Protein-coated poly(L-lactic acid) fibers provide a substrate for differentiation of human skeletal muscle cells

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Received 15 August 2003; accepted 1 October 2003
Published online 6 April 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30009

Abstract: Tissue engineering represents a potential method for repairing damaged skeletal muscle tissue. Extracellular matrix (ECM) proteins were evaluated for their ability to aid in cell attachment, whereas a poly(L-lactic acid) (PLLA) fiber scaffold was tested as a substrate for the differentiation of human skeletal muscle cells. In comparison to uncoated or gelatin-coated PLLA films, cell attachment increased significantly ($p < 0.001$) on PLLA films coated with ECM gel, fibronectin, or laminin. Myoblasts differentiated into multinucleated myofibers on ECM gel-coated PLLA fibers, and expressed muscle markers such as myosin and α-actinin. Oligonucleotide microarray analysis showed similar gene expression profiles for human skeletal muscle cells on ECM gel-coated PLLA fibers as to that observed for myofibers on tissue culture plates. Therefore, PLLA fibers coated with ECM proteins provide a scaffold for the development of skeletal muscle tissue for tissue engineering and cell transplantation applications. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 69A: 373–381, 2004

Key words: skeletal muscle; differentiation; tissue engineering; poly L-lactic acid fiber scaffold; extracellular matrix proteins

INTRODUCTION

Tissue engineering is an emerging field that provides an alternative solution to the treatment of diseases, in which donor organs or tissues are insufficient or unavailable. Biosynthetic constructs have been developed for skin,[1] bone,[2] cartilage,[3] kidney,[4] and liver[5] tissues. We propose to apply cell-scaffold technology to skeletal muscle.

Duchenne muscular dystrophy (DMD), an X-linked genetic disease, is one example in which cell-based therapies have been attempted. The absence of the structural protein dystrophin in the skeletal muscles of DMD patients causes injury to these muscles upon contraction. Because of repeated cycles of damage and regeneration, the satellite cell population is exhausted and muscle tissue is replaced by connective and adipose tissue, leading to muscle weakening and wasting.[6,7] Current therapies involving direct injection of muscle cells into the skeletal muscles of patients with DMD have proven inadequate because of low cell survival and a lack of an extracellular architecture to provide the appropriate cues for differentiation into muscle.[8–10] As a result, injected cells are unable to organize into a functional muscle unit.

The Supplementary Materials referred to in this article can be found at http://www.interscience.wiley.com/jpages/0021-9304/suppmat.

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Contract grant sponsor: National Institutes of Health; contract grant numbers: RO1 NS40592-01 and training grant T32-HL07360

Contract grant sponsor: M. R. & E. Hudson Foundation

Contract grant sponsor: P.O'B. Montgomery Distinguished Chair

Contract grant sponsor: Soldier Biological Chemical Command, APG to the Biological Chemical Countermeasures Program of the University of Texas; contract grant number: DAAD13-02-C-0079

Tissue engineering provides an alternative therapeutic approach by restoring both muscle architecture and function to damaged skeletal muscle tissue, either due to injury or disease. For skeletal muscle, cells must terminally differentiate into myofibers, which contract upon stimulation. A three-dimensional extracellular architecture aids in cellular organization and provides support during contraction. In addition, myofibers must be aligned parallel to each other to generate sufficient force for whole muscle contraction. The linear structure of a scaffold consisting of polymer fibers would support cell growth and differentiation in the proper orientation. The presence of extracellular matrix (ECM) proteins within a polymer fiber scaffold should facilitate muscle cell attachment and organization.

Satellite cells, located adjacent to myofibers, participate in the natural process of muscle regeneration after injury. After damage to skeletal muscle tissue, satellite cells are activated, proliferate, and migrate to the site of injury. These cells repair the muscle architecture by either fusing with partially damaged myofibers or fusing with each other to form new myofibers.[11] In certain disease states or with a large muscle-tissue loss, these cells are unable to fully repair the muscle tissue. Satellite cells or myoblasts are a logical choice for tissue engineering skeletal muscle, because of their endogenous role in repair of damaged skeletal muscle fibers. With the appropriate environmental cues, skeletal muscle satellite cells can be used to build skeletal muscle tissue within a polymer fiber scaffold.

In the current study, we investigated the effect of various protein coatings on human skeletal muscle cell attachment to a poly(L-lactic acid) (PLLA) surface. In addition, we evaluated the ability of skeletal muscle cells to differentiate into myofibers on ECM gel-coated PLLA fibers.

MATERIALS AND METHODS

PLLA films and fibers

Glass coverslips (12-mm diameter) were immersed in 30% (w/v) NaOH for 1 h at room temperature, rinsed with distilled water three times, incubated with 1.2*M* HCl for 1 h at room temperature, rinsed with distilled water three times, and then air dried 5 min. Coverslips were briefly dipped in Sigmacote (Sigma), air dried for 5 min, rinsed with distilled water, and then air dried for 5 min. A solution of 2% (w/v) PLLA (100,000 MW) in chloroform was spread across one side of the coverslips, where PLLA films formed upon evaporation of the chloroform in the hood overnight, and subsequent venting for 24–48 h.

