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Title: Role of Integrin $\alpha 7 \beta 1$ Signaling in Myoblast Differentiation on Aligned Polydioxanone Scaffolds

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Abstract

The aligned structural environment in skeletal muscle is believed to be a crucial component in functional muscle regeneration. Myotube formation is increased on aligned biomaterials, but we do not fully understand the mechanisms that direct this enhanced fusion. Previous studies indicate that the $\alpha 7$ integrin subunit is upregulated during myoblast differentiation, suggesting that signaling via $\alpha 7\beta 1$ mediates the effect of alignment. To test this hypothesis, we took advantage of an in vitro model using random and aligned polydioxanone (PDO) matrices and C2C12 myoblasts. We measured expression and production of myoblast markers: paired box-7 (Pax7), myogenic differentiation factor-1 (MyoD), myogenin (MyoG), myogenic factor-6 (Myf6), and myosin heavy chain (MyHC). To examine the role of $\alpha 7\beta 1$ signaling, we measured expression and production of $\alpha 7$, $\alpha 5$, and $\beta 1$ and myoblast markers in wild type cells and in cells silenced for $\alpha 7$ and assessed effects of silencing on myogenic differentiation. Downstream signaling via ERK1/2 mitogen activated protein kinase (MAPK) was examined using a specific MEK1/2 inhibitor. Alignment increased mRNAs and protein for early (MyoD) and late (MyoG, MyHC) myoblast markers in comparison to non-aligned matrices, and these levels corresponded with increased $\alpha 7$ protein. $\alpha 7$ -silencing reduced MyoG and MyHC protein in cells cultured on tissue culture polystyrene and aligned PDO matrices compared to wild type cells. Inhibition of ERK1/2 blocked effects of alignment. These data suggest that alignment regulates myogenic differentiation via $\alpha 7\beta 1$ integrin signaling and ERK1/2 mediated gene expression.

1. Introduction

Skeletal muscle wounds caused by blasts and pressurized fragments are diffuse, heterogeneous, and extensive [1]. These wounds account for 33% of all admissions to polytraumatic rehabilitation centers in the United States military [2]. The injuries heal slowly and improperly, resulting in fibrosis, denervation distal to the defect, and loss of function [3]. Surgical strategies only partially restore function and fail to regenerate muscle tissue [2].

The lack of adequate methods for addressing muscle trauma is a tremendous challenge for orthopaedics and rehabilitation engineering. Regenerative medicine strategies using adult muscle precursor cells and stem cells hold promise for building new muscle tissue, but their ability to do so has been limited by the availability of suitable delivery systems. One approach has been to inject the cells into the injured muscle. Unfortunately, the injected cells fail to engraft well [4,5], underscoring the need for a delivery matrix.

Extracellular matrices (ECM) such as decellularized muscle tissue [6] and ECM mimicking grafts, including polycaprolactone [7,8], polyglycolic acid [9], poly-L-lactic acid [10,11], and collagen [12], provide a potential solution. Although these materials support myocyte differentiation *in vitro*, their *in vivo* success has been limited. One reason for this is that we do not fully understand substrate-dependent interactions that regulate myogenic differentiation, fusion, and maturation.

Myogenic differentiation is a highly regulated process controlled by paired box (Pax) transcription factors and the myogenic regulatory factor (MRF) family. During early differentiation in adults, myogenic progenitors such as activated satellite cells or myoblasts express Pax3 and Pax7. Pax transcription factors switch the cells toward a myogenic fate, and

repress myocyte differentiation [13]. To form muscle, the family of MRFs is required to terminally differentiate myoblasts to myocytes and myofibers. These regulatory proteins belong to a superfamily of basic helix-loop-helix transcription factors that consists of myogenic differentiation factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin (MyoG), and myogenic factor 6 (Myf6). In the initial stages of myogenic differentiation, MyoD and Myf5 are the first MRFs to be expressed, and their expression triggers increased production of MyoG and Myf6 [14]. Increased intracellular MyoG and Myf6 induces terminal differentiation of myoblasts into myocytes, leading to fused myotubes.

MRFs are regulated by extracellular signals such as growth factors, ECM, and cell-cell interactions [15], but the contribution of the physical properties of the substrate has not received much attention. The interaction of cells with an ECM substrate is largely mediated through integrins, involving a complex set of interactions between trans-membrane and internal protein complexes [16]. Studies in our group show that stem cell differentiation on biomaterials involves integrin signaling via mechanisms different than those typically observed under common cell culture conditions [17]. Whether this is also the case for myogenic differentiation is not known.

Our previous work showed that aligned PDO scaffolds supported enhanced myotube formation, upregulating myosin heavy chain (MyHC) and desmin, and down-regulating vimentin production in cultures of myoblasts compared to unaligned PDO and to other polymers such as polycaprolactone [18]. These observations suggest that alignment has a positive effect on myocyte differentiation, but the mechanisms by which aligned PDO fibers mediate these effects on myoblast progenitor cells are not well understood.

