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Mechanical strain applied to human fibroblasts differentially regulates skeletal myoblast differentiation

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Hicks MR, Cao TV, Campbell DH, Standley PR. Mechanical strain applied to human fibroblasts differentially regulates skeletal myoblast differentiation. *J Appl Physiol* 113: 465–472, 2012. First published June 7, 2012; doi:10.1152/japplphysiol.01545.2011.—Cyclic short-duration stretches (CSDS) such as those resulting from repetitive motion strain increase the risk of musculoskeletal injury. Myofascial release is a common technique used by clinicians that applies an acyclic long-duration stretch (ALDS) to muscle fascia to repair injury. When subjected to mechanical strain, fibroblasts within muscle fascia secrete IL-6, which has been shown to induce myoblast differentiation, essential for muscle repair. We hypothesize that fibroblasts subjected to ALDS following CSDS induce myoblast differentiation through IL-6. Fibroblast conditioned media and fibroblast-myoblast cocultures were used to test fibroblasts' ability to induce myoblast differentiation. The coculture system applies strain to fibroblasts only but still allows for diffusion of potential differentiation mediators to unstrained myoblasts on coverslips. To determine the role of IL-6, we utilized myoblast unicultures ± IL-6 (0–100 ng/ml) and cocultures ± α-IL-6 (0–200 μg/ml). Untreated uniculture myoblasts served as a negative control. After 96 h, coverslips ($n = 6$–21) were microscopically analyzed and quantified by blinded observer for differentiation endpoints: myotubes per square millimeter (>3 nuclei/cell), nuclei/myotube, and fusion efficiency (%nuclei within myotubes). The presence of fibroblasts and fibroblast conditioned media significantly enhanced myotube number ($P < 0.05$). However, in coculture, CSDS applied to fibroblasts did not reproduce this effect. ALDS following CSDS increased myotube number by 78% and fusion efficiency by 96% vs. CSDS alone ($P < 0.05$). Fibroblasts in coculture increase IL-6 secretion; however, IL-6 secretion did not correlate with enhanced differentiation among strain groups. Exogenous IL-6 in myoblast uniculture failed to induce differentiation. However, α-IL-6 attenuated differentiation in all coculture groups ($P < 0.05$). Fibroblasts secrete soluble mediators that have profound effects on several measures of myoblast differentiation. Specific biophysical strain patterns modify these outcomes, and suggest that myofascial release after repetitive strain increases myoblast differentiation and thus may improve muscle repair in vivo. Neutralization of IL-6 in coculture significantly reduced differentiation, suggesting fibroblast-IL-6 is necessary but not sufficient in this process.

myotubes; coculture; interleukin-6

REPETITIVE MOTION STRAIN (RMS) in combination with forceful movements increases risk of muscle tears, ligament sprains, joint inflammation, and fascia disruption (5). In 2010 the U.S. Bureau of Labor Statistics reported 35,920 work-related RMS injuries, requiring a median of 24 missed work days per incident (7). RMS injuries expand extracellular matrix deposition (i.e., fibrosis) around skeletal muscle, causing fiber necrosis, muscle wasting, and pain (18). Patients seeking therapy from RMS injury often undergo surgery, even though procedures are invasive, require long recovery time, and result in worsened postoperative pain in 10% of individuals (6). From a biophysical perspective RMS induces cyclic short-duration stretches (CSDS). Manual manipulation offers a less expensive and noninvasive treatment for RMS injury. Manual manipulation applies extracorporeal forces to an affected tissue to restore mobility, decrease inflammation, and improve physical performance (20a, 38, 46). The most widespread manual manipulation modality directed at alleviating muscle fascia restrictions is myofascial release. This technique stretches the long and transverse axes of an affected area for typically ≥60 s for immediate as well as long-term pain relief (2, 17). Myofascial release may also be applied to areas anatomically distant to an injury, suggestive of a potential role for systemic mediators (24). Importantly, and in context to RMS, myofascial release induces an acyclic long-duration stretch (ALDS).

Mechanical forces act upon fascia-embedded fibroblasts to effect cytokine release that in turn mediates inflammation, cell migration, proliferation, and differentiation. Studying the mechanical induction of these cytokines in response to strain variations that modify these cellular processes could lead to clinical interventions that may halt the progression of chronic musculoskeletal disorders and disability (5). We have previously developed in vitro models of CSDS, ALDS, and a combined cyclic short-duration stretch with an acyclic longer-duration stretch (CSDS + ALDS) paradigm using fibroblasts seeded on flexible membranes. In a manner similar to that observed with muscle injury (23), we have shown that CSDS dramatically impacts fibroblast actin morphology and cellular condensation (21), and ALDS applied 3 h post-CSDS reverses this effect (21). We have also shown that our strain paradigms modify fibroblast cytokine output (13, 22). Fibroblasts secrete insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), interleukins (ILs), and nitric oxide (NO) involved in skeletal muscle regeneration (3, 8, 39). Of interest, we have shown IL-6 is mechanically upregulated 24 h after CSDS. IL-6 acts on the AMP-kinase pathway in skeletal muscle to activate glucose uptake and fatty acid oxidation needed for growth and repair (1, 25) and has been shown to stimulate skeletal muscle hypertrophy in vivo (32) and myoblast proliferation and differentiation in vitro (4).

