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Biophysical cues enhance myogenesis of human adipose derived stem/stromal cells

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ABSTRACT

Adipose-derived stem/stromal cell (ASC)-based tissue engineered muscle grafts could provide an effective alternative therapy to autografts – which are limited by their availability – for the regeneration of damaged muscle. However, the current myogenic potential of ASCs is limited by their low differentiation efficiency into myoblasts. The aim of this study was to enhance the myogenic response of human ASCs to biochemical cues by providing biophysical stimuli (11% cyclic uniaxial strain, 0.5 Hz, 1 h/day) to mimic the cues present in the native muscle microenvironment. ASCs elongated and fused upon induction with myogenic induction medium alone. Yet, their myogenic characteristics were significantly enhanced with the addition of biophysical stimulation; the nuclei per cell increased approximately 4.5-fold by day 21 in dynamic compared to static conditions (23.3 ± 7.3 vs. 5.2 ± 1.6, respectively), they aligned at almost 45° to the direction of strain, and exhibited significantly higher expression of myogenic proteins (desmin, myoD and myosin heavy chain). These results demonstrate that mimicking the biophysical cues inherent to the native muscle microenvironment in monolayer ASC cultures significantly improves their differentiation along the myogenic lineage.

1. Introduction

Skeletal muscle makes up almost half of the human body mass and is frequently subject to injury [1]. Roughly 35–55% of all sport-related activities and approximately 40% of all traumatic injury involve skeletal muscle damage while muscle tears account for more than 1 million visits to clinics each year globally. In total, this accounts for ca. $6 billion annually worldwide [2,3]. Skeletal muscle possesses robust self-regeneration capacity mainly by the activation of resident satellite cells. However, spontaneous regeneration of volumetric muscle loss (VML) is limited. In such cases, the fibrotic scar tissue formation proceeds more rapidly than *de novo* myogenesis [4]. Eventually, this fibrosis results in the failure of regeneration if the initial muscle loss is more than 20% of the tissue volume [5]. Currently, no effective drug therapies are available to induce healing of VML defects and the surgical treatments using autologous skeletal muscle transplantation (i.e., flaps) are limited by the availability of suitable donor tissues [6]. Numerous efforts have been made to overcome this problem, including blocking pathways of scar tissue formation to stimulate the intrinsic regeneration capacity [7,8] as well as cellular transplantation therapies using satellite cells [9], muscle-derived stem cells [10], bone marrow-derived mesenchymal stem cells (MSCs) [11] and adipose-derived stem cells (ASCs) [12]. However, there are drawbacks to each of these approaches such as deleterious side effects, insufficient incorporation, and rapid cellular clearance from the injury site. For example, when directly injected into the rat soleus muscle, ASCs provided better healing at 2 weeks compared to the untreated group, while no difference was observed at 4 weeks mainly due to insufficient retention at the defect site [12]. To this end, developing tissue engineered muscle grafts from autologous cells could provide an alternative effective therapy.

ASCs are obtained from adipose tissue which is an easily accessible, abundant autologous cell source associated with an acceptable level of patient discomfort, and therefore of great interest for tissue engineering and cell therapy approaches [13–15]. ASCs have demonstrated considerable multi-lineage capabilities, having the ability to differentiate along osteogenic [16], chondrogenic [17], vascular [15], neuronal [18] and myogenic [19] phenotypes. However, there remains room for further exploration of ASCs' myogenic differentiation capacity. Comparative studies have shown that ASCs exhibit better myogenic capacity than MSCs as well as a higher proliferation rate and a greater percentage of stem cell marker expression [20]. Additional reports have explored the myogenic use of ASCs using substrates that mimic muscle niche

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stiffness [21] and alignment [22] as well as blocking negative regulators of myogenesis such as myostatin [23]. While these methodologies all increased the efficiency of differentiation along the myogenic lineage, their overall differentiation efficiency remains relatively low (<15%); thus there remains need for alternative strategies.