During myocyte differentiation on tissue culture polystyrene (TCPS), expression of mRNAs for integrin $\alpha 7$ are up-regulated [19], $\alpha 5$ expression is down-regulated [20], and $\beta 1$ expression is down-regulated [21]. $\alpha 5$ associates with $\beta 1A$ whereas $\alpha 7$ associates with $\beta 1D$. During terminal differentiation, $\beta 1D$ displaces $\beta 1A$, contributing to downregulation of $\alpha 5\beta 1$ [22]. $\alpha 5\beta 1$ binds fibronectin and mediates cell attachment to many biomaterials in the presence of FBS [23] whereas $\alpha 7\beta 1$ binds laminin, an ECM protein [24]. Taken together, these observations led us to hypothesize that signaling via $\alpha 7\beta 1$ plays a role in differentiation. To test this hypothesis, we analyzed MRFs and integrin mRNA and protein levels in myoblasts using aligned and random polymer fiber orientations. The specific role of $\alpha 7\beta 1$ was determined using cells silenced for $\alpha 7$, which partners exclusively with $\beta 1$ [25]. Downstream signaling was assessed by specifically inhibiting extracellular-signal regulated kinase 1/2 (ERK1/2) mitogen activated protein kinase (MAPK) activity and examining gene expression of early and late MRFs.

2. Materials and Methods

2.1 Fabrication and Characterization of Polymer Matrices

Random and aligned fibers were electrospun using a 2 cm diameter, threaded mandrel with a 1 mm to 1 mm peak to peak thread width (Fig. 1A). Polydioxanone used in this study (RESOMER® X, $(C_4H_6O_3)_n$, Sigma Aldrich, St. Louis, MO) was a 100-120 kDa polymer with a 1.5-2.2 dl/g inherent viscosity, $-10^\circ C$ glass transition temperature, and $110-115^\circ C$ melting temperature. PDO pellets were dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (TCI America, Portland, OR) at a concentration of 120 mg/ml. Dissolved PDO was loaded into a 5 ml plastic Becton Dickinson syringe with an 18 gauge blunt tip needle, and dispensed at a rate of 5 ml/hr,

+22kV, and an air gap distance of 20 cm between the needle and mandrel. Random and aligned scaffolds were electrospun onto the mandrel rotating at a rate of 500 and 2000 rpm, respectively, as previously described [18,26].

Relative atomic concentration and chemical bonding information were obtained from the specimen surfaces by x-ray photoelectron spectroscopy (XPS) (Thermo K-Alpha XPS; Thermo Fisher Scientific, West Palm Beach, FL). The XPS instrument was equipped with a monochromatic Al K α X-ray source ($h\nu = 1468.6$ eV). Spectra were collected at 5×10^{-8} mbar or lower using an X-ray spot size of 400 μm and a pass energy of 100 eV, with 1 eV increments, at a 55° takeoff angle. Two specimens of PDO aligned and random fiber scaffolds were scanned three times each and all values were averaged.

Construct morphology was determined using scanning electron microscopy (SEM). Samples were coated with gold, and imaged using JEOL LV-5610 SEM. A fast Fourier transform (FFT) method was used to evaluate relative fiber alignment in electrospun scaffolds [27]. The FFT function converts information present in an optical data image from a “real” domain into a mathematically defined “frequency” domain. The resulting FFT output image contains grayscale pixels that are distributed in a pattern that reflects the degree of fiber alignment present in the original data image. For analysis, grayscale, 8-bit TIF SEM images were cropped to 2048 x 2048 pixels. FFT analysis was conducted using ImageJ software (NIH, <http://rsb.info.nih.gov/ij>) supported by an oval profile plug-in (created by William O’Connell, University of Texas at San Antonio). The FFT data were normalized to a baseline value of 0 and plotted in arbitrary units.

2.2 Cell Response

2.2.1 Cell Culture

C2C12 murine myoblasts (ATCC, Manassas, VA) were cultured and expanded under standard growth conditions using high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) and 100 µg/ml penicillin-streptomycin (Invitrogen). Aligned and random scaffolds were punched into 16 mm discs and placed in a 24-well plate. C2C12 cells were seeded onto TCPS or the two scaffolds at a density of 50,000 cells/cm² in DMEM containing 10% FBS and allowed to attach to the substrates for 24 hours. Media were removed and replaced with DMEM supplemented with 2% horse serum and 100mg/ml penicillin-streptomycin for periods of 3 and 6 days. Media were changed every 2 days. At the end of each time period, scaffolds were washed with phosphate buffered saline (PBS), and evaluated for gene expression or protein production.

2.2.2 Myogenic Regulatory Factor and Integrin Expression

Total RNA was extracted using TriZol (Invitrogen) according to the manufacturer's protocol and measured using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). 500 ng of RNA was reverse transcribed into cDNA (High Capacity cDNA Kit, Applied Biosystems, Carlsbad, CA) and the cDNA was amplified by real-time PCR with gene-specific primers using Power Sybr Green (Applied Biosystems). mRNA levels for Pax7, Myod, Myog, and Myf6, as well for integrin subunits α 7 (Itga7), α 5 (Itga5), β 1 (Itgb1), were quantified using C2C12 standard curves and normalized to Gapdh. The primers were designed using Beacon Designer Software and synthesized by Eurofins MWG Operon (Huntsville, AL). The Itgb1 primers recognize mRNAs for all isoforms for β 1 (β 1A, β 1B, β 1C, and β 1D) and primers specific for β 1A and β 1D

could not be separated in NCBI BLAST. For these experiments, mRNA was extracted from cells grown on 6 scaffolds per group.