Muscle fascia is anatomically juxtaposed to underlying skeletal muscle functioning in locomotion and as a molecular scaffold for cellular remodeling (28). Muscle fascia fibroblasts may also influence muscle hyperplasia and repair through paracrine cytokine activity (9, 29). The process begins when quiescent satellite cells, which reside at the periphery of myotubes, are activated through molecular signals to express

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myogenic markers of myoblasts (16). Myoblasts then proliferate extensively to self-renew the satellite cell population, or form terminally differentiated myotubes. Myoblasts that undergo differentiation exit the cell cycle (40) and fuse with other myoblasts to form multinucleated myotubes that repopulate the injured site with functional muscle (8a). Myoblasts' ability to optimally fuse and add nuclei to myotubes is crucial to restoring muscle architecture and will ultimately dictate myotube size and strength (27, 44). Signaling molecules direct each stage of muscle repair (15), and fibroblasts, via biomechanical strain-induced activation of mechanoreceptors (37), represent a likely source of these signals.

In this study, we investigated the mechanism by which fibroblast manipulation could be responsible for accelerated recovery from muscle injury. We additionally sought to determine whether ALDS without injury could influence muscle repair. C2C12 skeletal muscle myoblast differentiation was measured in response to fibroblasts strained with CSDS, ALDS, or a combined CSDS + ALDS, in vitro. We used fibroblast-conditioned media and constructed a novel myoblast-fibroblast coculture (Fig. 1*C*) that allowed us to directly measure paracrine effects of fibroblasts on myoblast growth and differentiation. We hypothesized that fibroblasts exposed to CSDS inhibit myoblast differentiation, and treatment with ALDS reverses CSDS-inhibited differentiation. We also hypothesized that ALDS alone increases differentiation vs. nonstrained through the release of IL-6. We sought to identify a role for IL-6 in mediating these effects and its incorporation into a potential molecular and cellular-based mechanism supporting the clinical efficacy of myofascial release.

MATERIALS AND METHODS

Cell Cultures

All studies were carried out using human dermal fibroblasts (NHDF) from Cambrex Laboratories (East Rutherford, NJ) and C2C12 skeletal muscle myoblasts from American Tissue Culture Collections (Manassas, VA). Cells were maintained at 37°C, 5% carbon dioxide, and 100% humidity in DMEM supplemented with 2% or 10% fetal bovine serum (FBS) depending on the experimental conditions (see below). Differentiation media (2% horse serum, American Tissue Culture Collections) was used as a positive control for all experiments, and to verify that our cells were capable of differentiating into myotubes (13a). Cell cultures were fed every day with fresh media and passaged when confluent. All experiments used passage numbers between 3 and 8.

Strain Apparatus and Strain Paradigms

The Flexercell FX-4000 Tension Plus System (Flexcell International, Hillsborough, NC) is a computer-controlled device designed to deliver vacuum-assisted stretch regimens to Bioflex culture plates. Bioflex plates have an elastomeric surface that allows the fibroblasts that are adherent to its surface to deform under vacuum. Strain paradigms are created by programming the strain magnitude, duration, and frequency of the negative pressure to create the desired regimen (45). We have used this apparatus to effectively model aortic pressure waveforms (35), repetitive motion strain (22), counterstrain (13), and myofascial release (21).

The following strain models and dimensions were used.

1) Nonstrain fibroblasts. Nonstrain fibroblasts were grown on Bioflex membranes but were not subjected to CSDS or ALDS.

2) CSDS. Cyclic short-duration stretches (CSDS) modeled 8 h of cyclic strain as would be experienced in a work day comprised of repetitive tasks such as hammering. It was designed to exceed the strain modulus experienced from exercise and impose the phenotype of an overuse injury (23). Fibroblasts were grown on Bioflex membranes and cyclically strained to 10% beyond resting length at loading and unloading rates of 33%/ s, and a frequency of 0.6 Hz, for 8 h.

3) ALDS. Acyclic long-duration stretch (ALDS) was designed based on videomorphometric analyses of clinically applied myofascial release and compilations of elongation and rate of displacement (21). Since myofascial release typically lasts 60–120 s, we selected a duration within this time frame. Fibroblasts were grown on Bioflex membranes and subjected to a single bout of acyclic strain that stretched cells 6% beyond their resting length at a loading rate of 3%/s. Strain was held for 60 s before being released at a rate of 1.5%/s back to resting length.

4) CSDS + ALDS. Cyclic short-duration stretches combined with an acyclic long-duration stretch (CSDS + ALDS) combined our two strain paradigms to model patients suffering from repetitive motion strain injuries seeking treatment (46). Fibroblasts were subjected to a CSDS paradigm for 8 h followed by a 3-h static period. They were then subjected to the ALDS paradigm for 60 s.

Conditioned Media Crossover

Conditioned media from uniculture fibroblast wells (Fig. 1*B*) were collected from all strain groups 24 h poststrain and stored at −80°C. Thereafter we sought to determine if these conditioned media were capable of inducing differentiation in uniculture myoblasts (Fig. 1*A*). Media were replaced with fresh aliquots of fibroblast conditioned media every day for 5 days. Untreated media (2% FBS in DMEM) served as a negative control. Three photomicrographs taken at 10× magnification (2.32 mm^2 per high-powered field) were obtained from myoblast wells after 48, 72, and 96 h in conditioned media and assessed for differentiation (multinucleated cells with ≥3 nuclei). Three experiments each with three independent cell cultures were analyzed ($n = 9$).

