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

Jens Isak Andersen [1], Morten Juhl [1], Thøger Nielsen, Jeppe Emmersen, Trine Fink, Vladimir Zachar, Cristian Pablo Pennisi *

Laboratory for Stem Cell Research, Dept. of Health Science and Technology, Aalborg University, Denmark

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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.

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

* Corresponding author. Address: Laboratory for Stem Cell Research, Dept. of Health Science and Technology, Aalborg University, Fredrik Bajers Vej 3B, 9220 Aalborg, Denmark.
E-mail address: cpennisi@hst.aau.dk (C.P. Pennisi).
[1] These co-authors contributed to the manuscript equally.

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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

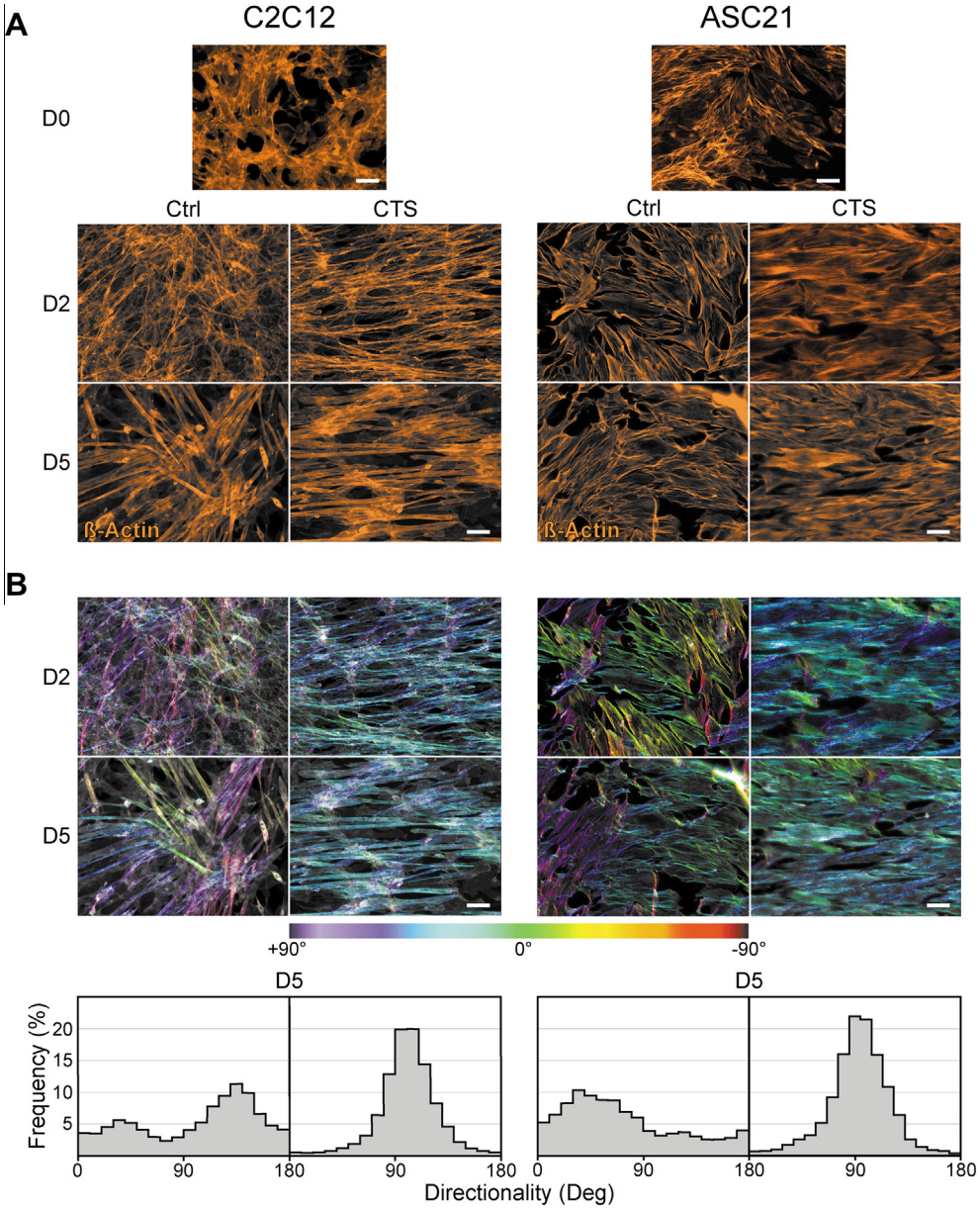

Fig. 1. Effect of uniaxial cyclic strain on directional organization of skeletal myoblasts and ASCs. (A) Cells were subjected to mechanical stimulation for 48 h, from D0 to D2, in differentiation conditions. The cultures remained stationary for further 3 days (D5). (B) Directionality analysis of the images shown in panel A provided a visual rendition based on the HSB color-coded map with hue indicating local orientation, saturation coherency, and brightness the original image. Plotting of the proportion of cells for a given angle interval produced significantly distinct population distributions ($P = 0.0024$) for each of the cell types. The scale bars denote 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