PLLA fibers were fabricated using a wet extrusion process.[12] Briefly, a 15% (w/v) solution of PLLA (100,000 MW) in chloroform was extruded from a 10-mL glass syringe through a 23-gauge needle into an isopropanol bath, at a rate of 0.03 mL/min. PLLA fibers of approximately 60-μm diameter were collected.

PLLA films and fiber wads (6–8 mm in diameter) were sterilized with 70% ethanol for 5 min and then rinsed in phosphate-buffered saline (PBS) three times, 5 min each. PLLA films and fibers were placed into 24- or 48-well plates, respectively, and coated overnight at 4°C with 1 mL of protein solution (ECM gel, gelatin, fibronectin, or laminin). ECM gel (Sigma) was diluted two-fold with Dulbecco's modified Eagle medium (DMEM). Gelatin was used as a 0.1% solution in distilled water, whereas fibronectin and laminin were used at 20 μg/mL in PBS.

Cell culture

The mouse skeletal muscle cell line (C2C12) was cultured on 100-mm plates in growth media containing DMEM, 20% fetal bovine serum, and 100 U/mL penicillin plus 100 μg/mL streptomycin, at 37°C and 5% CO_2. Differentiation media contained DMEM, 2% horse serum, 50 m*M* HEPES, 10 μg/mL Holo-transferrin, 10 μg/mL insulin, and 100 U/mL penicillin plus 100 mg/mL streptomycin.

The human adult skeletal muscle cell line (HSKM5) was established from satellite cells of a biopsy from human adult pectoral muscle. The LH121 cell line was generated from HKSM5 cells through infection with retroviruses expressing hTERT (the catalytic domain of telomerase) and cyclin-dependent kinase 4 (CDK4). LH121 cells were then selected for telomerase and CDK4 expression using hygromycin and neomycin (respectively).

HSKM5 and LH121 cells were cultured on 100-mm tissue culture plates (coated overnight at 4°C with 0.1% gelatin) at 37°C and 5% CO_2 in growth media containing DMEM and Media 199 in a 4:1 ratio (respectively), 20% fetal bovine serum, 0.02*M* HEPES, 1×10^{-6}*M* dexamethasone, 1.4 mg/L vitamin B_{12}, 0.03 mg/L zinc, and 0.05 mg/mL gentamicin, adjusted to pH 7.24. Hepatocyte growth factor was also added to the media at 2.5 ng/mL to stimulate cell growth. HSKM5 cells were grown under low oxygen conditions (2–5% O_2), whereas LH121 cells were grown at room oxygen conditions (21% O_2).

In a 24-well plate at a 45° incline, LH121 cells (1×10^4/well) were seeded onto either uncoated PLLA films or PLLA films coated with ECM gel, gelatin, fibronectin, or laminin, and cultured at 37°C in growth media overnight before being used for the cell attachment assay. In a 48-well plate, HSKM5 cells (2×10^5/well) were seeded onto ECM gel-coated PLLA fibers, and cultured overnight at 37°C in growth media. The following day, all PLLA fibers containing HSKM5 cells were transferred to new wells, cultured for another week in growth media, and then changed to differentiation media for 1 week. Differentiation media consisted of DMEM and Media 199 in a 4:1 ratio (respectively), 0.02*M* HEPES, 0.05 mg/mL gentamicin, 10 μg/mL insulin, and 100 μg/mL apo-transferrin. Samples were used for either immunohistochemistry, protein analysis, or microarray analysis.

Cell attachment assay

Attachment of LH121 cells to uncoated and protein-coated PLLA films was measured after 1 day in culture. Cell nuclei were stained with Giemsa (1:50 in distilled water) at room temperature for 30 min. Films were rinsed with distilled water and air dried. The number of cells (within the field of view) attached to uncoated and protein-coated PLLA films were counted along the center of the PLLA film, from one edge to the opposite edge. Cell counts were repeated in the perpendicular direction for each condition.

Scanning electron microscopy

Both uncoated and ECM gel-coated PLLA fiber wads were fixed with 5% glutaraldehyde (in PBS containing 50 m*M* Tris, at 7.4 pH) for 24 h at room temperature, gently shaking. PLLA fibers were rinsed four times in PBS (containing 10 m*M* Tris) for 30 min each and then overnight, at room temperature with gentle rocking. Samples were dehydrated through incubation for 20 min each at room temperature with gentle rocking in 50, 70, then 95% ethanol. PLLA fibers were incubated in 100% ethanol four times for 20 min each and then overnight in fresh 100% ethanol, at room temperature with gentle rocking. Samples were air dried, gold coated, and viewed using a JEOL JSM-840A scanning electron microscope with a DSG digital image acquisition system.