Coculture System

A novel coculture system was developed to allow for immediate interaction between soluble signaling molecules released by strained and unstrained fibroblasts and attendant nonstrained myoblasts. Our coculture was modeled after the myofascial junction as it exists in vivo. In two separate microenvironments, fibroblasts were seeded in

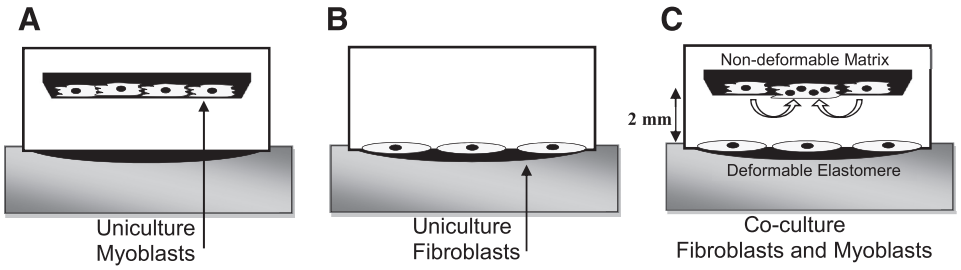

Fig. 1. Uniculture and coculture systems in Bioflex wells. Myoblasts (*A, C*) were seeded on nondeformable coverslips and situated 2 mm above Bioflex membranes. The orientation of the coverslips is such that the cell side faces the fibroblasts. Fibroblasts (*B, C*) were seeded on Bioflex membranes and subjected to mechanical strain paradigms as described.

Bioflex plates (25,000 cells/ml, 2% FBS) and skeletal myoblasts were seeded onto nondeformable glass coverslips (16,000 cells/ml, 10% FBS). After 24 h, the myoblast coverslips were transferred to the Bioflex plates containing fibroblasts. The orientation of the coverslips was such that the cell side faced the fibroblasts. Coverslips were custom made (Fisher Scientific, Houston, TX) to be fastened ~2 mm above Bioflex well membranes, allowing for fibroblasts but not myoblasts to be selectively strained (Fig. 1*C*). Immediately after establishing the coculture, fresh growth media (2% FBS in DMEM) was added and strain paradigms initiated. Media were not changed during the experiment, allowing strain-induced cytokines to remain in coculture and within diffusible range of the proliferating myoblasts. We selected a 96-h time point poststrain for myotube assessment since this duration produced sufficient differentiation without requiring replacement of media (data not shown). Uniculture myoblasts seeded on coverslips and placed into fibroblast-free Bioflex wells (Fig. 1*A*) served as controls. At the end of each experiment, myotube coverslips were gently removed with sterile forceps and assessed for differentiation (see details below). Seven experiments each with three independent cell cultures per treatment were completed ($n = 21$).

IL-6 ELISA

Fibroblast-derived IL-6 is upregulated in response to mechanical strain and has documented roles in skeletal muscle differentiation (32, 33). Therefore culture media from strained and nonstrained groups were collected at three time points: *1*) when our ALDS paradigm was applied 3 h post-CSDS, *2*) when conditioned media were collected at 24 h poststrain, and *3*) when myotube differentiation was assessed at 96 h poststrain. Media were analyzed for human IL-6 using DouSet ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer protocol. The ELISA specifically detected IL-6 from human cells only; hence all IL-6 data from coculture are fibroblast derived ($n = 9$).

Exogenous IL-6 in Uniculture

To test potential effects of IL-6 alone on myoblast differentiation, human recombinant IL-6 (hrIL-6; R&D Systems, Minneapolis, MN) was added to uniculture myoblasts at incremental doses of 0.0, 0.10, 1.0, 10.0, and 100.0 ng/ml. Myoblasts were seeded (at 16,000 cells/ml, 10% FBS) and then allowed to acclimate for 24 h after which time the media were replaced with 2% FBS supplemented with hrIL-6. Uniculture myoblasts remained in hrIL-6-containing media for 96 h after which differentiation was assessed microscopically. Four experiments each with three independent cell cultures were completed ($n = 12$). We also tested these IL-6 concentrations in coculture to determine if there were additive effects ($n = 4$). We verified that exogenous IL-6 was present at the conclusion of these experiments by measuring immunoreactive IL-6 via ELISA.

Neutralization of IL-6 in Coculture

To determine the potential role of IL-6 in fibroblast-mediated myoblast differentiation, an IL-6 antibody (ab6672, Abcam, Cambridge, MA) was used to neutralize IL-6 activity in coculture. Based on a combination of manufacturer-recommended dosing and our own preliminary experiments, 20 μg/ml of IL-6 antibody was used. Media were supplemented with the IL-6 antibody immediately upon establishing the coculture followed by initiation of strain paradigms. Myoblast differentiation parameters (see below) were then analyzed 96 h poststrain. As a negative control, 20 μg/ml of heat-inactivated rabbit serum (Abcam, ab7487) was added to untreated strain groups in coculture. Two experiments each with three independent cell cultures ($n = 6$) were completed.

Imaging Analysis

At the conclusion of each experiment, myoblast coverslips were fixed in Papfix (BBC Biochemical, Mt. Vernon, WA) and stained with hematoxylin and eosin. Coverslips were mounted on prelabeled microscope slides. Labels were then concealed and coded so that cells were imaged by blinded observer. A series of high-powered field (HPF) images at 20× (0.58 mm^2/HPF) were obtained using an Olympus IX-71 inverted microscope. Coded images were then assessed for myotube differentiation using three criteria: the number of myotubes per square millimeter (≥3 nuclei/cell); the number of nuclei within each myotube; and fusion efficiency, a measure that calculates the number of nuclei contained within myotubes as a percentage of the entire population of myoblast and myotube nuclei (Fig. 2). Myotubes with multiple branches were considered to be one large myotube when the cytoplasm was continuous. Myotubes that bordered the edge of an image were only counted if ≥3 nuclei were observed. Quantification of these large data sets was facilitated via CellProfiler (Broad Institute, Cambridge, MA). Utilizing this bioimaging software platform, we designed a unique algorithm to automatically quantify images of hematoxylin-stained nuclei to produce nuclei counts of 50,000–200,000 per experiment.

