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Mechanical derivation of functional myotubes from adipose-derived stem cells

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ABSTRACT

Though reduced serum or myoblast co-culture alone can differentiate adipose-derived stem cells (ASCs) into mesenchymal lineages, efficiency is usually not sufficient to restore function *in vivo*. Often when injected into fibrotic muscle, their differentiation may be misdirected by the now stiffened tissue. Here ASCs are shown to not just simply reflect the qualitative stiffness sensitivity of bone marrow-derived stem cells (BMSCs) but to exceed BMSC myogenic capacity, expressing the appropriate temporal sequence of muscle transcriptional regulators on muscle-mimicking extracellular matrix in a tension and focal adhesion-dependent manner. ASCs formed multi-nucleated myotubes with a continuous cytoskeleton that was not due to misdirected cell division; microtubule depolymerization severed myotubes, but after washout, ASCs refused at a rate similar to pre-treated values. BMSCs never underwent stiffness-mediated fusion. ASC-derived myotubes, when replated onto non-permissive stiff matrix, maintained their fused state. Together these data imply enhanced mechanosensitivity for ASCs, making them a better therapeutic cell source for fibrotic muscle.

1. Introduction

Regenerative musculoskeletal applications have been plagued with setbacks owing in part to the fibrosis present in degenerative muscle disorders, e.g. muscular dystrophy [1], which affects 1 in every 3600 male births [2]. Fibrotic muscle has increased collagen density and transglutaminase activity that stiffens the extracellular matrix (ECM) [1,3], making it more closely resemble the osteoid environment of bone [4]. Injection of undifferentiated stem cells into diseased muscle, i.e. cellular myoplasty, was thought to ameliorate the disease by restoring dystrophin expression and thus muscle contraction. Instead, aberrant stem cell differentiation in this fibrotic muscle causes calcified lesions to form [5] due at least in part to the osteogenic properties of abnormally stiff muscle [6]. Tissue engineered musculoskeletal systems will require significant efforts to understand the stem cell–microenvironment interaction and overcome the fibrosis problem for successful stem cell engraftment in damaged muscle.

Adult human bone marrow-derived stem cells (BMSCs) [7] are a commonly used cell source for cellular myoplasty since these cells differentiate into myocytes when exposed to myogenic growth factors and express functional dystrophin. They can also be transplanted and detected over an extended time period [8]. However, these cells have not been shown to be myogenic in fibrotic muscle *in vivo* [5,9,10] or even to form fused skeletal muscle *in vitro* [4]. While skeletal muscle precursors can engraft into dystrophic muscle [11], limited availability despite expansion capability on compliant matrices [12] may adversely impact their clinical translation. Adult human adipose-derived stem cells (ASCs) are readily available, easily isolated, can be chemically differentiated into myocytes, and are competent to engraft into fibrotic muscle whereas BMSCs are not [10,13,14]. While ASCs may appear better suited for translation, functional muscle recovery with these cells is still limited [10], possibly by the stiff diseased environment [1,6,15].

In recent years, ECM stiffness has been identified as another potent stem cell differentiation regulator; cell fate is regulated by contraction against their soft or stiff niche [12,16]. Successful stem cell-based therapies will require acclimating cells to the abnormally stiff ECM of muscular dystrophy [1,3] while inducing and/or maintaining myogenesis, fusion, and dystrophin delivery. Here we directly compare ASC to BMSC stiffness responsiveness and show myogenic differentiation of ASCs on matrices that mimic skeletal muscle. These data suggest that ASCs could serve as a viable cell source for fibrotic muscle therapies.

2. Materials and methods

2.1. Cell Isolation and culture

Human ASCs were isolated from freshly aspirated human subcutaneous adipose tissue (donor age between 26 and 31 years) according to the method described

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previously [17–19] with approval of UCSD human research protections program (Project #101878). Liposuction samples (300 ml) were washed extensively with equal volumes of phosphate-buffered saline (PBS), and then incubated at 37 °C for 45–60 min in 0.1% type I collagenase (Worthington Biochemical). Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Invitrogen), containing 10% fetal bovine serum (FBS; Thermo Scientific) and 1% antibiotic/antimyocotic (Invitrogen). Cells were centrifuged at 1200 rpm for 10 min to remove adipocytes. The pellet was resuspended in 0.16 M NH_4Cl and incubated at room temperature for 5 min to lyse red blood cells. Cells were collected by centrifugation at 1200 rpm for 5 min, filtered through a 100 μm nylon mesh to remove fissile debris, and incubated overnight on tissue culture plastic in complete medium at 37 °C and 5% CO_2. Plates were then washed extensively with PBS to remove residual non-adherent cells. To reduce donor to donor variation, cells from three different donors were pooled. BMSCs (Lonza Walkersville) were cultured in low glucose DMEM with either 10 or 20% FBS and 1% antibiotic/antimyocotic as indicated. C2C12 skeletal myoblasts (ATCC) were cultured in high glucose DMEM 10% FBS and 1% antibiotic/antimyocotic unless cell fusion was induced in which case serum concentration was reduced to 2%. Stem cells were used at low passage numbers between 4 and 7 and C2C12 cells were sub-cultured below passage number 10. Myoseverin (Calbiochem) was dissolved in dimethylsulfoxide (DMSO) and used at 20 μM for a period of 18 h before washout. ASCs were fed by new complete medium for another 7 days before fixation [20]. DMSO only was used as negative control.