Immunofluorescence microscopy

Differentiated HSKM5 cells on ECM gel-coated PLLA fibers were rinsed with PBS, and then fixed with 95% ethanol for 10 min at room temperature. Cells were air dried, permeabilized with 0.1% Triton X-100, and then blocked with 1% bovine serum albumin (BSA) in PBS for 20 min. The samples were incubated with a mouse anti-myosin primary antibody (MF20, 1:4 in 1% BSA in PBS) for 1 h at room temperature, rinsed with PBS twice, incubated with a goat anti-mouse fluorescein isothiocyanate (FITC) secondary antibody (Jackson Laboratories) (1:25 in 1% BSA in PBS) for 1 h (light protected), and then rinsed with PBS three times. Cell nuclei were labeled with 12 μg/mL Hoechst fluorescent dye (Molecular Probes) in PBS for 30 min at room temperature and samples rinsed with PBS three times for 2 min each. Labeled cells were viewed on an Olympus IMT2 inverted fluorescence microscope equipped with phase optics (at 100×). Images were taken with an Optronics VI-470 CCD camera, and then collected and archived on a Macintosh G3 computer equipped with a Scion CG7 Framegrabber using Scion Image 1.62 acquisition software.

Protein isolation

HSKM5 and C2C12 cells on 100-mm plates were rinsed with PBS twice and lysed with 200 or 350 μL, respectively, of triple detergent lysis buffer [50 m*M* Tris at pH 8.0, 150 m*M* NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, and a protease inhibitor mixture] for 2 min. Cell lysates were collected into microfuge tubes. HSKM5 cells on ECM gel-coated PLLA fibers (eight wads) were placed into 2 mL of triple detergent lysis buffer and incubated for 5–10 min. Samples were homogenized in two short intervals (30 s) to dislodge cells from the PLLA fibers, placed on ice for 5–10 min, vortexed briefly, and collected into a microfuge tube.

All cell lysates were centrifuged at 5000 rpm at 4°C for 1 min. Supernatants were collected and then concentrated to at least 1 mg/mL using a speed vac. Protein concentrations were measured using the BCA protein assay reagent kit (Pierce). DNA and debris were removed by mixing protein samples 1:1 with 2× SDS and spinning through glass wool at approximately 14,000 rpm for 30 s.

Western blot analysis

Protein samples were boiled in SDS-polyacrylamide gel electrophoresis loading buffer for 2 min before loading on acrylamide gels (7.5 or 15%). A volume of 20 μL was loaded per lane for all protein samples (10 μg), and gels were run at 25 mA per gel in running buffer (1× Tris/glycine and 0.001% SDS). Proteins in the gels were transferred to Hybond-C nitrocellulose membranes (Amersham Life Sciences) in transfer buffer (1× Tris/glycine plus 20% methanol) overnight at 45 V and 4°C. Membranes were rinsed briefly with distilled water, rinsed three times in TBS-T (1× Tris buffered saline plus 0.1% Tween-20) for 5 min each at room temperature, blocked for 1 h with 5–10% nonfat dry milk in TBS-T, and rinsed with TBS-T three times. Membranes were then incubated with primary antibody for 1 h, rinsed with TBS-T three times, incubated with a secondary antibody for 1 h, and rinsed with TBS-T three times. Protein expression signals were visualized by incubating each membrane with 1 mL of the SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 2 min, and then exposing membranes to HyperFilm ECL (Amersham) for up to 5 min. The primary antibodies used for Western blot analysis were mouse anti-myosin (MF20, 1:4), mouse anti α-actinin (1:800; Sigma), rabbit anti-myoglobin (1:3000; DAKO), and mouse anti-tubulin (1:2000; Sigma). The secondary antibodies used were goat anti-mouse horseradish peroxidase (1:20,000; Pierce) and goat anti-rabbit horseradish peroxidase (1:25,000; Pierce).

RNA isolation

Total RNA was isolated from HSKM5 cells using TriPure Isolation Reagent (Roche). HSKM5 myoblasts and myofibers on six-well tissue culture plates were incubated with 200 μL of TriPure per well, whereas cells on ECM gel-coated PLLA fibers (eight wads) were placed into a 14-mL tube containing 3 mL of TriPure reagent for 5 min at room temperature. Samples were precipitated with isopropanol and centrifuged at 12,000 rpm at 4°C. The RNA pellet was washed

with 75% ethanol and resuspended in diethylpyrocarbonate-treated water. The concentration and integrity of total RNA in each sample were evaluated using a 2100 Bioanalyzer (Agilent Technologies) by measuring absorbance at 260 and 280 nm and electrophoresis on an ethidium bromide 1% agarose gel.