One approach is to provide biophysical cues that mimic the physiological stresses acting on the cells *in situ*. Cells residing within physiologically active tissues (i.e. cardiac [24], skeletal and smooth muscle [25,26], tendon [27], ligament [28], cartilage [29], and bone [30]) experience unique forces that govern their native behavior. Mimicking these physical forces within the cellular microenvironment, leads to enhanced tissue specific gene and protein expression as well as structural changes in the extracellular matrix and 3D tissues. For example, application of cyclic strain to primary human myoblasts enhanced elasticity, myofiber diameter and area when compared to static culture conditions [25]. Similarly, enhanced metabolic activity of C2C12 mouse myoblasts were observed in response to 10% cyclic stretch [31] and, conversely, cessation of 12% stretch on C2C12 cultures caused myotube atrophy [32]. Thus, we hypothesized that biophysical cues (cyclic uniaxial strain) in conjunction with biochemical stimuli (azacytidine) would induce ASCs to exhibit enhanced myogenic outcomes (alignment, multi-nucleation, and expression of muscle-specific proteins) and significantly improve the efficiency of differentiation.

2. Methods

2.1. Cell isolation and culture

ASCs were isolated from human subcutaneous adipose lipoaspirates as described previously [15,16] at the Pennington Biomedical Research Center under an Institutional Review Board approved protocol with informed patient consent. Briefly, the lipoaspirate was digested for 1 h at 37 °C in media containing 1 mg/mL Collagenase Type I (Worthington Biochemical Corp., Lakewood, NJ), 10 mg/mL bovine serum albumin (Sigma, St. Louis, MO), and 2 mM $CaCl_2$ in PBS. After centrifugation and re-suspension in Stromal Medium composed of DMEM/F12 Hams Medium (Hyclone, Logan UT) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin/amphotericin (Antibiotic/Antimycotic, Fisher Scientific), the stromal vascular pellet was plated. The adherent population (passage 0) was harvested upon confluence and stored in liquid nitrogen prior to shipment to Johns Hopkins University. Incubation was performed in Culture Medium (CM) containing low-glucose DMEM with 10% FBS and 1% P/S.

2.2. Biochemical and biophysical stimulation

Passage 2 ASCs were seeded at 5000 cells/cm^2 in collagen I coated 6-well plates with flexible membrane substrates (UniFlex Culture Plate, Flexcell International Corp, Hillsborough, NC). Biochemical stimulation was done 24 h after seeding via myogenic induction medium (MIM) applied to the *Induced* group for 24 h. The composition of MIM was determined in preliminary experiments using head-to-head comparisons of several published protocols (data not shown), to include 1% FBS, 5% horse serum (HS; Invitrogen), 10 μM 5-Azacytidine (Aza; Sigma) and 1% P/S within low-glucose DMEM to maximize multi-nucleation. After 24 h, the wells were washed with PBS and fed with fresh CM for the rest of the culture. *Uninduced* cells were retained in CM throughout. ASCs in these two groups were either grown in *Static* or *Dynamic* conditions. *Dynamic* cultures were exposed to uniaxial cyclic strain using the Flexcell system (FX-5000™) between days 3–21 of culture for 1 h/day using 11% strain (the maximum limit of the equipment to provide uniaxial strain) at 0.5 Hz, to approximate the physiological conditions present during normal locomotion [33]. *Static* controls were not subjected to strain.

