ADIPOSE STEM CELLS ENHANCE MYOBLAST PROLIFERATION VIA ACETYLCHOLINE AND EXTRACELLULAR SIGNAL–REGULATED KINASE 1/2 SIGNALING

ROINE EL-HABTA, MSc, PAUL J. KINGHAM, PhD, and LUDVIG J. BACKMAN, PhD

Department of Integrative Medical Biology, Section for Anatomy, Umeå University, SE-901 87, Umeå, Sweden

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ABSTRACT: *Introduction:* In this study we investigated the interaction between adipose tissue–derived stem cells (ASCs) and myoblasts in co-culture experiments. *Methods:* Specific inductive media were used to differentiate ASCs *in vitro* into a Schwann cell–like phenotype (differentiated adipose tissue–derived stem cells, or dASCs) and, subsequently, the expression of acetylcholine (ACh)-related machinery was determined. In addition, the expression of muscarinic ACh receptors was examined in denervated rat gastrocnemius muscles. *Results:* In contrast to undifferentiated ASCs, dASCs expressed more choline acetyltransferase and vesicular acetylcholine transporter. When co-cultured with myoblasts, dASCs enhanced the proliferation rate, as did ACh administration alone. Western blotting and pharmacological inhibitor studies showed that phosphorylated extracellular signal–regulated kinase 1/2 signaling mediated these effects. In addition, denervated muscle showed higher expression of muscarinic ACh receptors than control muscle. *Discussion:* Our findings suggest that dASCs promote proliferation of myoblasts through paracrine secretion of ACh, which could explain some of their regenerative capacity *in vivo*.

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Long-term muscle denervation as a result of traumatic nerve injury is a worldwide problem, affecting hundreds of thousands of people each year.[1] Even after peripheral nerve repair has been performed, the often long distances required for axon regeneration mean that it is not uncommon for a muscle to remain denervated for years.[2] Muscle has some inherent capacity to regenerate and the formation of new muscle fibers peaks between 2 and 4 months after injury.[3] Nevertheless, return of function is compromised by the incomplete recovery from muscle atrophy, which affects muscle fiber number, muscle cross-sectional area, and muscle force.[4] Methods for delaying muscle atrophy and increasing the regenerative potential of denervated muscles are therefore much needed, but are so far limited in number. Most present-day surgical nerve repair treatments as well as more experimental approaches, such as implantation of microchips and bioengineered tissue conduits,[2,5] fail to directly address the denervated target organ. Given the increasing evidence showing the benefits of stem cells in enhancing axon regeneration,[6,7] we propose that stem cells may also have a role in treating the distal muscles.

Adipose tissue–derived stem cells (ASCs) are abundant multipotent cells that can be isolated from both visceral and subcutaneous adipose tissues.[8,9] Compared with Schwann cells, which are limited in number and hence time-consuming to culture, ASCs are easily accessible and relatively simple to differentiate.[9] Furthermore, a wide range of neurotrophic factors are produced by ASCs.[10] A number of investigators have studied the therapeutic potential of ASCs in nerve reconstruction,[11] but very few have looked at the ability of ASCs to facilitate recovery of denervated muscle. Schaakxs *et al.* studied the effect of ASCs injected into the denervated rat gastrocnemius muscle and found that stem cells differentiated toward a Schwann cell–like phenotype significantly reduced muscle atrophy and enhanced recovery in a walking-track test.[12] The mechanism of repair was not determined in that study, but others have shown that ASCs can spontaneously differentiate into skeletal muscle when co-cultured with primary myoblasts *in vitro*.[13] Furthermore, uncultured adipose tissue–associated cells showed a high regenerative capacity *in vivo* when incorporated into ischemic muscle.[13] Such myogenic conversion and fusion with myoblasts could be explained by the influence of secreted factors, or cell-to-cell contacts.[14,15]

In this study, as a follow-up to the work by Schaakxs *et al.*,[12] we hypothesized that the Schwann cell–like differentiated ASCs (dASCs), through a paracrine mechanism, could induce proliferation of myoblasts. We also hypothesized that the effect could be mediated by acetylcholine (ACh), as ACh is an

Additional supporting information may be found in the online version of this article.

Abbreviations: ACh, acetylcholine; ASC, adipose-derived stem cell; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; ChAT, choline acetyltransferase; dASC, differentiated adipose-derived stem cell; DMEM, Dulbecco's modified Eagle medium; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; MEM, minimal essential medium; MTS, [3-(4,5-dimethythiozol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; p-ERK1/2, phosphorylated extracellular signal–regulated kinase 1/2; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcript polymerase chain reaction; TBST, Tween-20 with Tris-buffered saline; uASC, undifferentiated adipose-derived stem cell

Key words: adipose-derived stem cells; denervation; myoblasts; non-neuronal acetylcholine; proliferation

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Correspondence to: L.J. Backman; e-mail: ludvig.backman@umu.se

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important neuromuscular modulator that can induce proliferation in skeletal muscle.[16] There is also evidence suggesting that ACh could work as a "molecular break" by controlling the balance between pro- and anti-apoptosis.[17,18] With this in mind, we investigated the expression of ACh-related genes in ASCs, and determined the expression of muscarinic acetylcholine receptors in denervated muscle.

METHODS

Animals. Ten- to 12-week-old female Sprague-Dawley rats (Taconic Europe A/S) were used for cell culture and *in-vivo* experimentation. The animal care and experimental procedures were carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes, and was also approved by the Northern Swedish Committee for Ethics in Animal Experiments (No. A186-12). Sciatic nerve transection was performed as described elsewhere.[10] Muscle biopsies (gastrocnemius muscle) were collected from rats 2 weeks after unilateral sciatic nerve transection. The contralateral muscle was used as a control. For each group, 6 animals were studied in total.

