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Fabrication of Skeletal Muscle Constructs by Topographic Activation of Cell Alignment

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Abstract

Skeletal muscle fiber construction for tissue-engineered grafts requires assembly of unidirectionally aligned juxtaposed myotubes. To construct such a tissue, a polymer microchip with linearly aligned microgrooves was fabricated that could direct myoblast adaptation under stringent conditions. The closely spaced microgrooves fabricated by a modified replica molding process guided linear cellular alignment. Examination of the myoblasts by immunofluorescence microscopy demonstrated that the microgrooves with subcellular widths and appropriate height-to-width ratios were required for practically complete linear alignment of myoblasts. The topology-dependent cell alignment encouraged differentiation of myoblasts into multinucleate, myosin heavy chain positive myotubes. The monolayer of myotubes formed on the microstructured chips allowed attachment, growth and differentiation of subsequent layers of linearly arranged myoblasts, parallel to the primary monolayer of myotubes. The consequent deposition of additional myoblasts on the previous layer of myotubes resulted in three-dimensional multi-layered structures of myotubes, typical of differentiated skeletal muscle tissue. The findings demonstrate that the on-chip device holds promise for providing an efficient means for guided muscle tissue construction.

Keywords

skeletal myogenesis; microtopography; cell alignment; tissue engineering

Introduction

Skeletal muscle accounts for 48% of the human body weight and is largely responsible for the voluntary control of movement in the body. The skeletal muscle is mainly composed of striated multinucleate myotubes that span the entire length of the muscles. The myotubes, organized in arrays parallel to one another along a common axis, provide muscles the ability to generate contractile forces required for body movement. Loss of muscle integrity due to inherited muscular dystrophies, accidents, or removal of tumor tissues, in many cases, leads to irreparable impairment of morphological and physiological function. This has necessitated development of tissue-engineered striated muscle implants to mimic the cellular organization and physiologic function of skeletal muscle, for the repair and replacement of

damaged muscle (Bach et al., 2004; Charge and Rudnicki, 2004; Fuller et al., 1992; Law et al., 1993). In particular, there has been a long-standing research interest to direct the fusion of muscle precursor cells (myoblasts) to generate myotubes, which mature into myofibers. The fusion of these immature skeletal muscle cells has also been used in cellular cardiomyoplasty in attempts to regenerate cardiac muscle tissues by injecting these cells into damaged myocardium (Chiu et al., 1995; Murry et al., 1996; Taylor et al., 1998).

While skeletal precursor muscle cells can be cultivated and differentiated in vitro, these cells invariably differentiate into unorganized and nonfunctional myotubes. Therefore, technologies are needed to direct the satellite cells/myoblasts to form highly organized assemblies that mimic the natural skeletal muscle morphology. To this end, a variety of engineering approaches have been developed to regulate the alignment of skeletal myotubes, including the use of aligned collagen gel system (Yan et al., 2007), microcontact printing of cell-adhesion domains (Parker et al., 2002), and cyclic mechanical loading (Powell et al., 2002). Topographic modulation is shown to be an effective method for regulating cell growth and proliferation. When being cultured in a microtopographic environment, the cells usually undergo a remodeling process and adapt to the local environments by contact guidance of the microstructures whose characteristic dimensions are comparable to those of the cells (Curtis and Wilkinson, 1997; Oakley and Brunette, 1993; Sniadecki et al., 2006; Snyder and Desai, 2001; von Recum and van Kooten, 1995). Such adaptation often leads to directional cell growth and proliferation along edges of the microstructures, which is largely due to the reorganization of the cytoskeletal structures (Hyun et al., 2006; Zhao and Zhang, 2007). Studies have shown that the skeletal myoblasts and myotubes can align to nano fibers (Neumann et al., 2003), microgrooves (Charest et al., 2007; Huang et al., 2004), microcantilevers (Das et al., 2007), and continuous wavy micropatterns (Lam et al., 2006). Nonetheless, most of these studies have focused on the alignment of cells, while little is reported on the fabrication of fully differentiated three-dimensional organized myotubes. The objective of this study is to develop a microfabricated platform to investigate the efficiency of microtopography activated myotube formation, and to facilitate on-chip fabrication of three-dimensional multilayered juxtaposed myotubes that mimics striated muscle with multinucleated myosin heavy chain positive myotubes. Figure 1 shows the schematic drawing of our work. A polymer microchip with periodic linear microstructures was developed. The linear structures with subcellular widths and medium height-to-width ratios were used to direct growth and proliferation of skeletal myoblasts along the longitudinal axes to form the primary cell layer. The primary layer directed growth and differentiation of subsequently seeded myoblasts to form additional cell layers. By alternating the cycles of cell growth and differentiation, multi-layered muscle like tissues with organized structures were successfully formed.