2.3. Cell morphology and immunocytochemistry (ICC)

The morphology of ASCs was assessed using phase contrast microscopy at days 1, 3, 7, 14 and 21. At 7, 14 and 21 days, the samples were fixed with 3.7% paraformaldehyde, rinsed in PBS and permeabilized with 0.2% triton X-100 for 10 min. After washing with PBS, samples were incubated with 10% normal donkey serum (Sigma) for 30 min to prevent non-specific binding. The samples were then either incubated for 1 h with TRITC-conjugated phalloidin (Sigma) for actin filament staining or primary antibodies rabbit anti-desmin (1:50, Santa Cruz Biotechnology, Dallas, TX), mouse anti-myoD (1:125, Sigma) and mouse anti-skeletal myosin (MHC, Sigma, 1:400) diluted in 1% donkey serum at 4 °C overnight. Following this, samples were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor®647-conjugated donkey anti-rabbit IgG or Cy™3-conjugated donkey anti-mouse IgG, 1:200) (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Nuclei were labeled using DAPI. All images were taken using a Zeiss Axio Observer inverted fluorescence microscope. Phase contrast images were used to quantify cell alignment ($n = 24$) while fluorescence images were used for the determination of multi-nucleation (phalloidin-TRITC) and extent of myogenic marker expression (immunostaining for desmin, myoD and MHC; $n = 6$) using Image J software (NIH). Quantification of cellular alignment was performed by measuring the angle of the major axes of the cells relative to the direction of strain, by 4 random angle measurements from 6 different images. Multi-nucleation was assessed by counting the number of nuclei within tubes with a continuous cytoskeleton. Staining intensity measurements were performed using 3 representative images from 2 separate culture wells.

2.4. Statistical analysis

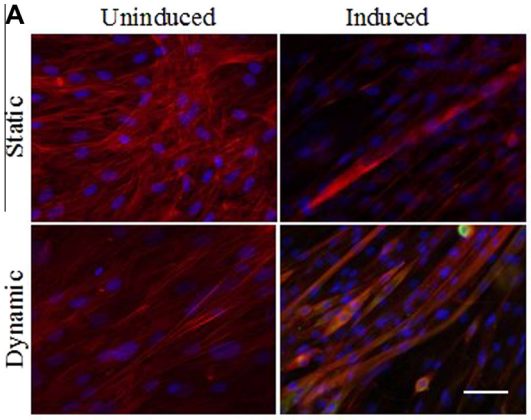
All quantitative results were expressed as means ± standard deviation ($n = 6$). Data was analyzed with statistically significant values defined as $p < 0.05$ based on one-way analysis of variance (ANOVA) followed by Tukey's test for determination of the significance of difference between different groups ($p \leqslant 0.05$).

3. Results

3.1. Biochemical stimulation is necessary to induce ASC multi-nucleation

The effect of biochemical and biophysical stimulation on the morphology and multi-nucleation of ASCs was assessed by actin cytoskeleton staining and ICC at specified time points. Actin staining on day 14 revealed increased cell fusion and multi-nucleation in response to the application of myogenic induction medium (MIM) under *Static* conditions, while *Uninduced* controls proliferated randomly (Fig. 1A). The effect of biochemical stimulation was also prevalent under *Dynamic* conditions; *Induced* cells form multinucleated myotubes by day 14, while no significant multi-nucleation was observed for *Uninduced* counterparts (Fig. 1A). Therefore, biochemical stimulation was found to be necessary to induce ASC multi-nucleation both under *Static* and *Dynamic* conditions and further studies to assess the effect of biophysical stimulation were conducted using *Induced* cells.

The number of myotubes and nuclei/myotube throughout the culture was quantified on days 7, 14 and 21 based on the staining images. The total number of myotubes in *Static* culture was 6.0 ± 1.4 on day 21 with 5.2 ± 1.6 nuclei/myotube (Fig. 1B). Both



B

Induced		Day 7	Day 14	Day 21
Static	*No of Myotubes*	0	3.5±0.7	6.0±1.4
	Nuclei / Myotube	-	2.7±1.1	5.2±1.6
Dynamic	*No of Myotubes*	6.5±2.1	15.5±3.5	32.0±2.8
	Nuclei / Myotube	4.7±2.2	9.9±3.5	23.3±7.3

Fig. 1. (A) Phalloidin (red) stains demonstrate the need for both biochemical and biophysical stimulation to induce ASCs to form multi-nucleated myotubes. (Mag: 40×). Blue: DAPI. Scale bar: 50 μm. (B) Quantification of myotube formation and multi-nucleation in response to uniaxial strain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