another 24 h to aid the myogenic differentiation. It is interesting that in the case of ASCs, the strain resulted not only in a specific directional organization of the cells, but the treatment appeared to modulate cell morphology, in that they acquired more slender, fusiform shape.

As expected, in the control groups, the cells remained randomly oriented, whereas strained cells oriented around the prevalent axis in a tight pattern that was very close to a model normal distribution (Fig. 1B). The difference between the two populations could be confirmed by cytomorphometric analysis with a high level of statistical confidence ($P = 0.0024$). The cells remained organized with the same directionality throughout the differentiation period, even as the cells elongated and fused to form multinucleated syncytia.

3.2. Effect of uniaxial cyclic tensile strain on myogenic differentiation

The uniaxial straining accelerated myogenic differentiation of C2C12 myoblasts as indicated by the formation of myotubes and expression of both early and late myogenic markers, the myogenin and myosin, respectively, already after 48 h (D2) (Fig. 2). The differentiation process also occurred in control cultures albeit with a delayed dynamics. Nevertheless, after additional 3 days of differentiation (D5), the control and strained cultures exhibited a comparable level of differentiation and differed only with respect to the directional organization of the myotubes.

The ASC cultures, on the other hand, did not undergo spontaneous myogenesis in the differentiation conditions, neither mechanical stimulation seemed to induce this process. More than 65 fields