Microarray analysis

Microarray analysis was performed using the Affymetrix Human GeneChip HU133A microarrays. RNA samples (10 μg for each) of myoblasts on gelatin-coated tissue culture plates (Control), myofibers on gelatin-coated tissue culture plates (Plate), and cells on ECM gel-coated PLLA fibers (Fibers) were processed on separate GeneChips following standard procedures for Affymetrix gene chip analysis. Results were exported from the Affymetrix scanning system and analyzed using GeneSpring 5.1 software (Silicon Genetics) to compare samples. Raw signals for each sample were normalized within GeneSpring before analysis: 1) raw signals below zero were set equal to zero, 2) signals were normalized to the 50th percentile of all measurements on that chip, 3) for each gene, signals were normalized to the median value for measurements in all samples, 4) signals for the Control sample were set equal to 1, and then signals for each gene in "Plate" and "Fibers" were normalized against Control. Using GeneSpring, a gene tree (along the *y*-axis) and experimental tree (along the *x*-axis) were created using hierarchical clustering. The Simplified Ontology tool (hierarchically grouped genes) was used to view genes within biological categories (based on the Gene Ontology Consortium Classifications). Implicit Relationship IDEntification by Software Construction of an Entity-based Network from Text (IRIDESCENT), an automated text data mining tool, was used to find direct and indirect relationships among genes up-regulated or down-regulated three-fold in both "Plate" and "Fibers."[13]

Statistical analysis

Comparison of cell attachment on uncoated and protein-coated PLLA films was performed by Student's two-tailed *t* test. A value of $p < 0.05$ was accepted as statistically significant. Values are expressed as mean ± standard error of the mean.

RESULTS

Cell attachment to PLLA films

To determine whether the presence of ECM proteins affected skeletal muscle cell attachment, PLLA films were coated with either ECM gel, gelatin, fibronectin, or laminin. The level of cell attachment to uncoated PLLA films and PLLA films coated with ECM gel, gelatin, fibronectin, or laminin was evaluated by counting the nuclei of attached LH121 cells (Fig. 1). After 1 day in culture, significantly more ($p < 0.001$) cells attached to ECM gel-, fibronectin-, and laminin-coated PLLA films compared with either uncoated or gelatin-coated PLLA films. The ECM gel coating was arbitrarily chosen for subsequent experiments with PLLA fibers.

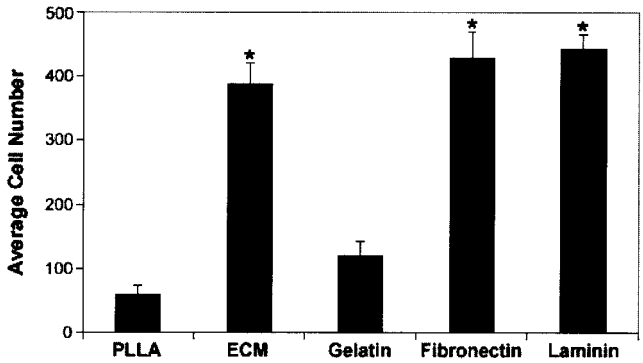

Figure 1. HSKM5 attachment to uncoated PLLA films (PLLA) and PLLA films coated with ECM gel (ECM), gelatin, fibronectin, or laminin at an incline. After 1 day in culture, cell nuclei were stained with Giemsa and the number of cells along the center of the film counted for each condition. Data are expressed as the average cell number per condition and represent the mean ± standard error of the mean ($n = 4$). The asterisk indicates a significant increase in cell number relative to uncoated PLLA films, at a level of significance of $p < 0.001$.

Surface morphology of PLLA fibers

The surface morphology of the PLLA fibers was viewed using scanning electron microscopy. Images revealed that the surface morphology of uncoated PLLA fibers [Fig. 2(A)] differed from that of ECM gel-coated PLLA fibers [Fig. 2(B)]. Uncoated PLLA fibers were rough with ridges and pores covering the surface. The ECM gel-coated PLLA fibers, however, exhibited a uniform coating, which appeared fibrillar in nature. The topography of the surface of ECM gel-coated PLLA fibers should provide cells with numerous points for attachment.

Cell differentiation on PLLA fibers

Immunofluorescence microscopy of HSKM5 grown on ECM gel-coated PLLA fibers showed the presence of elongated, multinucleated myofibers (Fig. 3). Cells, which stained positive for myosin heavy chain (FITC label), were seen as linear myofibers along the long