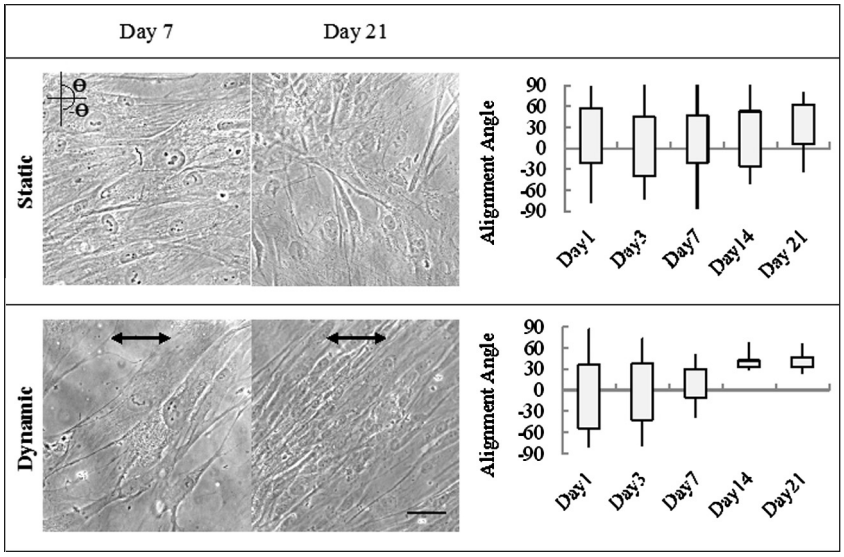


Fig. 2. Alignment of induced ASCs upon uniaxial cyclic strain. Bidirectional arrows indicate direction of strain. Scale bar: 50 μm.

the total number of myotubes (32.0 ± 2.8) and the nuclei/myotube (23.3 ± 7.3) were significantly enhanced under *Dynamic* conditions (5.3- and 4.5-fold, respectively) compared to the *Static* culture.

Cellular alignment was studied in response to strain using *Induced* ASCs as both *Static Induced* and *Dynamic Induced* revealed a global orientation at an angle to the direction of uniaxial strain. The substrate design facilitated unidirectional strain and allowed cell alignment in both *Static* and *Dynamic* cultures to be measured relative to the same axis. Under *Static* conditions, there was no observed preferential alignment of the cells between days 1 and 7 after induction (range: ca. −90° to 90°), while on day 14 the alignment angle narrowed to range from −45° to 85° and by day 21 cell alignment ranged from −30° to 80° (Fig. 2). In the *Dynamic* culture, there was a gradual narrowing in the distribution of the alignment angle over time; the angle at which the cells were positioned gradually trended toward 45° to the direction of strain and showed a very narrow distribution on days 14 and 21.

3.2. Biophysical stimulation enhances the expression of myogenic markers

The expression of muscle specific proteins was assessed by immunofluorescence staining of desmin, myoD and myosin heavy chain (MHC) as early and later stage myogenesis markers. No muscle specific protein expression was observed in *Uninduced* controls (not shown) confirming that biochemical induction is a necessary component of the differentiation protocol. Immunostaining data demonstrated that *Static-Induced* ASCs expressed desmin at days 7, 14 and 21 and the intensity of the staining increased with time as quantified by image analysis (Fig. 3A and B). The expression of desmin was significantly higher t with the application of uniaxial strain. The difference in desmin expression between *Static* and *Dynamic* conditions were statistically significant at all three time points. Moreover, a homogenous distribution of desmin positive cells was observed throughout the *Dynamic-Induced* cultures on day 21, indicating uniformly high myogenic differentiation efficiency in response to biophysical cues. Similar trends were observed for myoD expression. MyoD was observed both in the nuclei and in the cytoplasm of the cells. However, myoD appeared later in the culture than desmin: No myoD expression was detected in *Static-Induced* cultures on day 7 and only few, dimly stained cells were observed on Day 21. In *Dynamic* cultures, a small, but statistically higher percentage of cells (relative to *Static*) were myoD positive at Day 7, while almost all cells were myoD positive on Day 21 ($p < 0.001$ relative to *Static*).