Statistical Test and Data Acquisition

Replicate experiments, each with three independent cell cultures per treatment, were completed. Between 8 and 16 images were obtained per cell culture to acquire 72–180 images per treatment. Images for each cell culture were averaged and designated as $n = 1$. Image data of all independent wells in a treatment group ($n = 9$–21) were then combined in SPSS v.20 and analyzed for significance via two-way ANOVA with experiment and strain type as explanatory factors. Tukey post hoc tests were used for coculture and ELISA experiments and Dunnett's tests were used to compare IL-6 doses to vehicle, or strained-conditioned media to non-strain-conditioned me-

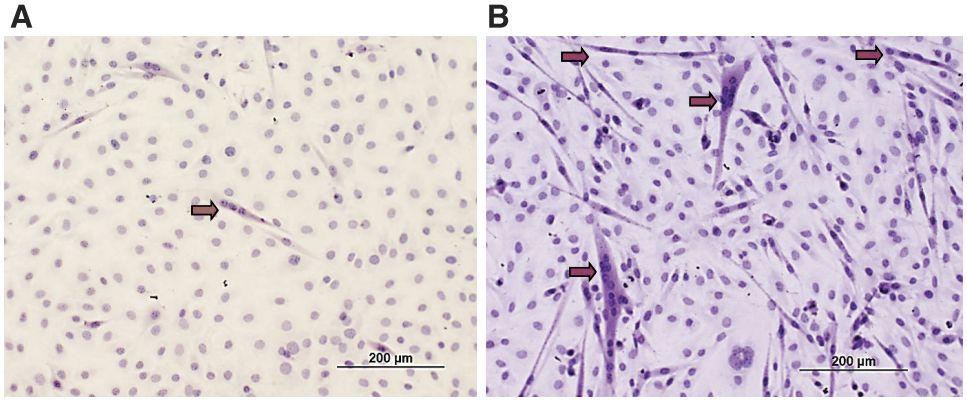

Fig. 2. Images of C2C12 skeletal muscle in uniculture (*A*) and coculture (*B*) 96 h after establishing the coculture. Quantification of myotubes per square millimeter (1.7, 6.9), nuclei per myotube (3.0, 7.2), and fusion efficiency (1.1%, 6.7%) demonstrate fibroblast's ability to induce myoblast differentiation. Examples of multinucleated myotubes are shown by arrows.

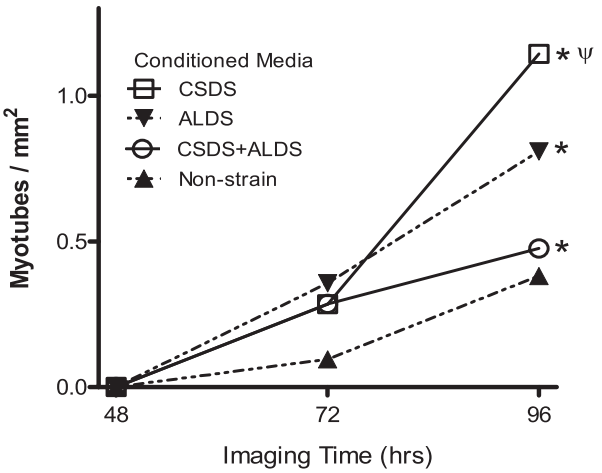

Fig. 3. Myotube densities in fibroblast-conditioned media after 48, 72, and 96 h. Three experiments with 3 cell cultures per strain group ($n = 9$) were combined. CSDS, cyclic short-duration stretches; ALDS, acyclic long-duration stretch; CSDS + ALDS, CSDS combined with ALDS. Symbols show: * > 48 h, ψ > nonstrain ($P < 0.05$).

dia. A repeated-measures ANOVA was used to analyze time-dependent myoblast differentiation for each conditioned media sample, and Student's *t*-tests were used for single comparisons between α-IL-6 treatments and their respective untreated group. Means were considered statistically significant if $P < 0.05$.

RESULTS

Fibroblast Media Crossover

We have previously shown fibroblasts secrete a diverse population of cytokines, and that CSDS upregulates IL-1α, IL-1β, IL-2, IL-6, IL-1rα, IL-16, and NO. We have also reported that ALDS modifies fibroblast cytokine secretion induced by CSDS (22). We used these same conditioned media to test for their abilities to induce myoblast differentiation. Conditioned media from all strain groups enhanced differentiation by 96 h ($P < 0.05$) (Fig. 3). However, the degree to which differentiation occurred varied among strain groups. CSDS-conditioned media produced a greater number of myotubes than non-strain-conditioned media after 96 h. However, when combine with ALDS, the conditioned media did not have an effect. These results demonstrate that fibroblast mediators in the conditioned media induce myoblast differentiation in a strain-dependent manner.

Coculture

In coculture, the presence of fibroblasts in coculture enhanced myotube numbers by 190% vs. uniculture ($P < 0.05$) (Fig. 4*A*). Resultant cocultured myotubes also displayed a greater number of nuclei than those derived from uniculture ($P < 0.05$) (Fig. 4*B*). We again observed that strained fibroblasts modify myoblast differentiation outcomes. Fibroblasts subjected to CSDS did not show a significant increase in myotube parameters vs. uniculture, suggesting an inhibitory role for CSDS in myoblast differentiation (Fig. 4*C*). ALDS alone had no effect on any parameter of myoblast differentiation vs. nonstrain coculture. However, when following CSDS, ALDS significantly increased myotube number by 78% and fusion efficiency by 96% vs. CSDS alone, suggesting a beneficial role for the combined CSDS + ALDS paradigm in myoblast differentiation. We seeded myoblasts on nondeformable coverslips above fibroblast-free Bioflex wells (Fig. 1*A*) and subjected them to strain paradigms to confirm that elastomeric strain and potential-associated fluid currents did not influence myoblast differentiation. No differentiation was observed (data not shown). Neither myoblast nor fibroblast proliferation rates were significantly affected by strain or coculture (data not shown).