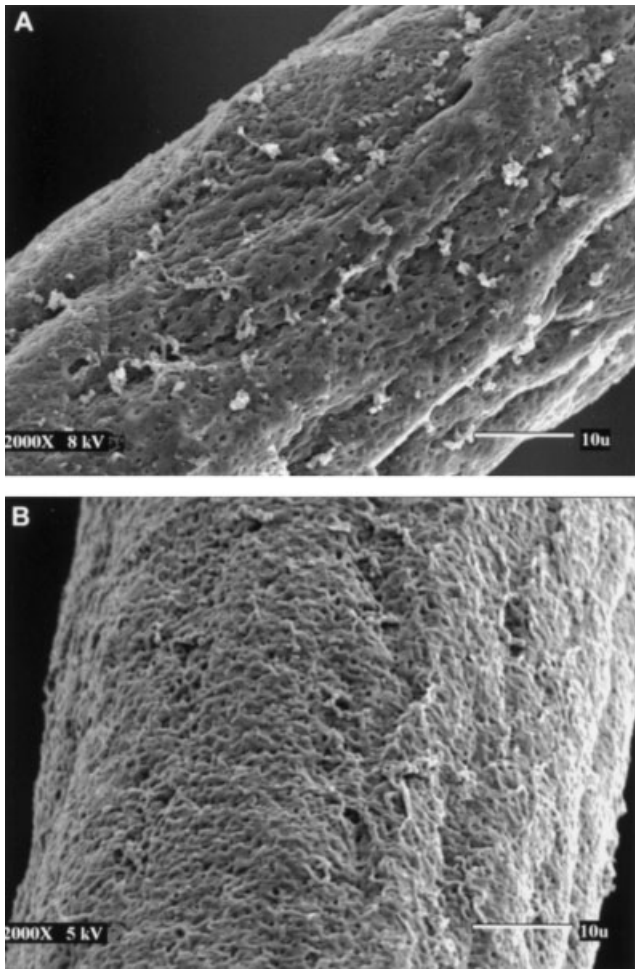

Figure 2. Scanning electron microscopy depicting surface morphology for PLLA fibers (A) and ECM gel–coated PLLA fibers (B), at an original magnification of 2000×. Scale bar represents 10 microns.

axis of ECM gel-coated PLLA fibers [Fig. 3(A)]. These myofibers were organized parallel to each other on individual PLLA fibers. In addition, visualization of nuclei using Hoechst dye demonstrated that myosin heavy chain positive myofibers contained multiple nuclei [Fig. 3(B)].

To further evaluate the expression of muscle markers, protein was isolated from myoblasts and myofibers growing on gelatin-coated tissue culture plates and from cells on ECM gel-coated PLLA fibers. HSKM5 cells expressed both myosin and α-actinin upon differentiation, whether grown on gelatin-coated tissue culture plates or ECM gel-coated PLLA fibers (Fig. 4). Undifferentiated HSKM5 myoblasts did not express these sarcomeric proteins. Myoglobin, however, was not expressed in HSKM5 myoblasts or myofibers on either gelatin-coated tissue culture plates or ECM gel-coated PLLA fibers, showing these cells were not fully differentiated or differentiated exclusively into glycolytic (Type IIb) myofibers. Protein expression in C2C12 myofibers serves as a positive control, whereas tubulin expression shown in all samples confirms that equal amounts of total protein were loaded per lane.

RNA expression and microarray analysis

An oligonucleotide microarray (Human Affymetrix HU133A GeneChip) containing 22,283 genes was used to examine the overall gene expression profile and state of differentiation of the cells on ECM gel-coated PLLA fibers as compared with tissue culture plates. RNA from three different culture conditions was used for the microarray experiment: 1) myoblasts on gelatin-coated tissue culture plates (Control), 2) myofibers on gelatin-coated tissue culture plates (Plate), and 3) cells differentiated on ECM gel-coated PLLA fibers (Fibers). Approximately 11,140 reliable gene signals were obtained for each sample and used for further analysis.

As the cluster analysis in Figure 5 demonstrates, the gene expression profile of skeletal muscle cells grown

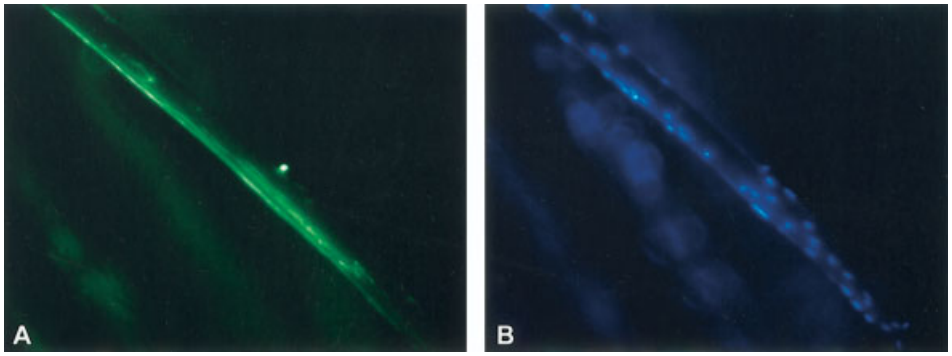

Figure 3. Immunofluorescence microscopy of HSKM5 on ECM gel–coated PLLA fibers exposed to differentiation conditions for 1 week. Expression of myosin heavy chain was detected using an anti-myosin primary antibody and FITC secondary antibody (A), whereas nuclei were labeled with Hoechst (B). Cells were viewed with a 10× objective.