Following the Introduction Section, the materials and methods used in the work are first described, including microchip fabrication, protocols of cell culture, cryosection, and immunofluorescence microscopy. In Results and Discussion Section, the effectiveness of the microchip in regulating myoblast adaptation and proliferation is examined, followed by the study of the microchip effectiveness in regulating directional myoblast differentiation. The formation of multi-layered muscle fibers is demonstrated by alternating myoblast growth and differentiation. Finally, the possibility of using the microchip for repeated fabrication of multi-layered muscle fibers is studied. The results suggest that the microchip has the promise for closely mimicking native skeletal muscle organization.

Materials and Methods

Microchip Fabrication

A polymer microchip with linear microstructures made of poly(dimethylsiloxane) (PDMS) (Sylgard® 184, Dow Corning, Midland, MI) were fabricated using a modified replica molding process. Figure 2a–d shows the fabrication process. First, periodic linear microstructures with 2 μm in the width and 2 μm in the spacing were created using computer-aided design software and printed on high-resolution e-beam chrome mask. In order to investigate the cell adaptation to the microtopography and compare that with conventional two-dimensional cultures, smooth surface topography were patterned between two neighboring arrays of linear microstructures. The mask was then used in photolithography to transfer the microfeatures to a thick positive photoresist layer (AZ resist 9260, Clariant Corp., Charlotte, NC) on a glass substrate using I-line (365 nm) UV illumination. The thickness of the photoresist ranged from 2 to 7 μm , depending on the spin rate (measured using scanning electron microscopy (SEM), Hitachi S-3000). The periodic patterns of the microstructures formed after exposure (EVG620, EV Group, Austria) and development (AZ 400K 1:4 developer, Clariant Corp.). Afterwards, the polymer microchip was molded by casting PDMS pre-polymer (mixing at 10:1) on the patterned substrate. The pre-polymer mixture was cured at 65°C on a hotplate surface for 60 min. The cured PDMS substrate was released after dissolving the AZ resist from the base substrate. This is to avoid possible structure tear during the peeling process and to allow the formation of intact microstructures with medium height-to-width ratios. Figure 2e and f show the optical and SEM micrographs of representative as-fabricated microstructures. In this work, microgrooves with subcellular widths were used for effectively directing the myoblast proliferation and differentiation. By making the width of microgrooves well below the characteristic dimension of a single skeletal myoblast and the height of the microgrooves comparable to the cell dimension, the cell in the primary layer needs to squeeze itself to sit either on the bottom of the microgroove or on the top of the ridge. This leads to a highly anisotropic cellular profile. Such anisotropic profile is useful to orient the neighboring cells along the same direction through their direct contacts, thus forming three-dimensional tissues with organized structures. To compare the alignment effectiveness between “shallow” microgrooves and “deep” microgrooves, microgrooves with small heights were also fabricated, where the cells in the primary layer can easily lie across several neighboring microgrooves and poorly aligned. Specifically, microgrooves with two different height-to-width ratios were patterned: one with 2 μm in width and 2 μm in height (height-to-width ratio 1:1, “shallow” microgrooves) and the other with 2 μm in width and 7 μm in height (height-to-width ratio 3.5:1, “deep” microgrooves). The resulting thickness of the PDMS substrate was about 0.5 mm. The PDMS substrate was then sealed to the bottom surface of a 100 petridish. Finally, the petridish carrying the PDMS substrate was autoclaved for sterilization, and kept sealed prior to the cell culture.