MHC is a late stage marker of myogenesis. We investigated its expression to validate the differentiated state of ASCs. ICC revealed

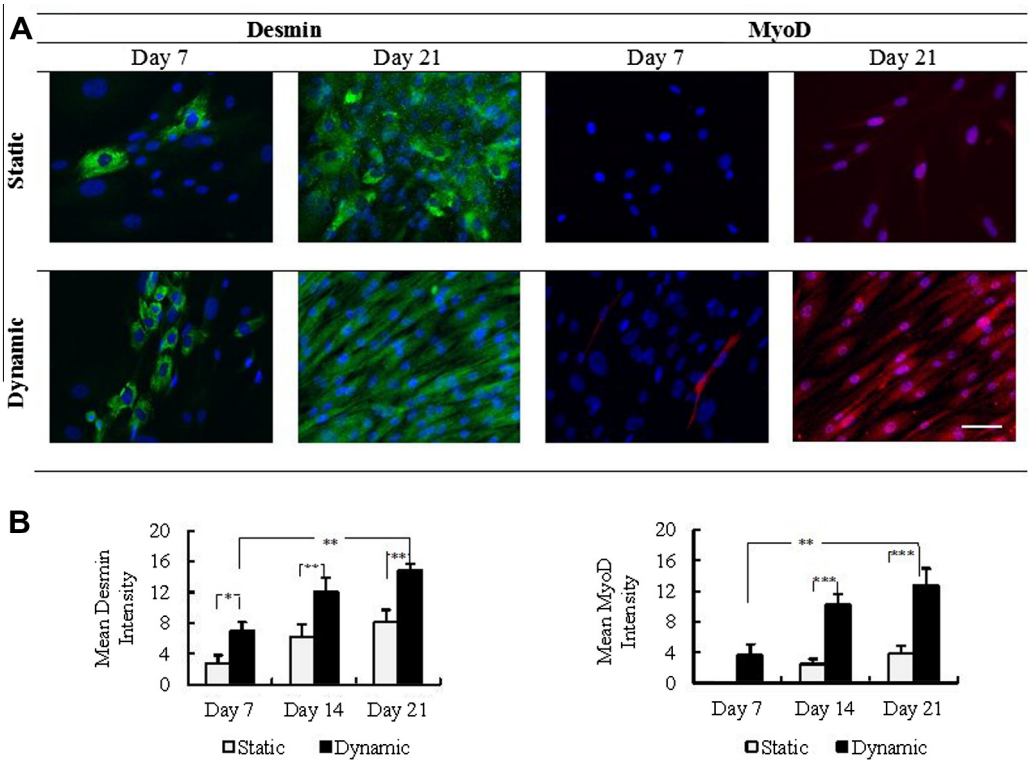


Fig. 3. (A) Desmin and myoD expression in *Static* and *Dynamic Induced* cells at days 7 and 21. (B) Quantification of staining intensity using Image J. Error bars represent standard deviation for $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar: 50 μm.

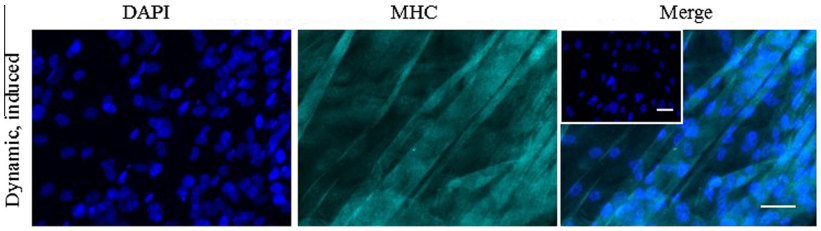


Fig. 4. Expression of myosin heavy chain on Day 21 of *Dynamic* cultures. (Left): DAPI, (Middle) MHC positive stains and (Right): merged image of DAPI and MHC staining. Scale bar: 50 μm. Inset: *Static* culture.

that multi-nucleated tubes derived from ASCs expressed MHC on the Day 21 of *Dynamic* culture only (Fig. 4). It was not detected at earlier time points. No MHC positive staining was observed on any time point of *Static* culture (see the inset image in Fig. 4 for the day 21).