2.2.3 Myogenic Regulatory Factor and Integrin Protein Production

At day 3 and 6, total protein was extracted using 200 μ l lysis buffer (Invitrogen) supplemented with 1 μ l of protease inhibitor (Sigma Aldrich). Samples were incubated in lysis buffer over ice for 5 minutes. Each well was then sonicated 3 times for 10 seconds each. Scaffolds were carefully removed from the lysis buffer and centrifuged at 13,000g at 4°C for 10 minutes. Samples were then analyzed for protein content using a BCA Assay (Fisher Scientific, Pittsburgh, PA). Next, 30 μ g of protein from each sample was placed into a 10-14% TGX Tris-HCl gel (Bio-rad, Hercules, CA) and run at a constant voltage of 100 volts. Following electrophoresis, gels were transferred to polyvinylidene fluoride membranes (PVDF, Biorad), washed with PBS-0.1 % Tween (PBST) 5 times for 5 minutes, blocked with LiCor Blocking Buffer and probed with monoclonal primary antibodies at 4°C overnight at a 1:1000 dilution: anti-rabbit α 5 (Santa Cruz, Santa Cruz, CA); anti-goat β 1 (Santa Cruz); anti-goat α 7 (Santa Cruz); anti-mouse MyHC (EMD Millipore, Billerica, MA); anti-rabbit myogenin (Abcam, Cambridge, UK); anti-rat MyoD (EMD Millipore); anti-rat Pax7 (EMD Millipore); and anti-mouse GAPDH (EMD Millipore). Membranes were washed again with PBST and incubated with IRDye 800 or IRDye 680 conjugated secondary antibody (Licor, Lincoln, NE) at a 1:10,000 dilution for 45 minutes. Membranes were washed again in PBST and imaged using a LiCor Odyssey CLx Imager (LiCor). β 1A and β 1D proteins were isolated on the blot according to their molecular weight (150kDa and 130kDa, respectively) and measured (Supplemental Fig. 2).

2.2.4 Immunocytochemistry

In order to assess cell morphology and response to aligned and random PDO, cultures were examined for production of myosin heavy chain and counterstained with actin and nuclear stains. 10 mm biopsy punches of PDO grafts were seeded with C2C12s and cultured for 6 days. Constructs were fixed using 4% paraformaldehyde for 24 hours at 4°C. Samples were then permeabilized using 0.25% Triton X-100 (Sigma Aldrich) for 10 minutes, washed in PBS, blocked with 1% bovine serum albumin in PBS-0.25% Tween with 0.3 M glycine for 30 minutes, incubated with anti-mouse MyHC (EMD Millipore) in 1% BSA PBST overnight at 4°C, washed with PBS, incubated with anti-mouse AlexaFluor 567 (Invitrogen) for 1 hr at room temperature, and washed again with PBS. Lastly, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) to identify nuclei and AlexaFluor Phalloidin 488 to image actin, placed on a coverslip, covered with 50 µl of mounting medium, and imaged. All samples were imaged using a Zeiss 710 laser scanning microscope equipped with a 20x and 63x objective.

2.2.5 Role of $\alpha 7$ Integrin

To examine the role of $\alpha 7\beta 1$ signaling, integrin $\alpha 7$ expression was silenced using MISSION shRNA lentiviral transduction particles (Sigma Aldrich, SHCLNV-NM_008398) for Itga7 (TRCN0000437567). mRNA levels for $\alpha 7$ were reduced by 80% (Supplemental Fig. 1A) in shC2C12 cells and protein levels were reduced by 50% (Supplemental Fig. 1B) compared to WT cells. Wild type (WT) and shItga7 C2C12 cells (shC2C12) were seeded at 50,000 cells/cm² on TCPS and aligned PDO substrates in high glucose DMEM supplemented with 2% horse serum. Total RNA and total protein were extracted as described above. mRNA levels for Myod, Myog, Myf6, Itga7, Itga5, and Itgb1 were determined by RT-PCR. Protein levels for MyoG, MyHC, and integrin subunits $\alpha 7$, $\alpha 5$, $\beta 1A$, and $\beta 1D$ were determined by western blot.

2.2.6 Role of ERK1/2

To study the effect of ERK inhibition, we used the MEK1/2 inhibitor, U0126 (Cell Signaling Technology, Danvers, MA) at 10 μ M in dimethylsulfoxide (DMSO) in high glucose DMEM supplemented with 2% horse serum. Cells were seeded at 50,000 cells/cm² on TCPS and aligned PDO scaffolds and cultured for 3 and 6 days. At the end of each time period, scaffolds were washed with PBS, and evaluated for protein production of MyoG and MyoD. New media with DMSO or inhibitor were replaced every 2 days. Samples were harvested for western blot and MyoD, MyoG, and MyHC were measured.