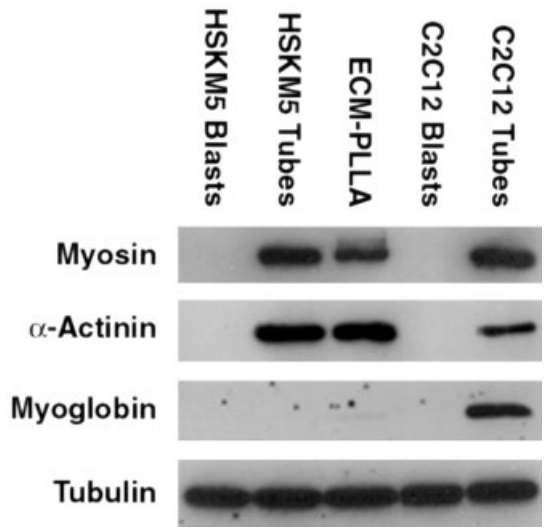

Figure 4. Muscle-specific protein expression by skeletal muscle cells. Cells were grown on either gelatin-coated tissue culture plates (HSKM5 Blasts and HSKM5 Tubes) or ECM gel–coated PLLA fibers (ECM–PLLA). Expression of myosin heavy chain (Myosin), α-actinin, myoglobin, and tubulin was evaluated for both undifferentiated myoblasts (HSKM5 Blasts) and differentiated myofibers (HSKM5 Tubes and ECM–PLLA). C2C12 myoblasts (C2C12 Blasts) and myofibers (C2C12 Tubes) acted as negative and positive controls, respectively.

on ECM gel-coated PLLA fibers (Fibers) resembled that of differentiated cells on gelatin-coated tissue culture plates (Plate) more closely than HSKM5 myoblasts (Control). In general, the majority of genes were either up- (red) or down-regulated (blue) in both Plate and Fibers when compared with Control. For both Fibers and Plate (relative to Control), 223 genes showed a three-fold or more increase in expression levels, whereas 323 genes showed a three-fold or more decrease in expression. Genes that were not up- or down-regulated showed comparable levels of expression as for Control (yellow). The expression pattern for some genes was different between Plate and Fibers, but most of these genes were either ESTs or hypothetical proteins. Syntrophin, β 2 was the only gene up-regulated marginally in Fibers (two-fold relative to Control) but down-regulated marginally in Plate (two-fold relative to Control). Syntrophin is associated with the dystrophin complex, which connects the contractile apparatus within myofibers to the cell membrane. This provides stability to the muscle fiber during contraction. The ECM gel coating on the PLLA fibers may have promoted the formation of cytoskeletal proteins which would be necessary for the development of functional myofibers. This indicates that the presence of ECM proteins within the PLLA fiber scaffold could enhance the formation of contracting myofibers.

To further evaluate the ability of cells to form myofibers on the ECM-PLLA fibers, expression levels of genes related to muscle fiber formation were inspected (Table I). Expression of myosin heavy chain increased in differentiated cells, both on gelatin-coated tissue culture plates (Plate) and ECM gel-coated PLLA fibers (Fibers). RNA expression levels varied for the various isoforms of α-actinin for Plate and Fibers relative to Control. Increases in gene expression for other key sarcomeric proteins (nebulin, titin, troponin, and tropomyosin) and muscle proteins (creatine kinase, desmin, and myogenin) were also seen. Expression of these muscle-specific genes demonstrated that after exposure to differentiation conditions for 1 week, HSKM5 cells were in the process of forming myofibers on both gelatin-coated tissue culture plates and the ECM gel-coated PLLA fiber scaffold.

DISCUSSION

This work demonstrates that a coating of ECM proteins aids in the attachment of skeletal muscle cells to a PLLA surface. These myoblasts can differentiate into multinucleated myofibers, which express muscle-specific genes and proteins, on a PLLA fiber scaffold. In addition, the PLLA fiber scaffold directs the organization of myofibers into a parallel orientation and provides a novel platform for tissue engineering skeletal muscle.

Previous work regarding tissue engineering of skeletal muscle has focused on either the ability of muscle cells to contract within a three-dimensional matrix or the formation of myofibers when implanted into host muscle tissue.[14–18] These studies have not addressed the extent to which skeletal muscle cells are able to differentiate on a three-dimensional matrix. The functional capacity and ability of tissue-engineered skeletal muscle to repair damaged tissue depends on the formation of fully differentiated myofibers organized into a parallel contractile unit. Skeletal muscle cells require sufficient cell density along with the appropriate architecture and cues to guide them in the process of differentiating into myofibers.

The surface morphology (i.e., topography and chemistry) of PLLA fibers has an important role in their ability to support the attachment and differentiation of muscle cells. Because the ECM provides cues for cells during development and repair, ECM proteins within a scaffold should aid in initial cell attachment and potentially cellular organization and differentiation. Recent reports demonstrate the significance of surface characteristics on cell attachment and growth. The presence of specific ECM proteins (fibronectin, laminin, collagen I and IV, and gelatin) enhanced epithelial cell attachment to PLLA or polyglycolic acid films,[19] whereas laminin increased the extent and direction of neurite growth from dorsal root ganglia on PLLA fibers.[20] Despite these findings,