Cell Seeding and Culture

All the procedures related to cell seeding and culture were carried out inside a Class II biosafety cabinet. Before cell seeding, the PDMS substrate was rinsed three times with phosphate buffered saline (PBS) and three times with the tissue culture medium [Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin]. Skeletal myoblasts C2C12 (ATCC, MD) were cultured according to the protocols described earlier (Lan et al., 2005), and seeded ($1 \times 10^5/\text{cm}^2$) on the pre-sterilized and pre-treated PDMS chips. Cells were grown in tissue culture medium until they reached a high level of confluence. Subsequently, fetal bovine serum in the tissue culture medium was replaced with 2% horse serum, to facilitate

myotube formation in the absence of insulin. The cell adaptation on the linear microgrooves was observed using optical microscopy every 24 h from the start of culture.

Cryosection Protocol

The histological samples were prepared by cryosection. Cells cultured on the PDMS microchip were fixed with 10% formalin for morphological observation. The fixed samples were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and then frozen at -80°C . The frozen samples were vertically cryosectioned along microgrooves to 12-mm sections using a cryostat (CM3050S; Leica Microsystems, Bannockburn, IL) to reveal their layered nature. The cryosectioned samples were rinsed four times with PBS to remove residual OCT and permeabilized with 0.2% TritonX-100 (Sigma–Aldrich, St. Louis, MO) for 30 min.

Immunofluorescence Microscopy

The differentiation of myoblasts into myotubes was monitored by immunofluorescence microscopy (Axioplan2, Carl Zeiss, MN) using mouse anti myosin heavy chain antibody (MF-20, Developmental Study Hybridoma Bank at University of Iowa) and CY3-conjugated goat anti mouse secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA). Beta-actin was stained with fluorescein isothiocyanate labeled phalloidin to assess the alignment of myoblasts and myotubes. The stained samples were counterstained with 4',6-Diamidino-2-phenylindole dilactate (DAPI; Sigma Aldrich, MO) to assess the multinucleate myotube formation. The cell morphology was observed using bright-field illumination and a phase contrast condenser.

Results and Discussion Cell

Adaptation and Proliferation

Microscopic examination of the microchips revealed that the cell adaptation highly depends on the height-to-width ratios of the microgrooves. In the microchips containing 2 μm deep microgrooves, the cells exhibited limited cell alignment after 24 h of cell culture by spreading across several microgrooves (Fig. 3a), which suggests that the shallow microgrooves (with approximate height-to-width ratios of 1) may be insufficient in providing topological guidance for myoblast alignment. In the microchips containing 7 μm deep microgrooves, after 24 h most cells preferentially descended into the grooves with cell membranes extending on the bottom surface. The cells thus exhibited a perfect alignment parallel to the longitudinal direction of the microgrooves (Fig. 3b). This is unlike the earlier reports where the cells preferred residing on the top of the ridges (Charest et al., 2007; Lam et al., 2006), which may be due to the fact that the subcellular width of the microgrooves provides limited area for the cell attachment on tops of the ridges.

In the initial culture, cells accommodated on the chip without forming contact points with the cells in the neighboring microgrooves. This affected subsequent cell proliferation (Fig. 4), where the dividing myoblasts remained within the same microgroove and formed arrays along the longitudinal axis. As the proliferation proceeded, while still remaining organized along the longitudinal axis, cells exhibited spreading on the transverse axis and started to proliferate and grow on the parallel ridges of the chips (Fig. 4c and d), which is practically due to the fact that the proliferated cells provided more adhesive areas around the tops of the microridges. After 96 h of culture, myoblasts cultured using the 7 μm deep microgrooves reach nearly 90% confluence, and exhibited highly organized parallel assembly of cells. Contrarily, myoblasts grown on the areas with smooth surface topography did not exhibit any obvious orientation. The experiments showed that the time it took the cells for reaching

the confluence on the smooth surface topography and on the microgrooves was approximately the same.