2.3 Statistical Analysis

Data from experiments examining cell response are presented as the mean $\pm$ standard error of the mean with six independent cultures per variable. Data were evaluated by analysis of variance, and significant differences between groups were determined using post hoc analysis by Bonferonni's modification to Student's T-test. A p-value below 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1 Characterization of Polymer Surfaces

SEM showed morphological differences between aligned and random scaffolds (Fig. 1B,C). FFT showed higher peak values for aligned PDO scaffolds in comparison to random fiber scaffolds (Fig. 1D). XPS identified no significant differences between aligned and random surfaces with respect to surface chemistry, and fiber diameters were comparable as well (Fig. 1E).

3.2 Myoblast Morphology

Actin filaments were parallel to fiber orientation in aligned PDO cultures whereas cells on random PDO exhibited a radial pattern (Fig. 2A). Immunofluorescence using anti-MyHC was more intense in myoblasts on aligned PDO fibers than on random PDO fibers (Fig. 2B).

3.3 MRF Expression and Production

Pax7 expression was greater on day 3 than on day 6 for both scaffold types (Fig. 3A). In contrast Myog and Myod were significantly higher at day 6 than day 3 and was greater on aligned fibers than on random PDO at day 6 (Fig. 3B,C). Myf6 expression decreased on the random PDO scaffolds on day 6, but remained unchanged on aligned PDO scaffolds (Fig. 3D). Measurement of protein confirmed the mRNA results. Pax7 protein was only detected on day 3 and levels on the aligned scaffolds were lower than on the random PDO (Fig. 3E). MyoD (Fig. 3F) and MyoG (Fig. 3G) protein increased on day 6 compared to day 3 and were greater in cells cultured on aligned PDO scaffolds. MyHC protein increased dramatically on day 6 on both materials, with the greatest effect on aligned PDO (Fig. 3H).

3.4 Integrin Expression and Production

Expression of Itga7 appeared to increase on day 6 compared to day 3 in cultures grown on aligned PDO and to be higher on aligned scaffolds compared to random scaffolds (Fig. 4A), although the variation among samples obscured statistical significance. In contrast, expression of Itga5 and Itgb1 decreased on both materials with time and to a comparable extent (Fig. 4B, C). There was a 10-fold increase in $\alpha 7$ on aligned PDO. $\alpha 7$ protein also increased on random PDO but the effect was less robust (Fig. 4D). $\alpha 5$ protein increased 2-fold on aligned PDO but did not change on random PDO as a function of time (Fig. 4E). $\beta 1D$ protein increased 5-fold on

the aligned constructs between days 3 and 6. C2C12 cells produced β 1D at low levels on random PDO and the levels did not change with time (Fig. 4F). β 1A protein was higher on aligned PDO than on random PDO, but did not change with time on either substrate (Fig. 4G).

3.5 Role of α 7

Silencing α 7 inhibited fusion of myoblasts into myotubes. WT C2C12 cells fused after 6 days in culture when grown on TCPS (Fig. 5A), and fluorescent actin and nuclear staining showed multinucleated myotubes, further demonstrating fusion (Fig. 5B). In contrast, C2C12 cells that were silenced for α 7 did not fuse (Fig. 5C), and fluorescent staining demonstrated C2C12 confluence but not fusion (Fig. 5D).

As expected, *Itga7* was reduced more than 80% in shC2C12 cells, regardless of culture surface (Fig. 6A). Expression of *Itga5* on TCPS was comparable in WT and shC2C12 cells (Fig. 6B). Both WT and shC2C12 cells exhibited reduced *Itga5* expression on aligned PDO compared to TCPS but the effect was less robust in the silenced cells. Expression of *Itgab1* was unaffected by α 7 silencing but was reduced on the aligned PDO fibers compared to TCPS in both cell types (Fig. 6C). Both WT and shC2C12 cells exhibited reduced *Myod* expression on aligned PDO, but silencing α 7 had no effect (Fig. 6D). Silencing α 7 reduced *Myog* and *Myf6* in cultures grown on TCPS but had no effect on either MRF when cells were grown on aligned PDO (Fig. 6E,F). However, when shC2C12 cells were cultured on aligned PDO, there was no effect on expression due to α 7 silencing.

Integrin protein levels were sensitive to the surface on which the cells were cultured. WT cells increased α 7 protein 3-fold on the aligned PDO fibers (Fig. 7A). There was a 2-fold increase in α 7 in the shC2C12 cells grown on PDO, although silencing caused a 50% reduction

in $\alpha 7$ on both surfaces. $\alpha 5$ protein was increased 2-fold when WT and shC2C12 cells were cultured on aligned PDO compared to TCPS (Fig. 7B). Moreover, silencing $\alpha 7$ caused a 54% increase in $\alpha 5$ on TCPS and a 64% increase on PDO fibers (Fig. 7B). $\beta 1A$ was unaffected by surface or $\alpha 7$ silencing (Fig. 7C). In contrast, $\beta 1D$ was increased in WT cells on PDO fibers (Fig. 7D). $\beta 1D$ was increased more than 2-fold in shC2C12 cells grown on TCPS compared to WT cells. However, this $\beta 1$ isoform was reduced in shC2C12 cells grown on PDO compared to $\alpha 7$ -silenced cells on TCPS or to WT cells on PDO. MyoG protein increased 4-fold in WT and shC2C12 cells on PDO compared to TCPS (Fig. 7E). MyoG protein was reduced by 50% in shC2C12 cells on both surfaces. MyHC protein was increased 10-fold in WT cells on aligned PDO compared to TCPS (Fig. 7F). Silencing $\alpha 7$ had no effect on MyHC protein in cultures on TCPS but caused almost complete suppression of the protein in shC2C12 cells on aligned PDO fibers. These experiments revealed substrate-dependent differences in gene expression and protein production.