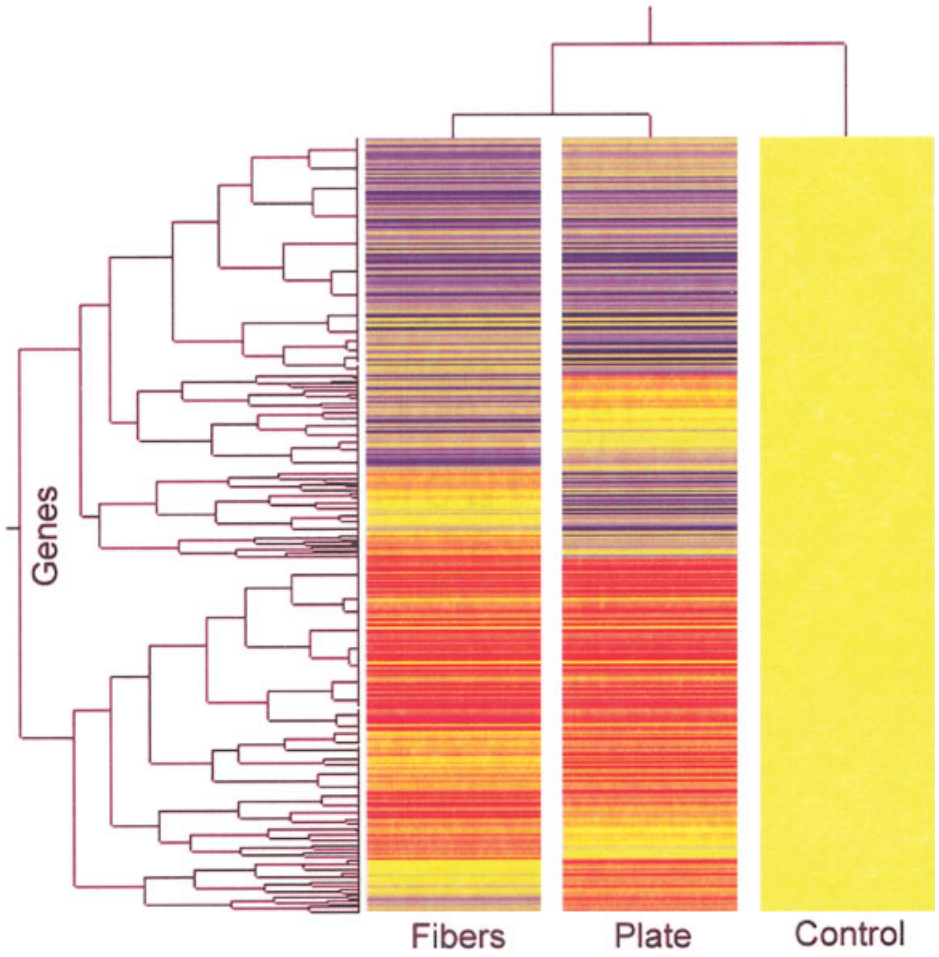

Figure 5. Cluster analysis of gene expression profiles for HSKM5 cells. RNA was isolated from HSKM5 cells exposed to differentiation conditions on gelatin-coated tissue culture plates (Plate) and ECM gel–coated PLLA fibers (Fibers). Undifferentiated myoblasts on gelatin-coated tissue culture plates were also collected (Control). Oligonucleotide microarrays (Human Affymetrix HU133A GeneChip) were used to measure gene expression profiles and results were analyzed using GeneSpring software. The Control sample was used as a reference standard for Fibers and Plate, where red indicated up-regulated genes and blue down-regulated genes relative to Control.

current research in cell-scaffold technologies have not utilized ECM proteins to enhance muscle-tissue formation.

The surface of PLLA fibers coated with ECM gel appeared fibrillar in nature and provided cells with numerous points of attachment. Because the ECM gel coating consisted of a combination of collagen, laminin, and proteoglycans, it resembled the ECM in skeletal muscle tissue. This ECM gel coating was effective in increasing LH121 cell attachment to uncoated PLLA films, and its gel-like consistency formed a thick coating on the PLLA fibers. A coating of ECM proteins provides a means for optimizing the scaffold environment for improved cell attachment and subsequent differentiation.

The appropriate genetic pathways for mature muscle fiber development were activated in skeletal muscle cells on ECM gel-coated PLLA fibers, as demonstrated by the expression of contractile proteins (myosin, actin, nebulin, titin, troponin, and tropomyosin) and genes related to muscle fiber formation (creatine kinase, desmin, myogenin). Using microarray analysis, it has been demonstrated that distinct functional categories of genes are differentially regulated as mouse myoblasts (C2C12 cells) differentiate into myofibers.[21] Aside from genes related to myogenesis and muscle contraction, categories such as cell adhesion, ECM function, cellular metabolism, and cell cycle control were also involved in the differentiation of C2C12 cells. The microarray results reported here, for skeletal muscle cells on ECM gel-coated PLLA fibers, followed the same pattern as that observed in C2C12 cells (Supplemental Data, Tables S1–S3). The up- or down-regulation of cytoskeletal, structural, and cell adhesion genes initiates the differentiation process by inhibiting proliferation, and maintains the integrity and function of muscle by aiding in cell fusion and the assembly of the contractile apparatus.