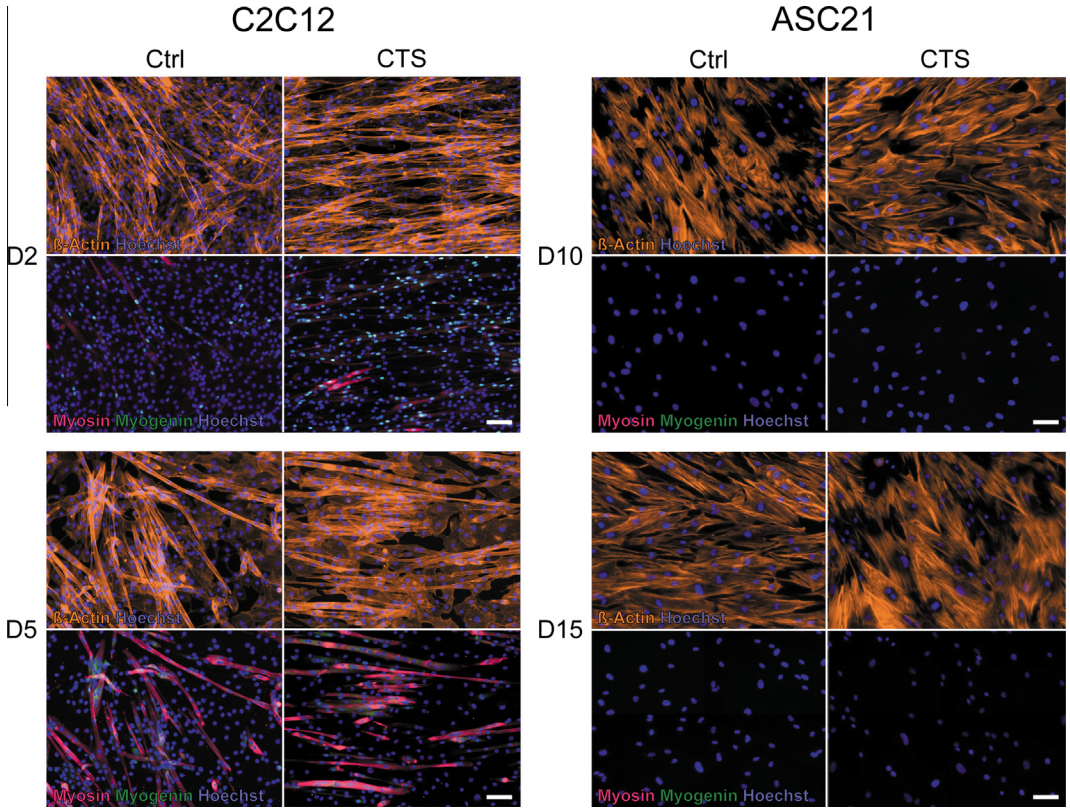

Fig. 2. Effect of uniaxial cyclic strain on myogenic differentiation of skeletal myoblasts and ASCs. Cells were subjected to mechanical stimulation for 48 h, from D0 to D2, in differentiation conditions. The cultures remained stationary for further 3 days (D5) for C2C12, and 8 (D10) or 13 (D15) days for ASCs. The scale bars denote 100 μm.

were analyzed but none of the investigated myogenic markers could be detected even though the cultures were allowed to differentiate for a total period of 15 days.

3.3. Mechanical stimulation enhances ASC myogenic potential on the background of myoblastic myogenesis

In both experimental groups, fusogenic activity of ASC21-EmGFP cells with the mouse cells was observed, as indicated by the appearance of green myotubes (Fig. 3A). This process became first obvious after a week, and peaked approximately after 10 days of differentiation. At this point, the percentage of multinucleated myofibrils expressing both EmGFP and myosin was significantly larger in the mechanically stimulated cocultures (4.98 ± 0.50% vs. 1.09 ± 0.23% in the control conditions, $P = 0.016$).

To determine the cell origin of individual myofibrils, lamin was used as a marker of human nuclei. Both lamin-positive and -negative nuclei could be identified in single myotubes, thus confirming participation of both the ASC and C2C12 cells (Fig. 3B). We did not observe instances of two-nucleated structures harboring single human nucleus, which indicates that the ASC myogenic contribution is most likely based on the fusion with cell syncytia already committed to differentiation rather than myoblastic precursors. It is also interesting, that although the mechanically induced ASC fusogenic activity translated into a relatively higher number of green myotubes per field (1.9 vs. 0.5), the contribution to individual myotubes, as reflected by the frequency of lamin-positive nuclei, did not seem to be influenced by mechanical stimulation (1.1 vs. 1.0).

4. Discussion

ASCs are multipotent mesenchymal stem cells that display skeletal muscle-specific phenotypical characteristics when exposed to myogenic microenvironmental cues [17–20]. Nevertheless, the limited efficiency of this process remains a problem. Although it has previously been shown that 5-Aza promotes their differentiation rate, the effects are non-specific, hampering a clinical translation [23]. Mechanical forces also constitute a significant part of the skeletal muscle microenvironment [32]. In particular, cyclic forces play an important role in the process of differentiation of myogenic precursors [27,28]. Following this concept, we hypothesized that cyclic mechanical stimulation would enhance the ability of ASCs to undergo skeletal muscle differentiation.