Differentiation of Myoblasts

Addition of tissue culture medium containing 2% horse serum to the confluent cultures of myoblasts on the microchip with 7 μm deep microgrooves resulted in the formation of multinucleated myotubes, well aligned along the longitudinal axis of the microgrooves (Fig. 5a). Figure 5b shows the histogram of myotube alignment in different areas, where the orientation of each myotube was normalized and denoted by an angle ranging from 0° to 90°. The angle 0° denotes the direction normal to the longitudinal axis, and the angle 90° denotes the direction along the longitudinal axis. The percentages of angular distribution showed the difference of myotube orientation in various areas. All the myotubes on the microgrooves were perfectly aligning at the angle of 90°. In the gap areas where smooth surface topography was patterned between two neighboring regions of microgrooves, aligned myotubes were also observed even without the initial oriented myoblasts. The myotube orientation in such areas was different from those aligned on the microgrooves, that is, at the two edges the myotubes aligned along the longitudinal axis of the microgrooves (90°), while in the middle of the gap area the myotubes slanted at an angle. The slanting angle varied with the width of the gap area. For the microchip with a gap width of about 200 μm , the median value of the slanting angle is about 70°. When the width increased to a value on the order of mm, the myotube formation became similar as those cultured in the open area with smooth surface topography, that is, the myotubes lost their preferable alignment and exhibited random orientation (Fig. 5c). Immunofluorescence staining to visualize myotubes further confirmed the formation of multinucleated cells arranged juxtaposed to each other in a linear fashion (Fig. 6). The transition of myotube orientation at the edge of the microstructure array can be clearly seen from the comparison of the actin filament staining and the phase contrast images showing the overlap of the cell nuclei and the underlying microstructures array.

Formation of Multi-Layered Muscle Fibers

To fabricate multi-layered muscle fibers, additional layers of myotubes were allowed to grow on the primary layer of myotubes. In these experiments, the additional myoblasts were added to the pre-existing layers of myotubes formed on the chips with 7 μm microgrooves. This was conducted by seeding myoblasts with the tissue culture medium, incubating for 48 h to allow proliferation, changing to the growth culture medium, and incubating for another 48 h to allow differentiation. The additional myoblasts attached to the adjoining myotubes and formed three-dimensional structures. Thicker tissue can be obtained by repeating the above steps. Although these additional cells did not directly contact with the microgrooves, it is interesting to note that they followed the similar highly organized parallel alignment as observed in the earlier deposited layers of myotubes. According to the observation, the secondary and subsequent layers of aligned myotubes morphologically differed from the primary layer of myotubes. Specifically, the myotubes in the primary layer were physically confined by the microstructures and exhibited a longitudinal geometry. The width of the myotubes was on the same order of the width of the microgrooves. The additional layers, however, were much wider and differentiated at a much more rapid pace than the primary layer.

To examine the thickness and the morphological differences in the layers of the myotubes constructed on the microchips, sectioning microscopic images were obtained by z-axis scanning. The results showed that after the additional myoblasts were allowed to differentiate for 2 days, the skeletal muscle myotubes were about 40 μm thick. The intensity traces of images indicated that the cell nuclei in the primary layer exhibited bright and dark