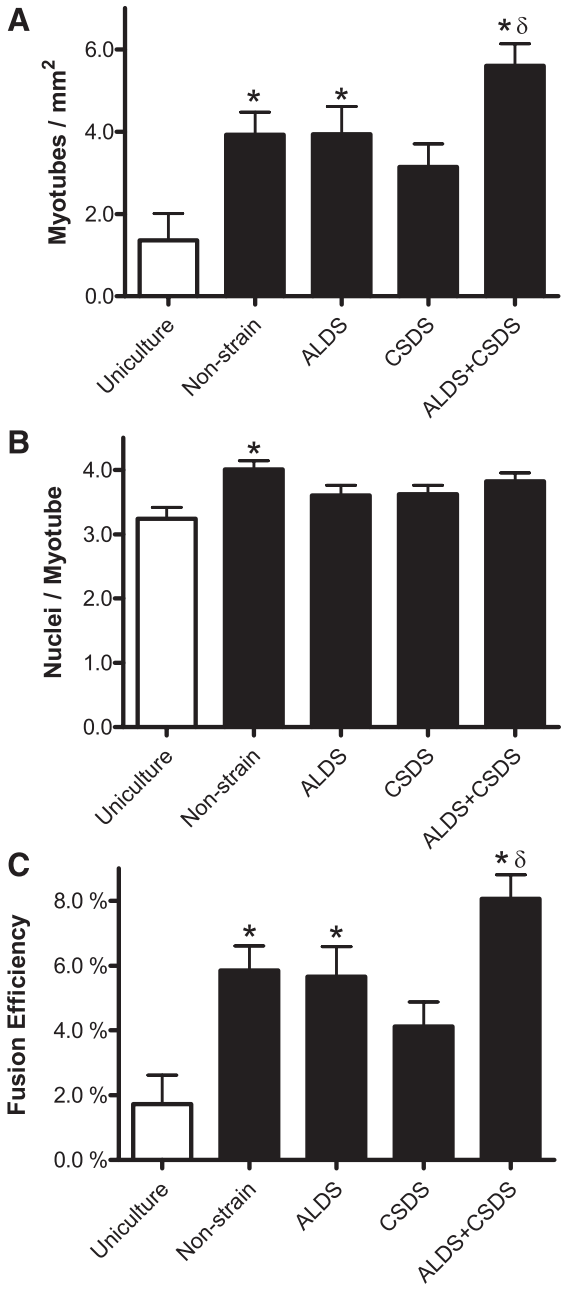

Fig. 4. Differentiation for myotubes per square millimeter (*A*), nuclei per myotube (*B*), and fusion efficiency (*C*) were combined from 7 experiments with 3 cell cultures per strain group ($n = 21$). The black bars designate coculture and white bars uniculture. Symbols show: * > uniculture, δ > CSDS ($P < 0.05$).

IL-6 ELISA

IL-6 secretion from fibroblasts was not significantly altered among uniculture or coculture groups at 24 h poststrain (Fig. 5*A*). However, after 96 h, coculture fibroblasts from the nonstrain and ALDS groups displayed a 10-fold increase in IL-6 secretion vs. uniculture fibroblasts ($P < 0.05$) (Fig. 5*B*). We determined that all detectable IL-6 was secreted from the fibroblasts as the C2C12 myoblasts did not secrete human IL-6 as measured by our ELISA (data not shown). Surprisingly, CSDS and CSDS + ALDS coculture groups did not display elevated IL-6 secretion at 96 h. Both groups were decreased compared with the ALDS-only group (Fig. 5*B*, $P < 0.05$). To determine whether IL-6 was upregulated at the time of ALDS treatment, IL-6 was measured 3 h post CSDS. There was no change in IL-6 secretion vs. nonstrain at this time point (data not shown).

Exogenous IL-6 Addition

Addition of hrIL-6to uniculture myoblasts tested whether IL-6 alone could reproduce fibroblast-enhanced myoblast differentiation. Doses of exogenous hrIL-6 tested failed to induce parameters of myoblast differentiation (Fig. 6). At the time of analysis, myoblasts had reached confluence for all groups and myoblast proliferation was equal among all hrIL-6 doses and vehicle (Fig. 6*D*). Preliminary experiments were also conducted in coculture to test for additive effects of hrIL-6 in the presence of fibroblasts, yet again, no effects on myoblast differentiation were observed (data not shown). To determine whether exogenous IL-6 was still present in media at the time of myotube analysis, we measured IL-6 via ELISA in all groups. Approximately 10% of the original exogenous IL-6 concentrations (0.10–100.0 ng/ml) was still detectable in all treatment groups (data not shown).