2.2. Fabrication of compliant substrates with varying stiffness

Acrylamide was polymerized on an aminosilanized, 25 mm diameter coverslips according to previously established protocol [21]. Briefly, a solution containing the crosslinker N,N′ methylene-bis-acrylamide, acrylamide, 1/100 volume of 10% Ammunium Persulfate and 1/1000 volume of N,N,N′,N′-Tetramethylethylenediamine was mixed. Three different combinations of acrylamide and bis-acrylamide made 1, 10 and 34 kPa (kPa; unit of stiffness) substrates based on a previous recipe [21]. Approximately 20 μl of the mixed solution was placed on the aminosilanized coverslip and a second coverslip pre-treated with dichlorodimethylsilane was added to ensure to ensure easy detachment and a uniform matrix surface once polymerized. Final matrices were 50 μm thick as measured by microscopy. 10 μg/ml fibronectin was chemically crosslinked using a photoactivating crosslinker, Sulfo-SANPAH (Pierce), and attachment was confirmed by fluorescence. Matrix stiffness was confirmed by atomic force microscopy (AFM; Asylum Research). Cells were seeded onto fibronectin-coated samples in 6-well plates at 5×10^2 to 2×10^4 cells per well as needed and cultured for the indicated duration.

2.3. Cell surface marker characterization by flow cytometry

ASC and BMSC cultures were trypsinized and resuspended in flow cytometry buffer (PBS supplemented with 2.5% FBS). Cells were incubated with fluorescent-conjugated antibodies against CD34 (Alexafluor 488), CD45 (Alexafluor 488), CD90 (FITC), CD105 (Alexafluor 488) for 30 min on ice. Unlabeled cells were incubated with the appropriate isotype control. Cells were analyzed using a FACScan (BD Biosciences) using a previous protocol [18].

2.4. Differentiation Assays

Samples were fixed in 3.7% formaldehyde at the indicated time point and selectively stained and imaged as indicated. Morphological changes in three groups (ASC, BMSC 10% FBS, BMSC 20% FBS) were examined by bright field images, and assessment of the development of branches (number of branch per cell) or spindle shapes (the major/minor axis of cell) were quantified using ImageJ software. To quantify matrix mineralization, cells were stained with alizarin red S, and staining was measured spectrophotometrically at 405 nm. Cells also were stained with lineage-specific marker antibodies: neurogenic differentiation with βIII tubulin (Sigma), myogenic differentiaion with Myogenesis Differentiation Protein 1 (MyoD; Santa Cruz Biotech.), and osteogenic differentiation with Runt-related transcription factor 2/Core Binding Factor α1 (Runx2; Alpha Diagnostic International). To measure mRNA transcription level, two specific primers were used per lineage: Microtubule-associated protein tau (MAPT) and Glial cell-derived neurotrophic factor (GDNF) for neurogenesis; myogenic factor 4 (Myogenin) and Myocyte-specific enhancer factor 2C (MEF2C) for myogenesis; Twist transcription factor 1 (TWIST) and bone gamma-carboxyglutamic acid-containing protein (Osteocalcin) for osteogenesis.

2.5. Fusion Assays

ASCs, BMSCs, and C2C12 cells were each divided into two populations, labeled either green (PKH67) or red (PKH26) hydrophobic dyes that localize to the plasma membrane, and seeded onto 10 kPa polyacrylamide (PA) matrices for one week. The continuous cytoskeletal structure of fused cells was confirmed by staining for β tubulin (Development Studies Hybridoma Bank) and proliferation marker Ki67 (Santa Cruz Biotech.) was used to identify proliferating bi-nucleated cells. Fusion frequency was determined for the indicated conditions. Myoseverin (Santa Cruz Biotech.) was used to depolymerize microtubules to cause cell fission. To quantify myotube formation, we observed more than 660 cells from four independent samples. Only binucleated cells with continuous cytoskeleton but lack of Ki67 staining were considered as myotubes.

2.6. Mechanosensing Assays

Vinculin antibody (Sigma) and rhodamine-phalloidin (Invitrogen) were used to visualize focal adhesions and actin, respectively. Strain energy and tangential stress were calculated by traction force microscopy (TFM) as previously described [22]. To knockdown integrin–fibronectin interaction, ASCs were treated by lipid-mediated siRNA (integrin α5 or αV) for 4 days and replated onto 10 kPa matrices for another week. Integrin knockdown effect was determined by flow cytometry using a FACScan (Becton Dickenson).