fields of fluorescence at the regular intervals (Fig. 7a). Such intervals were not seen in the secondary and subsequent layers of myotubes. It is reasonable to conclude that the alignment of the myotubes in the secondary and subsequent layers is not as organized as those in the primary layer since the alignment may not be a direct consequence of the topography of the linear PDMS microgrooves, but due to the periodic surface topology of the myotubes in the primary layer. In addition, it is plausible that the interlayer cell communication may also play a dominant role in the longitudinal alignment of the myotubes in the secondary and subsequent layers. As a result, most cell nuclei in the primary layer were stretched and well modulated by the microstructures, while those in the additional layers were more spherical without obvious spatial correlation with the geometry of the microstructures. This was confirmed by the visualization of cell nuclei staining in the primary and secondary layers (Fig. 7b and c). Further confirmation of the three-dimensional structures was provided by the histological (Fig. 8) and immunofluorescence (Fig. 9) images, which show the cross-sections of the multi-layered structures. As illustrated in the subfigure of Figure 8, the cross-section is along the longitudinal axes of the microgrooves (A-A). The difference of cell morphologies, myosin heavy chains and cell nuclei between the primary layer and the additional layers can be clearly seen, which supports our hypothesis that the myotube alignment in the primary layer and the additional layers may follow different mechanisms. Previous research showed that the additional cells in such cell-on-cell differentiation leads to a higher level of myosin striation (Engler et al., 2004), which may directly affect the contractile performance of the fabricated muscle tissue. Therefore, the findings of myotube morphological difference along the *z*-axis emphasize the importance of surface topography in the fabrication of muscle tissues *in vitro*, and indicate the necessity of its consideration in the future design of functional engineered skeletal muscles.

Stripping of the Muscle-Like Sheet

Finally, to test whether the PDMS polymer microchip containing linear microgrooves with subcellular widths and medium aspect ratios provides a viable approach for fabrication of skeletal muscle fibers, experiments were conducted to detach the as-fabricated multi-layered muscle fibers off the PDMS polymer substrates using a precision probe (5 μm in tip diameter). Given the fact that all the myotubes were aligned unidirectionally, the stripping was performed in the direction of myotube alignment to avoid tissue tear. To define the width of the stripping muscle tissue, two notches were first created by cutting at the edge of the tissue using the probe tip. The muscle tissue between these two notches was carefully lifted to detach from the polymer surface. Figure 10a shows a muscle tissue during stripping. It is evident that the detaching edges paralleled the longitudinal axis of the myotubes due to the highly unidirectional tissue alignment. This allows detachment with minimal peeling forces. A stand-alone and intact myotube bundle with the width about 150 μm was obtained without obvious structural damage. After the stripping, the microchip was examined using an immunofluorescence microscope. The image was compared with the immunofluorescence images taken immediately before the stripping (Fig. 10b). The result showed that very few cells remained on the polymer substrate after stripping (Fig. 10c). This indicates that the cells do not attach firmly to the surface of polymer microchip because the cellular adhesive forces between different myotube layers are greater than that between the PDMS substrate and the primary myotube layer. It thus allows efficient detachment to procure the tissue in a viable and transplantable state.

Conclusions

This work demonstrates the development and use of a polymer chip containing microstructures with subcellular widths and medium height-to-width ratios to fabricate

multi-layered skeletal muscle fibers with highly organized myotubes, by controlling the surface topographies conducive to longitudinal alignment of cells required for myotube formation. The optimally spaced linear polymer microgrooves (2 μm in width, 7 μm in height, and single spaced) not only accommodate cell alignment by creating a template to guide the immediate organization of myoblasts, but also support the proliferation and differentiation of additional layers into desired directions, forming three-dimensional organized structures. This chip can be used to alter the degree of alignment of the cells in thick muscle tissue by varying the heights (or height-to-width ratios) of the microgrooves, so that the contractile and physiological performance of the fabricated muscles could be controllable. The ability of the chip to allow detachment of fabricated tissues further validates the utility of this approach for on-chip muscle tissues fabrication. This work is expected to foster the development of engineered muscle tissues and add a new dimension to the understanding of microtopography-dependent cell adaptation.

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Figure 1.