In response to the 48 h CTS stimulation regimen, the ASCs were clearly organized with an orientation that was perpendicular to the direction of the applied strain. The robust realignment response was consistent with the effects observed on both myoblastic and non-myoblastic cells [33–35]. It is known that the actin reorganization process, as well as other cellular functions, depend on the cell type and the frequency and amplitude of the applied stretch [34,36]. Although a 10% strain at 1 Hz seems to be adequate to induce a maximal reorientation response of mesenchymal stem cells subjected to CTS [37,38], other studies have proposed a benefit of smaller values to reduce the risk of detachment of the cells [28]. However, a reduction in the strain amplitude and stimulation frequency may lead to adverse issues, such as incomplete realignment or activation of the osteogenic differentiation pathway [39]. Here, we found that application of the paradigm optimal for skeletal muscle myoblasts, 15% strain at 0.5 Hz, consistently provided the cues for efficient alignment of the ASCs, without causing a significant cell detachment.

Previously, it has been shown that transcriptional activity of skeletal myogenic markers is increased when ASCs are subjected to CTS in absence of myogenic supplements [40]. However, it is unclear if the ASCs could progress toward advanced stages of differentiation upon mechanical stimulation alone, given that

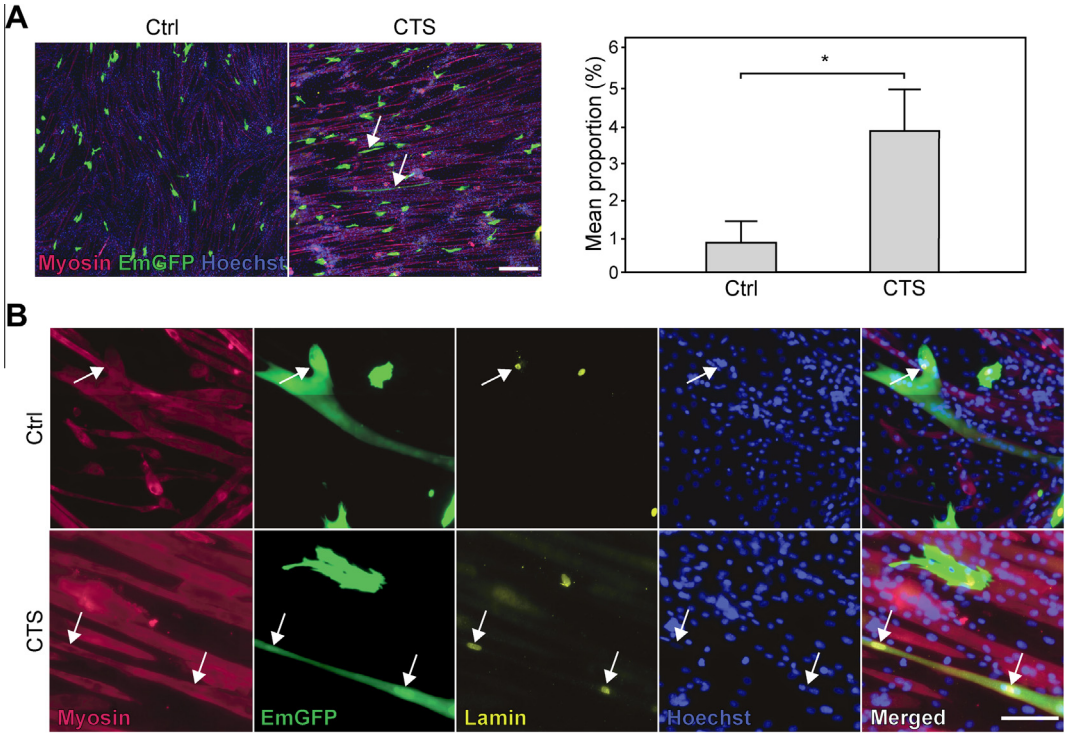

Fig. 3. Effect of uniaxial cyclic strain on fusogenic activity of ASCs. The ASC21-EmGFP cells were subjected to mechanical stimulation in coculture with the C2C12 cells in differentiation conditions for 48 h. The cultures were continued for further 8 days (D10) before analysis. (A) Representative micrographs demonstrating participation of ASCs on the myogenic fusion. The arrows indicate two multinucleated green myotubes. Enumeration of the proportion of ASC21-EmGFP contributing to myotubes confirmed the significant effect of mechanical stimulation (Asterisk, $P = 0.016$). The scale bar denotes 500 μm and the values are presented as a mean + SEM. (B) High power micrographs displaying ASCs participating in hybrid myotube formation. The arrows indicate lamin-positive ASC-specific nuclei. The scale bar denotes 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

myogenin positive nuclei, sarcomeric development, or formation of multinucleated structures have not been demonstrated yet. Here, we aimed to investigate the late stages of skeletal myogenic differentiation by assessing the expression of myogenin and myosin heavy chain. While nuclear presence of myogenin indicates commitment to myogenic differentiation and irreversible exit from the cell cycle, expression of myosin heavy chain is the basis for the formation of contractile apparatus [4]. Contrary to inductive effect of CTS on the mouse myogenic precursors [27], we were not able to detect the expression of myogenin or myosin