TABLE I
Muscle-Specific Genes Up-Regulated for Plate and Fibers Relative to Control[a]

Common Name	Description	Control Normalized	Fibers Normalized	Plate Normalized
ACTA1	Actin, alpha 1, skeletal muscle	1	18.75	42.37
ACTA2	Actin, alpha 2, smooth muscle, aorta	1	2.56	3.66
ACTN1	Actinin, alpha 1	1	1.06	0.76
ACTN2	Actinin, alpha 2	1	52.76	199.73
ACTN3	Actinin, alpha 3	1	0.97	1.09
ACTN4	Actinin, alpha 4	1	1.03	0.43
CKM	Creatine kinase, muscle	1	32.80	59.32
DES	Desmin	1	8.14	11.42
MYOG	Myogenin (myogenic factor 4)	1	9.17	18.24
MYO1D	Myosin ID	1	7.12	3.73
MYH1	Myosin, heavy polypeptide 1, skeletal muscle, adult	1	5.06	7.09
MYH2	Myosin, heavy polypeptide 2, skeletal muscle, adult	1	7.13	43.09
MYH3	Myosin, heavy polypeptide 3, skeletal muscle, embryonic	1	23.04	74.34
MYH6	Myosin, heavy polypeptide 6, cardiac muscle, alpha (cardiomyopathy, hypertrophic 1)	1	14.77	57.18
MYH7	Myosin, heavy polypeptide 7, cardiac muscle, beta	1	11.61	33.71
MYH8	Myosin, heavy polypeptide 8, skeletal muscle, perinatal	1	38.04	141.89
MYL1	Myosin, light polypeptide 1, alkali; skeletal, fast	1	3.13	6.00
MYL2	Myosin, light polypeptide 2, regulatory, cardiac, slow	1	5.73	20.08
MYL4	Myosin, light polypeptide 4, alkali; atrial, embryonic	1	5.64	15.55
NEB	Nebulin	1	14.62	74.36
SGCD	Sarcoglycan, delta (35-kD dystrophin-associated glycoprotein)	1	3.17	3.57
SGCE	Sarcoglycan, epsilon	1	2.04	2.64
SPTBN1	Spectrin, beta, non-erythrocytic 1	1	3.95	5.65
TTN	Titin	1	7.81	39.61
TTID	Titin immunoglobulin domain protein (myotilin)	1	2.23	4.18
TPM1	Tropomyosin 1 (alpha)	1	2.73	5.05
TPM2	Tropomyosin 2 (beta)	1	4.30	11.22
TPM4	Tropomyosin 4	1	3.17	2.12
TNNC2	Troponin C2, fast	1	19.93	71.42
TNNI2	Troponin 1, skeletal, fast	1	20.25	47.12
TNNI1	Troponin 1, skeletal, slow	1	189.32	355.51
TNNT2	Troponin T2, cardiac	1	11.21	14.99
TNNT3	Troponin T3, skeletal, fast	1	4.81	10.64

[a]Values represent normalized expression levels relative to Control, from GeneSpring analysis. Signals were averaged for gene duplicates.

Analysis of the microarray results was further supported by a data mining tool, IRIDESCENT. The IRIDESCENT software looked for either direct or indirect relationships among genes whose expression level changed by three-fold. Genes up-regulated in skeletal muscle cells on ECM gel-coated PLLA fibers showed relationships with the cytoskeleton, muscle differentiation, and the ECM (Supplemental Data, Table S4), whereas down-regulated genes were related to the cell cycle (Supplemental Data, Table S5). These observations indicate that cells on ECM gel-coated PLLA fibers were not only in the process of differentiating into myofibers but that structural supports required for functional muscle fibers were being formed.

The differentiation of myoblasts into multinucleated myofibers is required for functional muscle tissue to develop, and is therefore an important step in the engineering of skeletal muscle. Skeletal muscle cells were able to fuse together and form linear myofibers oriented parallel to each other along individual ECM gel-coated PLLA fibers, as seen during skeletal muscle development. In contrast to cell injection techniques, PLLA fibers within a scaffold provide the appropriate directional cues for proper myofiber formation and organization. For diseases such as DMD, the inherent architecture of the muscle tissue is damaged and injected cells are unable to form sufficient levels of organized, functional muscle fibers. A PLLA fiber scaffold, however, would aid in the three-dimensional growth, differentiation, and organization of cells by providing the necessary directional cues along with architectural support.

Our work provides a greater understanding of the level of differentiation of myoblasts into myofibers on a PLLA fiber scaffold, which will aid in the formation of mature myofibers on a polymer fiber scaffold. This

method of tissue engineering skeletal muscle can be used to: 1) study the incorporation of either vascular or neural cells through co-culturing, and 2) test the functional capacity of engineered skeletal muscle tissue. Knowledge gained from these *in vitro* studies can then be applied toward cell-scaffold transplantation efforts aimed at replacing damaged skeletal muscle tissue.

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