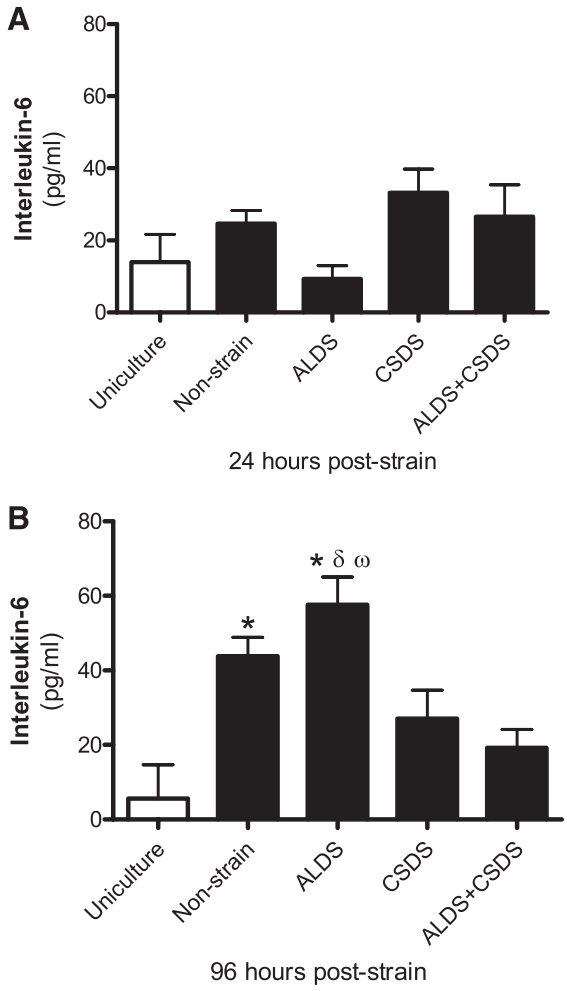

Fig. 5. Human IL-6 secretion from fibroblasts at 24 h (*A*) and 96 h (*B*) poststrain. The black bars designate coculture and white bars uniculture fibroblasts. Immunoreactive IL-6 was measured from 3 experiments with 3 cell cultures each ($n = 9$). Symbols show: * > uniculture, δ > CSDS, ω > ALDS + CSDS ($P < 0.05$).

Neutralizing IL-6 Antibody

A neutralizing IL-6 antibody was added to coculture to determine whether IL-6 was necessary for fibroblast-mediated myotube differentiation. All cocultured groups treated with 20 μg/ml IL-6 antibody and subjected to strain-decreased myoblast differentiation by 47–65% vs. untreated coculture strain groups ($P < 0.05$) (Fig. 7*A*). Fusion efficiency was also significantly reduced in all α-IL-6-treated strain groups by ~50–65%, but not in the nonstrain group (Fig. 7*C*), suggesting that mechanical strain may be affecting myotube sensitivity to IL-6. To ascertain whether IL-6 neutralization could also inhibit myoblast differentiation without fibroblasts present, we tested these same IL-6 antibody doses in myoblast unicultures in both growth media and differentiation media (data not shown). The IL-6 antibody had no effect on myoblast differentiation, suggesting that fibroblasts and their cosecretions are necessary for IL-6 action.

DISCUSSION

RMS is associated with a number of degenerative musculoskeletal disorders, including carpal tunnel syndrome, lower back pain, tension neck syndrome, rotator cuff syndrome, sciatica, epicondylitis, tendinitis, and carpet layer's knee. These disorders frequently result in muscle fibrosis and atrophy (5). Manual manipulation is commonly used to treat these disorders (31, 38, 43); however, cell-based studies evaluating the efficacy of treatment are still warranted. We therefore investigated the effects of applying a modeled repetitive motion strain, by inducing CSDS, and a modeled myofascial release, by inducing an ALDS, to fibroblasts on mediating skeletal muscle differentiation.

Our results support the idea that fibroblasts regulate skeletal muscle differentiation through paracrine actions of secreted mediators. We experimentally determined that both fibroblast conditioned media and fibroblasts in coculture were capable of stimulating myoblast differentiation. These data are supported by other researchers that concluded myotubes grown on a fibroblast feeder layer survived longer and displayed enhanced contractile activity. The authors attributed these results in part to unidentified secreted growth factors that assisted in the myotube development (9). Another study concluded that fibroblasts from bovine muscle secreted a soluble myoblast growth factor in conditioned media that increased myoblast proliferation and fusion to myotubes (29). These researchers sought to identify a potential growth factor mediating this process, and determined it was neither bFGF nor IGF-1. They further noted myotrophic activity only occurred when fibroblasts were pro-

