Optimizing the structure and contractility of engineered skeletal muscle thin films

Y. Sun [a,b,c], R. Duffy [a,b], A. Lee [a,b], A.W. Feinberg [a,b,*]

[a] Regenerative Biomaterials and Therapeutics Group, Department of Biomedical Engineering, Carnegie Mellon University, 700 Technology Dr., Pittsburgh, PA 15219, USA
[b] Regenerative Biomaterials and Therapeutics Group, Department of Materials Science and Engineering, Carnegie Mellon University, 700 Technology Dr., Pittsburgh, PA, USA
[c] Key Laboratory for Biomechanics and Mechanobiology of Ministry of Education, School of Biological Science and Medical Engineering, Beihang University, Xueyuan Road No. 37, Haidian District, Beijing 100191, China

ABSTRACT

An experimental system was developed to tissue engineer skeletal muscle thin films with well-defined tissue architecture and to quantify the effect on contractility. Using the C2C12 cell line, the authors tested whether tailoring the width and spacing of micropatterned fibronectin lines can be used to increase myoblast differentiation into functional myotubes and maximize uniaxial alignment within a 2-D sheet. Using a combination of image analysis and the muscular thin film contractility assay, it was demonstrated that a fibronectin line width of 100 μm and line spacing of 20 μm is able to maximize the formation of anisotropic, engineered skeletal muscle with consistent contractile properties at the millimeter length scale. The engineered skeletal muscle exhibited a positive force–frequency relationship, could achieve tetanus and produced a normalized peak twitch stress of 9.4 ± 4.6 kPa at 1 Hz stimulation. These results establish that micropatterning technologies can be used to control skeletal muscle differentiation and tissue architecture and, in combination with the muscular thin film contractility, assay can be used to probe structure–function relationships. More broadly, an experimental platform is provided with the potential to examine how a range of microenvironmental cues such as extracellular matrix protein composition, micropattern geometries and substrate mechanics affect skeletal muscle myogenesis and contractility.

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1. Introduction

Skeletal muscle forms the primary actuator system for a wide range of multicellular organisms from the nematode on up to humans. This application across diverse length scales, environments and force regimes demonstrates that striated muscle is highly scalable and adaptable; exceeding what is capable with artificial actuators [1,2]. However, it has proved challenging to tissue engineer skeletal muscle in vitro and leverage these properties in the design of new materials and devices. It has also proved challenging to tissue engineer skeletal muscle for the repair or replacement of muscle tissue damaged by injury and disease [3]. While the process of myogenesis is relatively well understood in vivo [4], recapitulating this process in vitro has been limited by an incomplete understanding of the microenvironmental cues that are required to drive muscle formation [5]. Researchers have successfully used established cell lines such as C2C12 or primary-derived cells and differentiated these into myotubes to study the electrical and mechanical properties of muscle tissue. However, in vitro differentiated myotubes in a petri dish do not spontaneously organize

themselves into aligned muscle bundles as occurs in skeletal muscle in vivo. Without this uniaxial alignment, the muscle tissue cannot generate maximal force.

To address this, a number of strategies have been developed to organize myotubes by engineering anisotropic alignment cues in the cell microenvironment. For example, topography-based approaches using nanofiber scaffolds [6,7], microabrated surfaces [8] and wavy micro-ridges [9] have been able to orient myoblasts and myotubes. Though these techniques induce alignment, the variability in fiber/feature size and shape has made it difficult to directly compare the effectiveness of specific topographical guidance cues across experiments. Another approach is soft lithography, which enables creation of high-fidelity extracellular matrix (ECM) protein micropatterns and has been successfully used to align myotubes [10–14]. With both topographical and protein micropatterning, however, a major limitation is that myotubes are constrained to a two-dimensional (2-D) substrate, making it difficult to measure contractile force generation, especially at the tissue scale. Moving to three-dimensions, researchers have used cell-generated compaction of collagen, fibrin and Matrigel hydrogels to generate uniaxial stresses and engineer constructs with aligned myotubes [15–18]. These three-dimensional (3-D) muscle tissues enable measurement of the contractile force and assess-

* Corresponding author. Tel.: +1 (412) 268 4897; fax: +1 (412) 268 9807.
 E-mail address: feinberg@andrew.cmu.edu (A.W. Feinberg).

ment of muscle physiology by attachment to force transducers or deformation of elastic pillars [18,19]. However, the control of myotube alignment in 3-D tissues is not as precise as with 2-D lithographic techniques and, in most cases, the myotubes do not form highly aligned bundles throughout the full cross-section of the engineered muscle, as they do in vivo. Fujita et al. have begun to address this by engineering C2C12 myotubes on 2-D collagen gel strips of defined width, quantifying alignment and measuring contractile force generation [20]. While this demonstrates that forces can be measured in 2-D skeletal muscle tissues, this system did not micropattern myotube alignment. This is important, because recent studies in engineered cardiac muscle and vascular smooth muscle have shown that micropatterning of tissue alignment can have significant impact on contractile function [21,22]. Therefore, there exists the need to engineer microscale tissue architecture in 2-D and measure contractility in 3-D in order to quantify how microenvironmental cues affect structure–function relationships in skeletal muscle tissue.