4. Discussion

The myogenic capacity of ASCs was first demonstrated by Mizuno et al. [19] and later confirmed by other researchers [34]. In these cases, the differentiation was induced by adding horse serum to the culture media. Alternatively, following protocols developed for MSC myogenesis [35], some researchers induced the differentiation using a methylation inhibitor – 5-azacytidine (Aza) – to obtain multi-nucleated cells [20,23]. Based on these previous findings, we screened the effect of various myogenic induction agents and assessed the cocktail providing maximal fusion and multi-nucleation of ASCs as 1% FBS, 5% HS, and 10 μm Aza. We demonstrated that biochemical induction is a necessary preliminary step in ASC myogenesis as *Uninduced* ASCs proliferate randomly and they do not fuse or produce myotubes even under *Dynamic* conditions (Fig. 1A).

Skeletal muscle is a dynamic tissue responding to the mechanical stimuli by atrophy with decreasing mechanical stimulation or, conversely, with hypertrophy following the application of increased mechanical stresses [32]. We hypothesized in this study that mechanical stimulation might also be a key inducer of the myogenic phenotype and that ASCs would exhibit enhanced myogenic capacity in response the application of biomimetic uniaxial strain. The mechanical loading parameters used in this study (11% strain applied for 1 h/day, at 0.5 Hz) were estimated from the literature based on loads provided to primary myoblasts [25] or to MSCs induced to become smooth muscles cells [36]. However, the dose-dependence on percent strain or frequency, the timing and duration of the strain are the important parameters regulating the ASC myogenesis. Therefore, more detailed investigations need to be done to better understand the mechanotransduction governing the dynamic culture of ASCs. Further, it should be noted mechanical strain was applied from days 3 to 14. The timing was delayed to enable cells to adhere and produce matrix and minimize any potential detrimental effects that might occur if loaded prematurely.

Several studies have demonstrated that ASCs' responsiveness to mechanical loading enhanced their ability to differentiate along other lineages. These include enhanced osteogenesis under shear

[37] and stretch loading [38], as well as enhanced chondrogenesis in response to cyclic hydrostatic pressure [39]. In a separate study, 10% uniaxial cyclic strain induced a decrease in ASC proliferation as well as an increase in their expression of vascular smooth muscle markers [26]. Similar responses were seen with vascular smooth muscle cells, where it was reported that cessation of cell proliferation was due to the upregulation of apoptosis-inducing nitric oxide in response to cyclic strain [40].

Reports vary in the literature in terms of actin cytoskeletal orientation with respect to uniaxial strain. ASCs have been shown aligned perpendicular [26,40] while MSCs aligned either perpendicular [41] or parallel [36] to the direction of strain. In our system, ASCs aligned approximately 45° to the direction of strain in *Dynamic* cultures suggesting that within the system, we might unwittingly be providing additional mechanical cues such as shear forces as cells pull on the membrane substrate. Interestingly, this is supported by the observation that cells cultured on the membrane under *Static-Induction* conditions also exhibited some alignment (Fig. 2). It may be possible that the cells are capable of sensing the intrinsic anisotropy built into the membrane to accommodate uniaxial stretching.

Our data demonstrate conclusively that expression of muscle specific proteins – desmin, myoD and MHC – were significantly enhanced by the application of strain (Fig. 3). These markers are expressed with spatiotemporal specificity during satellite cell differentiation to myotubes. We therefore explored the kinetics of expression of these proteins, and observed that while the timing of their expression differed from that of satellite cells, the sequence mimicked the myogenic pathway followed by satellite cells (i.e. desmin is expressed early followed by myoD and ultimately, expression of the late marker, MHC) [42]. The prolonged expression of both the structural protein, desmin, and the transcription factor, myoD, (which normally is detected a few days after onset of differentiation in canonical myogenic cells), over the culture period suggests that differentiation is progressing over this three weeks period and eventually leads to the expression of MHC positive multi-nucleated tubes by day 21.

In conclusion, this study demonstrates significantly enhanced ASC differentiation along the myogenic lineage by mimicking the biophysical cues present within the native muscle microenvironment through the application of cyclic strain. Our results demonstrate that ASCs align and fuse upon biochemical and biophysical stimulation and express muscle specific proteins desmin, myoD and MHC with spatiotemporal specificity.

Acknowledgments

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