3.6 Role of ERK1/2

Inhibition of ERK1/2 differentially affected MRF protein levels in C2C12 cells cultured on aligned PDO scaffolds. MyoD was increased (Fig. 8A), MyoG was decreased (Fig. 8B), and MyHC (Fig. 8C) was unchanged in C2C12 cultures treated with U0126. Similar effects were observed on day 3 and day 6. Whereas levels of MyoD and MyoG were reduced on day 6 compare to day 3, there was a 4-fold increase in MyHC.

4. Discussion

In this study, we demonstrated that myogenic differentiation of C2C12 cells on PDO is sensitive to fiber alignment and that the effects of alignment on differentiation are mediated via

$\alpha 7\beta 1$ signaling. Expression of the early myogenic differentiation marker Pax7 mRNA and synthesis of Pax7 protein were down regulated in response to alignment, whereas late differentiation markers MyoD and MyoG increased. In addition, we showed that myoblasts cultured for 6 days responded to alignment with a robust increase in MyHC protein, a marker of fusion and maturation. Taken together, these data indicated substrate-dependent interactions between myoblasts and aligned PDO scaffolds that altered levels of myogenic regulatory factor expression.

Importantly, $\alpha 7$ protein was increased on day 6 on aligned PDO, commensurate with an increased myogenic differentiation. This is further supported by the fact that $\beta 1A$ is associated with $\alpha 5$ and $\beta 1D$ is associated with $\alpha 7$. Mouse primers for $\beta 1$ mRNA could not differentiate between Itgb1A and Itgb1D. Therefore, we investigated $\beta 1$ protein isoforms using their specific molecular weights in western blot. $\beta 1D$ significantly increased over time and when compared to random PDO scaffolds, while $\beta 1A$ increased only when compared against random PDO scaffolds. Further evidence that $\alpha 7\beta 1$ signaling mediated the effect of alignment on myogenic differentiation was the observation that $\alpha 7$ suppression reduced MyoG and MyHC protein levels even in response to alignment. MyoG and MyHC are late markers of myogenic differentiation and signal the commitment of myoblasts to myocytes. Inhibition of ERK1/2 signaling also increased MyoD and reduced MyoG protein synthesis when myoblasts were grown on aligned PDO scaffolds, supporting the hypothesis that membrane-associated signaling pathways, like those due to integrin binding, are involved.

Muscle repair is highly regulated, and alignment might play a role in healing. *In vivo*, satellite cells respond to changes in signaling after injury [28]. These changes synergistically activate

satellite cells to become myoblasts marked by an increase in Pax7 and MyoD expression [29]. Simultaneously, these cells up-regulate $\alpha 5\beta 1$ to improve their mobility. Using $\alpha 5\beta 1$, these cells travel along existing aligned myofibers to the injury and participate in muscle repair. Without alignment, muscle regeneration might be impaired.

The alignment present in muscle fibers acts as a guide for myoblasts during early differentiation. The myoblasts migrate on large 10 μm diameter fibers that are coated with ECM proteins. We previously showed that electrospun PDO scaffolds fiber diameters range from 0.8-1.5 μm [26], which is more typical of ECM proteins [29,30]. PDO is a poly(ester-ether) degradable polymer and exhibits excellent biocompatibility as a biomaterial. In this study, surface characteristics were determined by SEM and XPS. We confirmed that scaffolds were aligned or random using FFT, and that average diameter was similar between aligned and random fibers. We previously showed that fiber alignment increased scaffold moduli in the direction of alignment, but elastic modulus was reduced in the transverse direction [31]. While fiber orientation, packing, and mechanical properties are different between random and aligned PDO scaffolds, cells are more sensitive to the physical properties of individual fibers [32,33]. Studies have demonstrated that fiber diameter and polymer composition are important determinants of single fiber stiffness [34,35]. Given that fiber diameters were comparable, XPS demonstrated that surface chemistry of the fibers were comparable, and the same material was used to generate the fibers, it is likely that single fiber moduli were similar between aligned and random scaffolds. Moreover, it is likely that adsorption of serum proteins like fibronectin to the fibers was comparable. Taken together, differences in cell responses to the aligned and random fiber scaffolds can be ascribed to alignment.

Scaffold alignment has been a key ingredient in the development of polymer technologies for muscle differentiation. Studies have examined the interaction of muscle precursors with aligned scaffolds such as polycaprolactone, polyglycolic acid, poly-L-lactic acid, and collagen [7,11,36-38]. These studies used MyHC and myotube fusion as outcome measures. However, these late myogenic differentiation factors do not provide a full assessment of the effect of alignment on myogenesis.