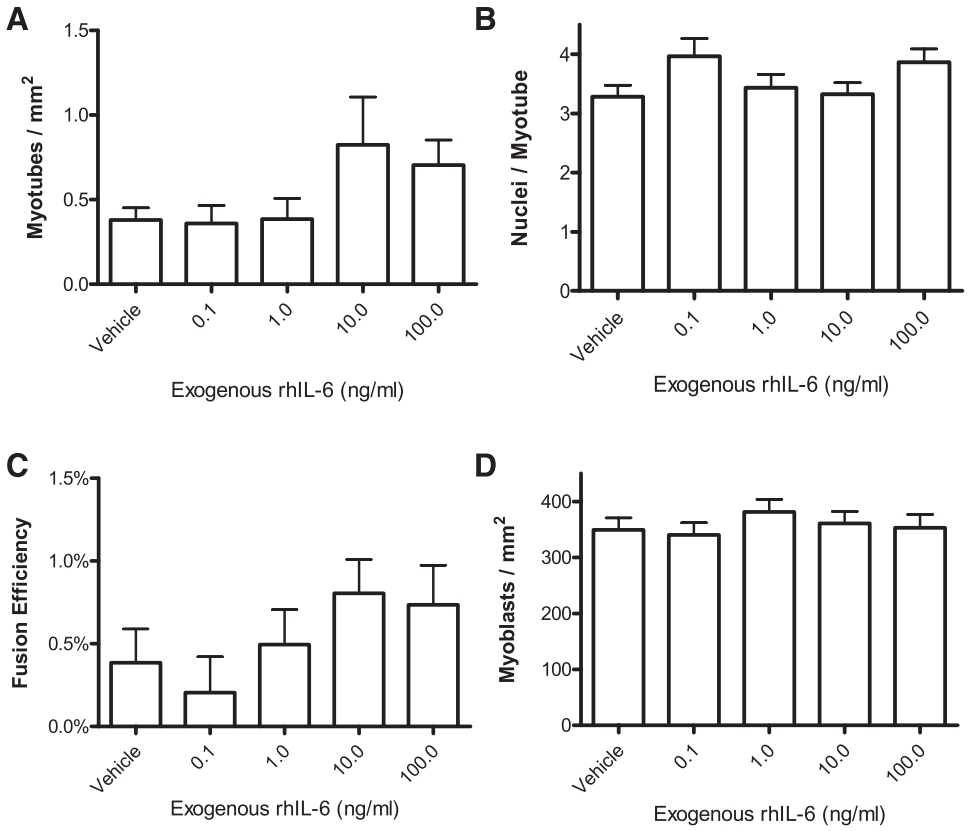

Fig. 6. Differentiation measures in the presence and absence of exogenous IL-6 doses (0–100 ng/ml) after 96 h in uniculture. The vehicle was untreated media (2% FBS in DMEM). Data for myotubes per square millimeter (*A*), nuclei per myotube (*B*), fusion efficiency (*C*), and myoblasts per square millimeter (*D*) were combined from 4 experiments with 3 cell cultures per IL-6 dose (n = 12). hrIL-6, human recombinant IL-6.

liferating. It was recently demonstrated that fibrogenic/adipogenic progenitor cells (FAPs) obtained from murine hindlimbs represent an inducible-source of IL-6. When induced to proliferate, FAPs not only secreted IL-6, but also facilitated myoblast fusion efficiency (19).

IL-6 is a known stimulator of muscle growth and differentiation (25, 32), and is secreted by fibroblasts in a strain-dependent manner (14, 33). We therefore sought to identify whether increased myoblast differentiation caused by the presence of fibroblasts in coculture was due to fibroblast-derived IL-6. We showed that human fibroblast IL-6 secretion increases by 10-fold when in coculture (Fig. 5*B*). This suggests that the C2C12 myoblasts likely influence the secretory behavior of fibroblasts. Similarly, other research has shown that fibroblast IL-6 secretion increases 100-fold in the presence of T-cells (34), leading to the speculation that cellular cooperation between fibroblasts and other cell types could be a powerful mediator of the inflammatory response and wound healing. These IL-6 data correlate well with our finding that fibroblasts induced myoblast differentiation when in coculture. For the first time, we also showed that fibroblasts directly regulate skeletal muscle differentiation through soluble IL-6. When IL-6 was neutralized in coculture myoblast differentiation was inhibited (Fig. 7). However, exogenous IL-6 alone could not induce differentiation (Fig. 6), indicating that IL-6 is necessary but not sufficient for myoblast differentiation. We speculate that fibroblasts and their cosecretions are required in addition to IL-6 for myoblast differentiation. While mechanical strain regulates many fibroblast-derived cytokines, it could not rescue the inhibitory effects of IL-6 neutralization (Fig. 7). At the time of myotube analysis we did not measure a change in myoblast number in response to exogenous IL-6 doses, although others have shown that C2C12s overexpressing IL-6 increase proliferation (32). It is likely that the time of analysis or the constitutive overexpression of IL-6 in transfected cells vs. a single additive dose used in our model account for this disparity.

We demonstrated that myoblast differentiation can be differentially regulated by conditioned media of mechanically strained fibroblasts (Fig. 3) and by strained fibroblasts in coculture (Fig. 4). Interestingly, these two experimental approaches uniquely regulated differentiation outcomes. For instance, conditioned media from fibroblasts subjected to CSDS produced the greatest number of myotubes, but in coculture CSDS treatment resulted in the least myotube differentiation among groups. Previously, we have shown CSDS conditioned media is a rich source of cytokines 24 h poststrain (22), and that myotube differentiation is also intensified from this CSDS-conditioned media may suggest differentiation-favorable cytokines are temporally upregulated by fibroblasts and strain. The coculture system we designed ensured that myoblasts were exposed to fibroblast cytokines at all stages pre- and poststrain. CSDS inhibited myotube differentiation in coculture, which may suggest that at a later time point the secretion of an inhibitory molecule occurs in the RMS paradigm. Contrasting with these data, CSDS + ALDS treatment in coculture significantly enhanced myotube differentiation and reversed the deleterious effects brought on by CSDS alone. Notably, we reported that ALDS downregulates several CSDS-induced cytokines 24 h poststrain (22). Together these data suggest that CSDS-induced cytokines which regulate myoblast differentiation are also regulated by the subsequent ALDS treatment.

The increased number of myotubes resulting from the CSDS + ALDS paradigm shows that a single sustained slow-loading strain