The aim of this study is to engineer the microscale geometric patterning of ECM proteins on a surface in order to maximize differentiation of C2C12 myoblasts into aligned myotubes in 2-D and determine the effect on tissue-scale contractile function. To do this, the present authors have developed a platform for tissue engineering skeletal muscle thin films that enables control over ECM protein composition and geometric patterning with quantitative assessment of muscle tissue structure and contractility. Specifically, the microcontact printing (μCP) of ECM proteins used to engineer 2-D anisotropic cardiac muscle [21,23,24] and vascular smooth muscle [22,23,25] has been adapted to engineer anisotropic 2-D skeletal muscle tissues. While myotubes have been patterned on surfaces previously, the present authors' unique criteria were to maximize myotube differentiation and alignment over millimeter length scales in order to maximize muscle mass within a 2-D tissue. To do this, a high-content screen was conducted by μCP fibronectin (FN) in 12 different line width and spacing combinations in order to determine the optimal pattern geometries. Then, using the muscular thin film (MTF) technique to engineer muscle on a flexible elastic film, the force generation was quantified, and the basic muscle physiology was determined as a function of tissue architecture. Thus, by combining 2-D patterning techniques with tissue-scale contractility measurement, the authors developed an experimental platform that enables systematic engineering and analysis of microenvironmental cues that influence myogenesis and contractility in skeletal muscle.

2. Materials and methods

2.1. Substrate fabrication

Engineered substrates with defined micropatterns of FN were fabricated by modification of previously published techniques (Fig. 1A) [21,23,24,26]. Briefly, Sylgard 184 polydimethylsiloxane (PDMS; Dow Corning Corp.) was mixed in a 10:1 base-to-curing agent ratio using a Thinky-Conditioning mixer (Phoenix Equipment Inc.) for 2 min at 2000 rpm followed by 2 min of defoaming at 2000 rpm. For immunofluorescent studies, the PDMS was spin-coated onto 25-mm-diameter glass coverslips at 4000 rpm to create films 15 μm thick. For contractility studies, first 1 g of poly(*N*-isopropylacrylamide) (PIPAAm, Polysciences Inc.) was dissolved in 10 ml of 1-butanol and spin-coated at 6000 rpm onto the 25-mm-diameter coverslips. Then PDMS was spin-coated at 4000 rpm on top of the PIPAAm layer. PDMS stamps used for μCP were fabricated using photolithographic techniques. Glass wafers were spin-coated with SPR 220.3 positive photoresist (MicroChem Corp.), exposed through a transparency-based photomask with

UV light, developed using MF-319 developer (MicroChem Corp.) and then post-baked at 115 °C for 90 s. PDMS pre-polymer was then poured on the topographically patterned photoresist and cured for 12 h at 65 °C to form the PDMS stamps. For patterning, the stamps were sonicated in 50% ethanol for 30 min, dried under sterile nitrogen, incubated with FN (BD Bioscience) at 50 μg ml^{-1} in DI water for 1 h and then dried again under sterile nitrogen. The PDMS-coated substrates were UV/ozone treated for 15 min (PSD Pro, Novascan) to increase the hydrophilicity of the PDMS and sterilize the surface. The FN-coated PDMS stamps were brought into conformal contact with the PDMS-coated substrates for 10 min in order to create the FN micropatterns used for the high-content screen of line width and spacing. FN was μCP on the PDMS substrates in 12 different patterns consisting of FN lines 20, 50, 100 and 200 μm wide, separated by 10, 20 and 30 μm spacing (Fig. 1B). The area between the FN lines was backfilled by incubating with 1% Pluronic F-127 to prevent cell adhesion. Coverslips were then washed three times with phosphate-buffered saline (PBS) and seeded with C2C12 cells.

2.2. Cell culture

The murine C2C12 cell line (CRL-1722, ATCC) was cultured and differentiated according to the supplier's directions at 37 °C and 5% CO$_2$. Cells were expanded in growth media (DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM) solution and 1% 10,000 unit penicillin–streptomycin solution) in T-75 flasks and split 1:3 at 80% confluence. C2C12 cells were seeded as a single cell suspension at a density of 2×10^4 cells cm^{-2} on the micropatterned substrates and cultured for 1 day in growth media. Note that it is important to ensure a single cell suspension in order to avoid cell bridging on the 20- and 30-μm-spaced lines. Cells were then cultured in differentiation media (DMEM supplemented with 2% horse serum, 1% L-glutamine (200 mM) solution and 1% 10,000 unit penicillin–streptomycin solution) for an additional 6 days to induce myotube formation. This is consistent with the differentiation time reported to reach maximal force generation in other studies [20,27]. Further, longer differentiation time points were avoided, because myotubes would begin to delaminate from the surface, making tissue quality inconsistent and quantitative analysis impossible. Differentiation media exchanges were performed every 24 h.

2.3. Immunofluorescent staining and image analysis

Myotubes were fixed, fluorescently stained and then imaged in order to quantify the structure of the engineered muscle tissue. All reagents were purchased from Life Technologies unless otherwise specified. After 6 days in differentiation media, myotubes were rinsed with PBS (with 0.625 mM Mg^{2+} and 0.109 mM Ca^{2+}) and then fixed in 0 °C methanol for 2 min. After fixation, samples were washed three times in PBS for 5 min per rinse, blocked with 5% goat serum for 1 h and then incubated for 1 h in 1:100 dilutions of monoclonal myosin heavy chain (MHC) antibody and DAPI in PBS. Samples were then washed three times in PBS and incubated for 1 h in a 1:200 dilution of Alexa Fluor 488 conjugated with goat anti-mouse antibody. Finally, samples were washed three times with PBS and mounted on glass slides using Prolong Gold antifade. Myotubes were imaged using a Zeiss LSM 700 laser scanning confocal microscope to acquire z-stacks and tiled images.

All images were post-processed with ImageJ [28] to quantify the muscle tissue structure. The percentage area of myotubes on the substrates was determined from the MHC staining by thresholding the images, converting to 8-bit binary, and counting the percentage of MHC-positive pixels. The percentage area of myotu-