Our study used multiple outcome measures to assess the effects of polymer alignment on myogenic differentiation, including both early and late markers. Our findings support our conclusion that alignment contributed to enhanced myogenic differentiation. Some studies have demonstrated enhanced MyHC using immunohistochemistry [39], while others report that MyHC is unaffected by alignment on patterned substrates and aligned electrospun poly L-lactide scaffolds using similar techniques [40,41], suggesting that alignment alone may not be sufficient to enhance myogenic differentiation. The differences in our findings from these earlier reports may have resulted from differences in experimental design, including differences in the chemistry of the polymers used or differences in the physical and mechanical properties of the scaffold fibers.

Our data showing changes in myogenic markers suggest that the aligned fibers promote myogenic differentiation. For example, Pax7 levels were reduced in myoblasts cultured on aligned PDO scaffolds, indicating that myoblasts had begun to differentiate toward myocytes [42,43]. Changes in Pax7 have also been shown to trigger altered levels of MyoD using normal and Pax7-deficient satellite cells [44]. MyoD is considered a key early differentiation marker, and it functions by activating a feed-forward loop to regulate myogenic gene expression. As a result

of intracellular signaling during myoblast differentiation, MyoD synthesis initiates p38 mitogen activated protein kinase to produce more Mef2 and MyoD gene expression [45], resulting in overall increased levels and MyoD accumulation. Our study displayed a significant increase in MyoD gene and protein expression due to alignment, indicating that accumulation of MyoD was mediated by the aligned surface. Pax7 and MyoD are important early regulators of myogenic differentiation, fusion, and maturation and could alter expression of later markers in response to alignment.

Elevated MyoD induces MyoG expression and commits the cell to differentiation [43,45], and it has been shown that MyoG is dependent on MyoD expression in MyoD deficient mice that cannot express MyoG [46]. However, MyoD deficient mice are still able to form skeletal muscle, indicating signaling pathways different from MyoD can promote muscle growth. Myf6 has been shown to play a role in these cases. Research has shown that Myf6 is able to drive normal muscle differentiation and can activate MyoG protein production in double knockout MyoD/Myf5^{-/-} zebrafish [46]. Taken together, MyoD and Myf6 regulate different pathways that affect MyoG in myoblasts.

In this study, Myog, and Myf6 increased in response to alignment. Protein production of late differentiation markers MyoG and MyHC, which depends on Myf6, was highly up regulated when myoblasts were cultured on aligned PDO scaffolds. This indicated that alignment accelerated both mRNA and protein levels for these key myogenic factors.

These results suggest that myoblast signaling on aligned PDO scaffolds is different from random scaffolds. Our results support the hypothesis that integrin signaling plays a major role. $\alpha 5$ and $\beta 1$ mRNAs were expressed at higher levels on day 3 compared to day 6, as has been

previously reported [25,47]. It has been suggested that *Itga5* decreases are caused by increases in $\alpha 7$ [25]. $\alpha 7$ is an essential tool for myofiber development and is involved in myoblast immobilization, fusion, and terminal differentiation [19,48], but how it is involved still remains unknown. Studies found expression of $\alpha 7$ to be suppressed during myoblast proliferation, but after differentiation, $\alpha 7$ mRNA increased more than 20-fold. This same study suggested a relationship between $\alpha 7$, c-myc, and MyoD. However, data to support this has not been determined, and the exact mechanisms are still poorly understood. Our study demonstrated a significant increase in $\alpha 7$ protein levels when myoblasts were cultured on aligned PDO scaffolds.

Studies using cells cultured on TCPS have shown that increased MyoD triggers increased $\alpha 7$ mRNA and protein. Our study demonstrated increased MyoD and increased $\alpha 7$ on aligned PDO scaffolds, supporting the hypothesis that alignment enhanced myogenic differentiation and maturation. In addition, we observed no change in laminin- $\alpha 2$ production in response to alignment. Studies have determined that laminin production occurs prior to differentiation and maturation [49]. Our results suggest alignment triggers a developmental cascade. Increased MyoD stimulates $\alpha 7$ expression and $\alpha 7\beta 1$ binds to laminin- $\alpha 2$ [50] to enhance myotube formation [51].

Although *Itga5* decreased with time, we observed increased $\alpha 5$ protein on aligned fibers whereas no change in protein was seen on the random fiber scaffolds. $\alpha 5$ and $\alpha 7$ associate with $\beta 1A$ and $\beta 1D$, respectively, and research has demonstrated that $\beta 1A$ is displaced by $\beta 1D$ during myoblast differentiation and maturation. While we did not observe $\beta 1A$ displacement during culture, we did find an increase in $\beta 1D$ that is consistent with other reports [52]. Differences in

expression for $\alpha 7\beta 1D$ and $\alpha 5\beta 1A$ presented an interesting dynamic in integrin signaling that appears to play a role in myoblast differentiation. We measured mRNA levels at the same time point as protein levels; thus, discrepancies between the two data sets may reflect transcription v. translation at a given time point.