Schematic drawing of topographic activated muscular tissue fabrication. The microgrooves with subcellular widths and medium height-to-width ratios align the primary layer of skeletal myotubes with high efficiency. This primary layer serves as a seed layer to affect the growth and differentiation of additional cell layers. By alternating cycles of cell growth and differentiation, three-dimensional muscle like tissues with organized structures are formed. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 2.

Fabrication process of the PDMS polymer microchip. **a:** A thick layer of AZ resist was spin coated on a glass substrate; **(b)** The resist was patterned using a standard photolithography process; **(c)** PDMS pre-polymer was applied to the patterned resist layer, and cured; **(d)** Single spaced PDMS microstructures were obtained by dissolving the AZ resist; **(e,f)** the optical and electron micrographs of the as-fabricated PDMS microstructures array. The line width and the spacing between neighboring lines are both 2 μm . [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 3.

The myoblast alignment varies with the height-to-width ratios of the microstructures. The cell morphology after 24 h culture showed that **(a)** With the height-to-width ratio about 1 (2 μm in width and 2 μm in depth), the myoblasts exhibited limited alignment and spreaded across several parallel microstructures; **(b)** With the height-to-width ratio about 3.5 (2 μm in width and 7 μm in depth), the cells propagated into the grooves exhibiting in-groove nuclear alignment and cytoplasmic spreading on the bottom surface (arrows).

Figure 4.

Closely spaced PDMS microgrooves with subcellular widths and appropriate height-to-width ratios lead to directional myoblasts proliferation. The myoblasts cultured in 7 μm deep microgrooves showed directional proliferation at (a) 24 h; (b) 48 h; (c) 72 h; and (d) 96 h, respectively. The surface topography dependent morphological differences are shown by comparing the cells grown on chips with microgrooves and the control cells grown on smooth PDMS surface printed in between the grooves. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 5.

The PDMS microchip with linear microgrooves directed the accommodated myoblasts into highly organized myotubes upon differentiation. **a:** The myotubes formed on the microstructures were highly aligned along the longitudinal axis of the structures, while the myotubes formed in the gap area exhibited directional differentiation at a slanting angle; **(b)** The histogram showing the angular distribution of myotube alignment; **(c)** The myotubes formed in the open area did not exhibit obvious alignment. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 6.

The immunofluorescence images showing the myotube alignment with exposure to different surface topographies. **(a)** The actin filament staining showed that the myotubes in different areas aligned at different angles. **(b)** The stained cell nuclei overlapped with the underlying microstructures showing the spatial correlation between the myotubes alignment and the microtopography (myosin heavy chain is stained red, actin is stained in green, cell nuclear is stained in blue). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 7.

The sectional microscopic scanning revealed that the myotube morphology varied along the *z*-axis. **(a)** *z*-axis scanning of nuclei from the primary layer of cells demonstrating regular intervals of bright and dark fields. Relative fluorescence of nuclei in the secondary layers demonstrating lack of fluorescence distribution at regular intervals. **(b)** cell nuclei of the myotubes in the primary layer showing the stretched nuclei with spatial correlation with the microstructures; and **(c)** cell nuclei of the myotubes in the secondary and subsequent layers showing more spherical nuclei without obvious spatial periodicity. The spacebars denote 10 μm . [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 8. H&E staining of the cross-section cut along longitudinal axes of the microgrooves. A-A indicates the cross-section. The spacebar demotes 5 μm . [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 9. Immunofluorescent images showing the cross-section of the multilayered structure. (a): cell nuclei; (b) myosin heavy chains; and (c) nuclei + myosin heavy chains. It is seen the cells in the primary layer and in the additional layers have difference morphologies.

Figure 10.

The PDMS microchip allows the detachment of the fabricated multilayered skeletal muscle. (a) Directional alignment of highly oriented myotubes facilitating the linear detachment of cells from the polymer microchip (myosin heavy chain is stained red and actin is stained in green); Cell nuclei staining of muscle scaffold (b) before detachment of myotubes; and (c) after detachment of myotubes exhibiting minimal residual myotubes remained on the microchip. The space bars indicate 100 μm .