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Role of integrin α7β1 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 α7 integrin subunit is upregulated during myoblast differentiation, suggesting that signaling via α7β1 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 α7β1 signaling, we measured expression and production of α7, α5, and β1 and myoblast markers in wild type cells and in cells silenced for α7 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 α7 protein. α7-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 α7β1 integrin signaling and ERK1/2 mediated gene expression.

Statement of Significance

Muscle regeneration in severe muscle injuries is complex, requiring a sequence of events to promote healing and not fibrosis. Aligned biomaterials that recapitulate muscle environments hold potential to facilitate regeneration, but it is important to understand cell-substrate signaling to form functional muscle. A critical component of muscle signaling is integrin α7β1, where mice lacking α7 exhibit a dystrophic phenotype and impaired regeneration. Here, we report the role of α7β1 signaling in myoblast differentiation on aligned biomaterials.
α7-silenced myoblasts were found to regulate myogenic differentiation and demonstrate defective fusion. Our data shows reduced levels of myogenin and myosin heavy chain protein, while MyoD remains unchanged. These results support the hypothesis that α7β1 signaling plays a role in substrate-dependent tissue engineering strategies.
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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 $\alpha7$ are up-regulated [19], $\alpha5$ expression is down-regulated [20], and $\beta1$ expression is down-regulated [21]. $\alpha5$ associates with $\beta1A$ whereas $\alpha7$ associates with $\beta1D$. During terminal differentiation, $\beta1D$ displaces $\beta1A$, contributing to downregulation of $\alpha5\beta1$ [22]. $\alpha5\beta1$ binds fibronectin and mediates cell attachment to many biomaterials in the presence of FBS [23] whereas $\alpha7\beta1$ binds laminin, an ECM protein [20]. Taken together, these observations led us to hypothesize that signaling via $\alpha7\beta1$ 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 $\alpha7\beta1$ was determined using cells silenced for $\alpha7$, which partners exclusively with $\beta1$ [24]. 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 °C glass transition temperature, and 110–115 °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/h, +22 kV, 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 [18,25].

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 Kα X-ray source (hv = 1468.6 eV). Spectra were collected at $5x10^{-8}$ 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 [26]. 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 × 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

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