Further evidence that $\alpha 7\beta 1$ signaling plays an important role in myogenic differentiation, particularly on aligned fiber scaffolds, was provided by our $\alpha 7$ -silencing results. shC2C12 myoblasts showed a fusion defect when cultured on TCPS, supporting the requirement for $\alpha 7$ in mediating differentiation. Moreover, myoblasts silenced for $\alpha 7$ demonstrated a different response when grown on either aligned PDO scaffolds or TCPS. After 6 days in culture on either TCPS or aligned PDO scaffolds, Myod was unaffected by $\alpha 7$ -silencing, suggesting that changes in Myod are not mediated through $\alpha 7$. Other studies have shown that Myod is required to induce $\alpha 7$ expression, indicating that dysregulated $\alpha 7$ signaling may affect pathways downstream of Myod [19]. Myog and Myf6 mRNA levels were significantly reduced on TCPS, but levels were recovered to normal WT when shC2C12 myoblasts were grown on aligned PDO scaffolds. These data demonstrated that the aligned PDO scaffolds mediated expression of late differentiation markers when $\alpha 7$ was suppressed, and that $\alpha 7$ is a key element in myoblast fusion, maturation, and self-alignment.

Whereas Myog was reduced by $\alpha 7$ -silencing in cultures grown on TCPS it was unaffected on aligned PDO fibers. This suggests that $\alpha 7$ does not mediate the effect of aligned PDO on MyoG, but it does play a critical role in cultures grown on TCPS. Conversely, MyoG and MyHC protein were dysregulated in both TCPS and aligned PDO cultures. Interestingly, $\alpha 5$ protein levels were markedly increased due to silencing, and appeared to be compensatory. As previously

discussed, $\alpha 5$ and $\alpha 7$ have exhibited interplay during differentiation that is required for migration and fusion, and data presented in this study support this relationship. Decreased MyoG and MyHC with altered $\alpha 5$ levels indicated $\alpha 7$ -dependent signaling to mediate these muscle markers required for fusion, and demonstrated a role for $\alpha 7$ to regulate myogenic factors.

Absence of $\alpha 7$ in mice effects muscle function and force output [50,53,54], leads to dystrophic muscle in mice, and is a known human congenital myopathy. One particular consequence of muscular dystrophy is increased fibrosis over time. Studies have investigated some of the functional roles for $\alpha 7\beta 1$ in muscular dystrophy, and found that it plays a protective role against sarcolemma damage [53-55]. Sarcolemma damage has been suggested to perpetuate muscle fibrosis in muscular dystrophies, and targeting therapeutics that ameliorate fibrosis and promote muscle healing like $\alpha 7$ hold promise. $\alpha 7\beta 1$ signaling has demonstrated importance in muscle healing and repair by also promoting satellite cell locomotion along the myofiber. During muscle repair, satellite cells are guided by healthy myofibers to the injury, but when satellite cell function is dysregulated muscle healing is affected. $\alpha 7$ -deficient mice have demonstrated impaired muscle healing after cardiotoxin injury in association with reduced satellite cell populations [55]. Based on the results of our study, it appears that $\alpha 7\beta 1$ deficiencies might impair progenitor cell fusion with injured myofibers, contributing to delayed healing.

Integrin-mediated signaling pathways may also play a role in the effects due to alignment. Integrins have an important role in regulating the efficiency of focal adhesion kinase-Ras-MAPK kinase-ERK (FAK/Ras/MEK/ERK), and since aligned PDO scaffolds stimulated $\alpha 7$ and $\beta 1$ D protein production other signaling pathways like Ras/MEK/ERK might be affected. FAK is an

enzyme that is activated after integrin-mediated adhesion to the ECM. In turn, the interactions between FAK and $\beta 1$ integrins can stimulate the cell signaling cascade that regulates Ras-MEK-ERK [56,57]. Our results indicate an ERK-dependent effect due to alignment with increased MyoD, decreased MyoG, and no change in MyHC. The ERK pathway has been implicated in myogenesis, but its functions are controversial. Reports have proposed an inhibitory role finding that ERK2 suppression using siRNA reduced MyoG [58]. Other studies have shown that activation of MEK1 reduces MyoD [58]. Taken together, our data suggest that repression of MEK1 might have increased MyoD while inhibiting MEK2 reduced MyoG. However, we did not compare the effects of $\alpha 7$ -silencing on responses to aligned and random fibers, so a definitive role for $\alpha 7\beta 1$ signaling in alignment cannot be determined.

5. Conclusion

In summary, our data showed that aligned PDO scaffolds enhanced myogenic differentiation with an increase in $\alpha 7$, $\beta 1$ D, $\alpha 5$, MyoD, MyoG, and MyHC. Integrin $\alpha 7$ was shown to mediate Myog and Myf6 gene expression on TCPS while MyoG and MyHC protein were reduced on TCPS and aligned PDO scaffolds. This study implicated $\alpha 7$ in late differentiation, maturation, and myocyte alignment using shC2C12 myoblasts. These data suggested that our aligned PDO scaffolds provided a suitable model for future investigations into integrin-mediated myogenic differentiation. In addition, we determined that aligned PDO scaffolds use ERK-dependent signaling to enhance MyoG and MyHC. Myoblasts cultured on aligned scaffolds use signaling that is different from TCPS to enhance myofiber formation. Using these scaffolds in vitro we can determine the role of $\alpha 7$ signaling in regenerative medicine. This has the potential to improve

technologies targeting skeletal muscle regeneration by recapitulating native microenvironment components present in skeletal muscle.

