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Uniaxial cyclic strain enhances adipose-derived stem cell fusion with skeletal myocytes

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ABSTRACT

Although adult muscle tissue possesses an exceptional capacity for regeneration, in the case of large defects, the restoration to original state is not possible. A well-known source for the *de novo* regeneration is the adipose-derived stem cells (ASCs), which can be readily isolated and have been shown to have a broad differentiation and regenerative potential. In this work, we employed uniaxial cyclic tensile strain (CTS), to mechanically stimulate human ASCs to participate in the formation skeletal myotubes in an *in vitro* model of myogenesis. The application of CTS for 48 h resulted in the formation of a highly ordered array of parallel ASCs, but failed to support skeletal muscle terminal differentiation. When the same stimulation paradigm was applied to cocultures with mouse skeletal muscle myoblasts, the percentage of ASCs contributing to the formation of myotubes significantly exceeded the levels reported in the literature hitherto. In perspective, the mechanical strain may be used to increase the efficiency of incorporation of ASCs in the skeletal muscles, which could be found useful in diverse traumatic or pathologic scenarios.

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1. Introduction

Skeletal muscle fulfills important physiological roles, such as voluntary movement and metabolic activity, but if pathologically affected, gives rise to a great number of diseases [1,2]. Healthy adult muscles possess an exceptional capacity for regeneration, which depends mainly on a pool of resident precursors known as the satellite cells [3]. Satellite cells become activated in response to factors secreted by injured muscle fibers and proliferate as myoblasts, which are committed to myogenic differentiation and serve to repair the muscle either by rescuing the existing muscle fibers or forming new ones [3,4]. However, the self-regenerating capacity of the muscle is severely compromised in case of volumetric muscle loss, as e.g. after surgical resection of tumors, representing a significant clinical problem.

Early studies explored the feasibility of expanding satellite cells *ex vivo* to allow their application in the treatment of degenerative muscle disorders or fabrication of engineered tissue for repair of muscle defects. Although initial results from the myoblast transfer therapies appeared promising for the treatment of Duchenne muscular dystrophy, the approach encountered several issues, such as short-term cell survival or immune rejection [5,6]. Moreover, obtaining clinically relevant amounts of satellite cells proved to be a formidable obstacle not only due to the limited amount of cells that may be obtained from biopsies, but also because the cells undergo senescence after few passages [7].

In recent years, alternative sources of cells with skeletal myogenic potential have been investigated for the regeneration of skeletal muscle [8]. Among them, adipose-derived stem cells (ASCs) represent an attractive source [9]. ASCs can be readily isolated and expanded in sufficient quantities, and differentiated into cell types of the mesodermal lineage [10–14]. In addition, ASCs possess regenerative properties that may be of advantage when transplanted at sites of injury [15,16]. The ability of ASCs to participate in skeletal myogenesis has been demonstrated both *in vitro* and *in vivo* [17–20]. These studies have demonstrated that ASCs participate in myotube formation by fusion with differentiating myoblasts. However, the number of stem cells integrated into myotubes remained below 1%. *In vitro* studies have demonstrated that the rate of incorporation and differentiation of the ASCs could be substantially enhanced by including a 5-Azacytidine (5-Aza) in the differentiation medium [21,22]. However, the use of a methylation inhibitor such as 5-Aza

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for skeletal myogenic differentiation remains controversial, due to the non-specific role of the drug in the regulation of several transcription factors, including those associated with cardiomyogenic specification [23].

Apart from biochemical factors, physical microenvironmental cues are crucial for stem cell differentiation [24]. In particular, ASCs have shown an enhanced rate of skeletal myogenic differentiation on substrates that mimic skeletal muscle stiffness, [25]. In addition, the fusion rate appears to be increased when the cells are guided to align on topographically patterned matrices [26]. While the adjustment of substrate properties such as topography and stiffness provides static mechanical cues, cyclic tensile strain (CTS) allows dynamic variation of the applied stimulus. As studies have shown, CTS is a versatile approach for the parallel assembly and enhanced differentiation of skeletal myogenic precursors [27,28]. However, the effects of dynamic mechanical stimulation on the skeletal myogenic differentiation of ASCs have not been well described as yet. In this work, we aim to study the potential of mechanical stimulation in the form of CTS on the skeletal myogenesis of ASCs without using differentiation supplements, alone or in co-culture with committed myogenic precursors.

2. Materials and methods

2.1. Cell sources and culture

C2C12 mouse myoblasts were obtained from LGC-ATCC (LGC Standards, Sweden). The ASCs were derived from adipose tissue from a healthy patient undergoing elective liposuction, as described previously [10]. The cell line used in this work (ASC21) has been extensively characterized in regards to its multilineage differentiation capacity [29–31]. C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. The ASC growth medium consisted of MEM Alpha Medium + GlutaMAX, 10% FCS, and antibiotics. Cells were used between passages 7 and 9. All culture reagents were from Gibco (Life Technologies, Denmark).

