics devices with primary myoblast-derived myotubes, while preserving the advantages that microfluidics devices offer, such as providing alignment for skeletal muscle cells to achieve a biomimetic anisotropic morphology as well as control of fluid flow. In this study, we introduced topological constraints in microfluidics channels to obtain aligned myotubes derived from primary myoblasts, mimicking the anisotropic organization of native skeletal muscle. Myotube area and the number of nuclei per myotube are indicators of myotube maturity and are critical variables in the development of in vitro models of skeletal muscle. We investigated the effects of introducing dashed walls as topographic constraints in microfluidics channels to improve the uniformity of cell seeding compared to solid walls in the devices with the goal of obtaining enhanced maturity of the resulting myotubes. We observed that a seeding density of 20K in the devices led to lower myotube area and the number of nuclei per myotube compared to seeding densities of 5K and 10K. However, compared to
solid walls, primary myoblast-derived myotubes differentiated in devices with dashed walls generally showed higher numbers of multinucleated cells, and larger myotube areas. Differentiation of primary myoblasts into multinucleated myotubes in vitro is particularly sensitive to the seeding uniformity and density and is undermined by densities that are too low or too high. We suggest that a cell seeding density of 20K was inhibitory for cell differentiation and maturation. Furthermore, we propose that channels with dashed walls resulted in more uniform cell seeding, leading to improved cell maturity, due to the low-velocity zones created by the dashed walls. In addition, it is possible that cell-cell communication through soluble factors, facilitated in channels with dashed walls compared to solid walls, had an impact on cell differentiation and maturation.

Myotubes derived from primary myoblasts were maintained in the microfluidic devices for up to 5 days. This was enabled by the fact that the microfluidic devices were mounted directly on TCPS, which is the standard substrate for myotube differentiation (Ruas et al., 2012), instead of glass that is generally used as a substrate in microfluidic devices. However, the use of TCPS instead of glass hindered analysis by confocal microscopy. Although we observed the formation of multinucleated myotubes through the cultured area of the devices, we also detected mononucleated primary myoblasts in all conditions, signaling a need for further improvements to promote the maturation of myotubes.

5 | CONCLUSIONS

We investigated the effects of topological constraints integrated in microfluidic models on the alignment and the maturity of multinucleated myotubes derived from primary myoblasts. We demonstrated that topological constraints in the form of dashed and solid walls were able to direct the alignment of myotubes derived from primary myoblasts. Importantly, dashed walls promoted the maturity of myotubes, resulting in larger numbers of nuclei per myotube and larger myotube areas. Together, these data show that dashed walls as topographic constraints integrated with microfluidic devices promote the maturation of myotubes derived from primary myoblasts.

ACKNOWLEDGMENTS

This study was supported by grants from the Human Frontier Science Program Young Investigator Award (A1T, JLR, RGY0082-2014) and the Swedish Research Council (A1T; VR 2015-03520 and JLR; VR 2016-00785).

AUTHOR CONTRIBUTIONS

Ki-Young Song planned, performed, and analyzed experiments. Jorge C. Correia provided primary myoblasts. Jorge C. Correia and Jorge L. Ruas provided expertise in muscle biology. Ana I. Teixeira supervised the study. Ki-Young Song and Ana I. Teixeira wrote the manuscript. All authors contributed to manuscript revision and gave approval to the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Song K-Y, Correia JC, Ruas JL, Teixeira AI (2021). Effects of topological constraints on the alignment and maturation of multinucleated myotubes. Biotechnology and Bioengineering, 118, 2234-2242. https://doi.org/10.1002/bit.27731