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Figure Legend

Figure 1. Characterization of electrospun fibers. PDO fibers were electrospun onto a 2 cm diameter mandrel (A). SEM images of aligned and random fiber orientations (B,C). FFT analysis showed differences in fiber alignment (D). No differences in surface chemistry were detected by XPS (E). Scale bars are 10 μm in B and C.

Figure 2 Fluorescence microscopy of C2C12 cells on aligned and random PDO. Cells on aligned PDO were elongated and had actin filaments (green) that were oriented parallel to the fibers (A). MyHC staining confirms its localization to the cytoplasm (B). All samples were imaged at 63x oil immersion. Scale bars are 20 μm .

Figure 3. Myogenic gene expression and protein production are sensitive to PDO fiber alignment. Gene expression and protein results were similar and showed lower Pax7 (A), but increased MyoD (B,E), MyoG (C,F), Myf6 (D), and MyHC (H) levels by day 6 when myoblasts were grown on aligned PDO. * indicates a difference from Day 3 Aligned, # indicates a difference from Day 6 Aligned, and \$ indicates a difference from Day 3 Random ($p < 0.05$).

Figure 4. mRNA and protein levels for $\alpha 7$, $\alpha 5$, and $\beta 1$ at day 3 and 6. mRNA levels did not change in response to alignment, although time dependent changes in $\alpha 7$, $\alpha 5$, and $\beta 1$ expression were observed on both scaffolds. Protein levels did change. $\alpha 7$ (D) was higher on aligned PDO and increased from 3 to 6 days in culture on both fiber types. $\alpha 5$ (E) was unaffected by alignment on day 3 but significantly increased by day 6. $\beta 1$ D (F) increased between 3 and 6 days on aligned PDO only. $\beta 1$ A (G) protein levels were higher on day 3 and 6 compared to random PDO scaffolds but did not change with time. * indicates a difference from Day 3 Aligned, # indicates a difference from Day 6 Aligned, and \$ indicates a difference from Day 3 Random ($p < 0.05$).

Figure 5. Silencing Itga7 causes a fusion defect in myoblasts. WT C2C12 and shC2C12 myoblasts were grown to confluence on tissue culture polystyrene and treated with 2% horse serum over 6 days. Myotubes formed in WT C2C12 cells (A), but not shC2C12 cells (B). Arrow points to fused myotube. Scale bar on brightfield images are 50 μm , and scale bar on fluorescent images are 20 μm .

Figure 6. mRNA levels for $\alpha 5$, $\alpha 7$, $\beta 1$, Myod, Myog, and Myf6, were measured at day 6 using real-time PCR in WT and shC2C12 myoblasts. Itga7 in shC2C12 cells was suppressed on both TCPS and aligned PDO (A); Itga5 decreased on aligned PDO (B). Itgb1 was not affected by $\alpha 7$ -silencing (C). Myod decreased on aligned PDO but was unaffected by $\alpha 7$ -silencing (D). Myog (E) and Myf6 (F) decreased when cultured on TCPS, but was not affected by $\alpha 7$ -silencing on PDO scaffolds. * indicates a difference from TCPS WT, # indicates a difference from TCPS shC2C12, and \$ indicates a difference from Aligned WT ($p < 0.05$).

Figure 7. Silencing $\alpha 7$ affected $\alpha 5$, $\beta 1$ D, MyoG, and MyHC protein. $\alpha 7$ protein was reduced on both TCPS and aligned PDO (A). $\alpha 7$ -silencing increased $\alpha 5$ protein on TCPS and aligned PDO

(B). β 1A was unaffected by α 7 silencing or surface (C). β 1D protein increased due to α 7-silencing on TCPS, but was unaffected on aligned PDO (D). α 7-silencing reduced MyoG protein on both TCPS and MyoG, and MyoG increased in WT and shC2C12 cells on both surfaces (E). MyHC protein was not affected by α 7-silencing on TCPS but was reduced on aligned PDO (F). * indicates a difference from TCPS WT, # indicates a difference from TCPS shC2C12, and \$ indicates a difference from Aligned WT ($p < 0.05$).

Figure 8. Inhibition of MEK1/2 increased MyoD (A), decreased MyoG (B), and had no effect on MyHC (C) when tested on aligned PDO grafts. These data demonstrated that myoblasts cultured on aligned PDO grafts increased expression of myogenic regulatory factors, in part, via an ERK-dependent pathway. * indicates a difference from vehicle day 3, # indicates a difference from vehicle day 6, and \$ indicates a difference from U0126 day 3 ($p < 0.05$).

2 cm

Aligne

Rando

E

XPS			
	Oxygen (%)	Carbon (%)	Nitrogen (%)
PDO Aligned	35.7 $\pm$ 0.8	63.9 $\pm$ 0.8	-
PDO Random	36.1 $\pm$ 0.5	62.7 $\pm$ 0.9	-
Fiber Diameter			
PDO Aligned	1.1 $\pm$ 0.4 μ m		
PDO Random	1.2 $\pm$ 0.4 μ m		

Figure 1.

Figure 2.

mRNA

Protein

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Silenced *Itga7* → ↓ $\alpha 7$ Transcription

Key