2.2. Tagging of ASCs with emerald green fluorescent protein

To obtain ASC line constitutively expressing emerald green fluorescent protein (EmGFP), a commercially available pLenti6.2-GW/EmGFP system was used (Invitrogen, Denmark). The viral particles were produced according to manufacturer's instructions and used to transduce the ASC21 cell line. For infection, the cells were seeded at 5000 cells/cm^2 and incubated with supernatant containing lentiviral particles in the presence of 6 μg/ml polybrene (Sigma–Aldrich, St. Louis, MO) for 24 h. Selection of positive cells was done by the addition of blasticidin in a final concentration of 5 μg/ml. The resulting homogenous cell population was designated ASC21-EmGFP and was maintained in ASC growth medium.

2.3. Myogenic differentiation and mechanical stimulation

The cells were seeded on collagen I coated flexible-bottom BioFlex culture plates (Dunn Labortechnik, Germany) at a density of 5000 cells/cm^2, and were allowed to reach 90–95% confluence before the straining was initiated. For coculture experiments, the ASC21-EmGFP to C2C12 ratio was 1:5. At the point the mechanical stimulation was initiated, the medium in both experimental and control cultures, regardless of the cell type, was replaced with a myogenic differentiation medium based on the DMEM supplemented with 2% horse serum (Invitrogen), and antibiotics. Mechanical stimulation consisted of pulses of 15% uniaxial strain at a frequency of 0.5 Hz using custom-made rectangular pistons for 48 h, as described previously [27]. After the stimulation was accomplished, the cultures were continued for a variable period of time. Differentiation medium was replenished every other day.

2.4. Immunocytochemistry

Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde, followed by permeabilization with 0.1% Triton X-100 and blocking with 1% bovine serum albumin (Sigma–Aldrich). Next, the cells were incubated with a mixture of an anti-myogenin mouse monoclonal antibody conjugated with Alexa Flour 488 (1:100, eBioscience, San Diego, CA) and an anti-myosin mouse monoclonal antibody (1:500, Sigma–Aldrich) labeled with Zenon Alexa Flour 647 (Molecular Probes). After a second round of fixation with formaldehyde, filamentous actin was stained with phalloidin-Bodipy 558/568 (1:40, Invitrogen). Following this, the nuclei were counterstained with 1 μg/ml Hoechst 33,342 (Molecular Probes). The stained preparations were kept in PBS at 4 °C until analyzed. To analyze the fusogenic activity of ASC21-EmGFP cells, the samples were first stained simultaneously for myosin and lamin using mouse monoclonal antibodies, as described above, and the anti-human lamin rabbit IgG (1:2000, Abcam, UK) labeled with Zenon Alexa Flour 555 (Molecular Probes).

2.5. Microscopy and cytomorphometry

Phase contrast and fluorescence images were obtained with a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam MRm camera and a motorized stage using AxioVision software package (Carl Zeiss, Germany). For high resolution imaging of large areas, mosaics from 4 to 25 fields were obtained using 20×/0.8 Plan-Apochromat objective. These compound images were used for the analysis of directionality, differentiation, and the fusogenic activity. Cell alignment was quantified using the OrientationJ (Daniel Sage, EPFL, Lausanne, Switzerland) plugin for ImageJ (NIH, Bethesda, MD) based on the evaluation of the structure tensor in a local neighborhood using actin. Cell count was performed by two independent observers using the Cell Counter plug-in for ImageJ (Kurt De Vos, University of Sheffield, UK). The percentage of fused ASCs was calculated as the number of lamin positive nuclei inside myosin/GFP positive myotubes divided by the total number lamin positive cells.

2.6. Statistical analysis

The data are presented as a mean + SEM. Statistical analysis was performed using SPSS 18 (SPSS, Chicago, IL). Kolmogorov–Smirnov test was used to test for normality, and unpaired *t*-test to compare the means. Statistical significance was assigned to differences with $P < 0.05$.

3. Results

3.1. Effect of uniaxial cyclic tensile strain on cell alignment

Prior to application of mechanical stimulation, the ASCs as well as C2C12 myoblasts assumed a random orientation (Fig. 1A). As a result of straining, the cells rearranged to assume a uniform orientation in a direction perpendicular to that of the strain field. The response of the cells appeared quite rapid, as the first signs of realignment could be observed already after 12 h, irrespective of the cell type. After further 12 h, the cultures became fully organized, but the stimulation was continued for

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