heavy chain on ASCs. Furthermore, no multinucleated cells could be found in the ASCs subjected to mechanical stimulation. Taken together, these results indicate that reduction of the mitogenic signals by serum deprivation in combination with uniaxial CTS is not sufficient to support the terminal skeletal myogenic differentiation of ASCs. This is in agreement with a recent report, in which myogenic differentiation was absent in monocultures of ASCs, irrespective of the application of cyclic strain [41]. Apparently, biochemical cues in the form of growth factors are essential to support myogenic differentiation of mesenchymal stem cells [28]. There remains though possibility that our mechanical stimulation paradigm has the capacity support specification into other lineages, as strain alone has shown to drive ASC commitment towards smooth muscle differentiation [38].

While the CTS stimulation did not promote the terminal myogenic differentiation of ASCs that were cultured alone, it dramatically enhanced their rate of fusion with myoblasts. In contrast, a concurrent study by Dugan et al. showed that CTS did not affect the fusion rate of ASCs with myoblasts [41]. This discrepancy might be explained by the dissimilar ASC to C2C12 ratio (5:1 vs. 1:5 used here), indicating that a sufficient number of myoblasts is required to sustain an efficient myogenic background. Contact-mediated and non-contact-mediated effects might explain our results. As for the contact-mediated effects, the fact that ASCs become significantly more elongated after mechanical stimulation may facilitate a tight contact with the myogenic precursors, increasing the probability of cadherin interaction that is required for cell fusion. This is also supported by a study of Choi et al., who has shown that the fusion rate increased when ASCs were aligned with myoblasts on mechanically patterned substrates, suggesting that strain might affect the expression of adhesion and fusion related proteins [26]. As for the non-contact-mediated mechanisms, it is known that muscle secretes a wide range of regulatory molecules, such as interleukins and growth factors [42]. For instance, both bFGF and IGF-1 are upregulated in skeletal muscle when subjected to stretch [43,44]. Thus it is conceivable that these growth factors released from C2C12 act as paracrine myogenic signals for the ASCs. In particular, bFGF is a key component of the differentiation media used for myogenic induction of ASCs [18,45] and BMSCs [20,28].

Our data show that application of mechanical cues in the form of CTS is clearly advantageous for the alignment and fusion of ASCs on the backdrop of appropriate myogenic niche. The mechanical strain thus could be conveniently exploited as means to increase the incorporation of ASCs into damaged or diseased muscles, and consequently found useful in applications, where the correction of gene defect or regeneration at large is desirable.

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