2.7. Morphological measurements

Quantitative cell measurements were made using brightfield images from a Nikon Eclipse TE2000-U microscope with a motorized, programmable stage using a CoolSnap HQ camera controlled by Metamorph 7.6 software. ImageJ software (NIH) was used to analyze images and quantify cell morphology. Specifically, the number of branch points per cells was counted if the branch was longer than the length of the cell's major axis by modified method form previous protocol [23]. The spindle factor was calculated for cells on the indicated matrices as the length of the cell's major divided by its minor axis [4].

2.8. Alizarin Red S and immunofluorescent staining

Cells were fixed with 3.7% formaldehyde (Sigma) at room temperature for 15 min. Samples to be stained by alizarin red were then washed once with dH_2O and 2 ml alizarin red S (ARS) solution (2%, pH 4.2) was added to each well. ARS solution was removed 1 h later and each well was washed with dH_2O for 4–5 times. Images were taken at this point and the plate was then air dried at room temperature. The amount of matrix mineralization was determined by dissolving the cell-bound ARS in 10% acetic acid and quantifying it spectrophotometrically at 405 nm. Samples to be stained immunofluorescently were treated with PBS containing 1% Triton X-100 for 15 min and washed with a staining solution of PBS containing 1 μM $MgCl_2$. The primary antibodies listed below with their indicated dilutions in a staining solution containing 2% bovine serum albumin were then added to samples: βIII tubulin (1:100; Sigma), MyoD (1:100; Santa Cruz), Runx2 (1:100; Alpha Diagnostic International), β tubulin (1:100; Developmental Studies Hybridoma Bank), vinculin (1:100; Abcam), and Ki67 (1:100; Santa Cruz Biotechnology). Antibodies were incubated with samples for 30 min at 37 °C. After washing three times with the staining solution, samples were incubated with Alexa fluor 488- or 568-conjugated secondary antibody (1:400; Invitrogen) for 30 min at 37 °C. After washing three times with staining solution, nuclei were stained by Hoechst 33342 (1:1000; Invitrogen) for 2 min. All samples were examined by a CARV II confocal microscope (BD Biosciences) mounted on a Nikon Eclipse TE2000-U microscope with a motorized, programmable stage using a CoolSnap HQ camera controlled by Metamorph 7.6 software.

2.9. Quantitative polymerization chain reaction (qPCR)

RNA from cells was extracted using a previously published Trizol, chloroform, and isopropanol-based protocol [24]. cDNA was synthesized from the isolated RNA template using Superscript II Reverse transcriptase (Invitrogen), 2.5 mM of random hexamer mix (Invitrogen), and 1 μg of total RNA. The reverse transcription reaction was performed with the following conditions: 37 °C for 60 min, 99 °C for 5 min, and 5 °C for 5 min. The SYBR Green PCR Master Mix (Applied Biosystems) was used with 800 nM of each primer. Primers were custom designed from IDT: MAPT (NM_005910.5) F: 5′ ATT ACT GCC AAC AGT TTC GGC TGC 3′, R: 5′ TAA GAA GGC CCA TGG TGC TGA AGA 3′; GDNF (NM_000514.3) F: 5′ TCC CAT TCA GAG AAC CTT GGC AGT 3′, R: 5′ ACC TGC TTG TGG TGT GTA GGT GAT 3′; Myogenin (NM_002479.4) F: 5′ GCC TTG ATG TGC AGC AAC AGC TTA 3′, R: 5′ AAC TGC TGG GTG CCA TTT AAA CCC 3′; MEF2C (NM_002397) F: 5′ AGT GGG TGG GAA AGG GTC ATT ACA 3′, R: 5′ TAG CCA AGG CTT CTG CTG GTA CTT 3′; TWIST (NM_000474.3) F: 5′ ACC ATC CTC ACA CCT CTG CAT TCT 3′, R: 5′TTC CTT TCA GTG GCT GAT TGG CAC 3′; Osteocalcin (NM_199173.7) F: 5′ TCC AGG CAC CCT TCT TTC CTC TT 3′, R: 5′ GAG TTT ATT TGG GAG CAG CTG GGA 3′. The real-time reaction was done in duplicate in an ABI Prism 7900 HT detection system (Applied Biosystems) using the following reaction profile: 2 min at 50 °C and 10 min at 95 °C for one cycle, followed by 15 s at 95 °C and 1 min at 60 °C for 40 cycles. Values were analyzed using SDS 2.3 software (Applied Biosystems), which calculated expression based on a standard curve generated by a fibronectin plasmid [24]. GAPDH was used to normalize all data, which was plotted as a fold change from undifferentiated ASC or BMSC control samples [24].

2.10. Traction force microscopy

Strain energy and tangential stresses were calculated by TFM as previously described [22]. Briefly, 30 μL of carboxylate-modified 0.1 μm diameter yellow-green fluorescent microspheres (Invitrogen) were added to 1 mL of solution that