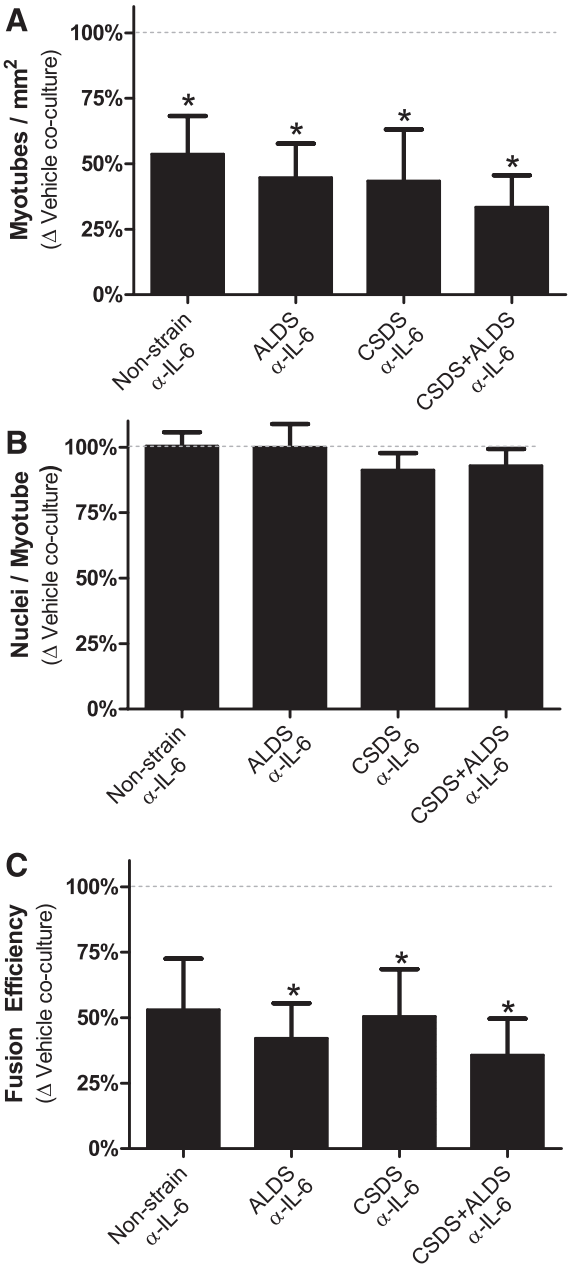

Fig. 7. Differentiation measures in coculture supplemented with 20 μg/ml IL-6 neutralizing antibody 96 h poststrain. Data for myotubes per square millimeter (*A*), nuclei per myotube (*B*), and fusion efficiency (*C*) were combined from 2 experiments with 3 cell cultures per treatment ($n = 6$). Results were normalized to respective untreated co-culture strain group and shown as a percent change from untreated (%CFU). Symbols show: * > untreated coculture ($P < 0.05$).

has profound impact on myoblast differentiation. If clinically translatable, restorative strain regimens such as myofascial release applied to a fibroblast-rich tissue following injury may facilitate muscle repair. While ours is the first study to measure the in vitro effects of mechanical strain and fibroblasts on myoblast differentiation, others have shown beneficial effects of slow-loading strains on skeletal muscle. For instance, chronic static stretching after exercise has been shown to increase knee flexion and extension repetition maximums (20). The authors proposed that increased muscle strength is the most likely mechanism of this effect as evidenced by several studies of stretch-induced muscle hypertrophy (36). Other studies have used external fixator devices to lengthen rabbit limb tibiae 1 mm/day for 10 days. The muscle lengthening consequently induced satellite cells to activate and fuse to generate de novo skeletal muscle (12, 42). The muscle fascia close anatomical interaction with the skeletal muscle, and similar exposure to mechanical loads during strain may help to explain a mechanism for increased muscle during these processes.

Biomechanical strain affects fibroblast mechanoreceptors such as integrin-cytoskeletal connections to activate several signaling pathways— MAPK, SAPK, NF-κB, and EGR—which then influence cytokine secretion (10). Upon mechanical stimulation, the p38 pathway is activated which subsequently upregulates IL-6 secretion (26). Surprisingly, our data did not show increased IL-6 in any group 24 h poststrain (Fig. 5*A*) and revealed CSDS and CSDS + ALDS attenuated fibroblast IL-6 secretion 96 h poststrain (Fig. 5*B*). We speculate that other cytokines are likely involved in the skeletal muscle differentiation process and coregulate IL-6 secretion, and we have designed experiments to test this. Strain-regulated cytokines may also affect the sensitivity of skeletal muscle to IL-6 by regulating IL-6 receptor expression, downstream intracellular proteins, or other means; however, this study did not investigate those pathways.

While repetitive motion strain and clinical myofascial release direct mechanical forces to fascial fibroblasts, indirect strains applied to nerves, blood vessels, the lymphatic system, and preexisting muscles (41) are not considered in our model. Preexisting muscles also greatly contribute to the muscle repair process by undergoing hypertrophy or fusing with adjacent myotubes; however, we were uniquely interested in the effects of stretched fibroblasts on myoblast differentiation and therefore did not make these measurements in the present study. We therefore do not want to overreach on interpretations of these data, and support the future use of these strain paradigms and analysis parameters in animal and human models to validate our findings. Our simple system allows soluble signaling molecules to interact in an in vitro environment modeled after a simple myofascial junction to investigate direct interactions between two cell types. Despite the limitations of in vitro modeling, data from our laboratory suggest that cultured human fibroblasts adapt specifically to mechanical loading in manners dependent upon strain magnitude, duration, and frequency. The unique response of fibroblasts to mechanical strain and consequent effects on myoblast differentiation should be further investigated to optimize strain protocols to best facilitate skeletal muscle repair. Our cellular strain models may prove useful in further investigation of mechanisms germane to muscle injury, as well as help to build a cellular evidence base describing the positive outcomes of applied manual treatments in the clinical setting.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.R.H., T.V.C., and P.R.S. conception and design of research; M.R.H., T.V.C., and D.H.C. performed experiments; M.R.H. and D.H.C. analyzed data; M.R.H., T.V.C., and P.R.S. interpreted results of experiments; M.R.H. prepared figures; M.R.H. drafted manuscript; M.R.H., T.V.C., D.H.C., and P.R.S. edited and revised manuscript; M.R.H., T.V.C., D.H.C., and P.R.S. approved final version of manuscript. M.R.H. performed the lead research for this study and drafted the original manuscript. T.V.C. helped with experiments, contributed intellectually to the design of the experiments, and assisted in manuscript revisions. D.H.C. developed a computer algorithm to quantify myotube parameters, performed much of the imaging, and completed several control experiments. P.R.S. conceptualized the basis for this study, provided oversight for the completion of this project, and finalized the manuscript revisions.

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