Cells. *L6 Myoblasts.* Myoblasts were cultured in T-175 flasks (Sarstedt, code: 83.3912.002) in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, code: 31966021) containing L-alanyl-L-glutamine (GlutaMAX), 10% fetal bovine serum (FBS; Thermo Fisher Scientific, code: 10500064), and 1% penicillin–streptomycin (Thermo Fisher Scientific, code: 15140122). Cultures were maintained at the subconfluent level to prevent the loss of myoblastic component as the cells were passed.

Primary Myoblasts. Muscles from human donors (Regional Ethics Committee Approval No. 2016-250-32M) were cut into small blocks (approximately 1 mm^3) in a culture dish containing DMEM with 10% FBS and 1% penicillin–streptomycin. The culture dish was incubated in a 37°C, 5% CO_2 incubator, with media exchange every 2 days for approximately 2 weeks. When the dish reached about 80% confluence the tissue blocks were discarded and the cells were moved to a collagen-coated dish (Thermo Fisher Scientific, code: A11428-01) for 15 min. This step was repeated 2 more times to leave behind the rapidly adhering cells, predominantly fibroblasts. The resulting media containing primary myoblasts were then transferred to a new flask for further culturing.

Adipose-Derived Stem Cells. Using a sterile razor blade, visceral and subcutaneous fat from adult rats was minced in 0.15% collagenase type I (Invitrogen) in Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, code: 14170088). The homogenate was placed in a 37°C water bath for 60 min or until most of the fat had been digested. The solution was then neutralized using minimal essential medium (MEM; Thermo Fisher Scientific, code: 32561029) supplemented with 10% FBS, and centrifuged at 650 × *g* for 5 min. The stromal pellet was resuspended in MEM with 10% FBS; the solution was filtered through a 70-μm filter, centrifuged at 200 × *g* for 5 min, and then resuspended in complete growth medium containing MEM with 10% FBS and 1% penicillin–streptomycin. ASC cultures were maintained at the subconfluent level in T-75 flasks at 37°C and 5% CO_2. Over the next few days, the cells were washed thoroughly with HBSS to remove all non-adherent cells. At passage 2, the cells were differentiated into a Schwann cell–like phenotype in 2 steps: (1) by replacing the growth medium with medium supplemented with 1 mmol/L β-mercaptoethanol (Scharlau Chemicals, code: ME00950250) for 24 h; (2) by treating the cells with 35 ng/ml all-*trans*-retinoic acid for 72 h. The dASCs were then expanded in differentiating medium consisting of growth medium supplemented with 5 ng/ml platelet-derived growth factor-AA (Pepro Tech, code: 100-13A), 10 ng/ml basic fibroblast growth factor (FGF; Pepro Tech, code: 100-18B), 14 μmol/L forskolin (FSK; R&D Systems, code: F6886-10MG), and 252 ng/ml neuregulin-1 (R&D Systems, code: 396-HB-050). Figure S1 (refer to Supplementary Material, available online) shows the induction of the Schwann cell phenotype, as previously reported.[19] The dASC cultures were stained with antibodies specific for the glial cell markers S100B (DAKO, code: Z0311; 1/2,000), glial fibrillary acidic protein (DAKO, code: Z0334; 1/500), and p75 neurotropin receptor (Alomone Labs, code: AN-170; 1/500).

Indirect Co-Culture of Myoblasts and ASCs. Myoblasts (L6 rat myoblast cell line *or* human primary myoblasts) were seeded at a density of 10,000 cells per well in a 24-well plate (Corning, code: 353504) and ASCs were seeded at a density of 10,000 cells per culture insert (pore size 0.1 μm; Corning, code: 353104). All cells were seeded in their respective growth medium as follows: DMEM GlutaMAX with 10% FBS for myoblasts; MEM GlutaMAX with 10% FBS for undifferentiated adipose-derived stem cells (uASCs); and differentiating medium for dASCs. Seeding was done 24 h before starting the experiment, to ensure proper attachment. Before the co-culture started the medium was removed and replaced with medium containing 1% FBS. The cells were co-cultured for 72 h at 37°C and the metabolic activity and proliferation rate were measured using MTS [3-(4,5-dimethythiozol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and bromodeoxyuridine (BrdU), respectively. When used, atropine (10^{-5} mol/L; Sigma-Aldrich, code: A0132), or Mitogen-activated protein kinase (MEK) inhibitor (25 mmol/L; Calbiochem, code: 513001), was added to myoblasts 30 min before starting the experiment.

Proliferation Assays. *CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay.* A CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay was performed according to instructions provided by the manufacturer (Promega, code: G3581). After the cell culture inserts (containing 10,000 cells/insert) were removed, MTS tetrazolium was added to the culture wells (20 μl of reagent in 400 μl medium) and incubated for 1 h at 37°C. The product, MTS formazan, which is proportional to the number of living cells, was recorded in a 96-well plate reader at 490 nm.

Cell Proliferation Enzyme-Linked Immunoassay. BrdU assay was performed according to instructions of the manufacturer (Roche, code: 11647229001). BrdU labeling solution was added to the culture wells (10,000 cells/well in a 24-well plate) to a final concentration of 10 μmol/L and incubated for 2 h at 37°C. The cells were fixed for 30 min at room temperature and treated with anti–BrdU-peroxidase (POD) working solution for 60 min. The antibody conjugate was then removed and the cells were washed 3 times in phosphate-buffered saline (PBS). For color development, substrate solution was added for 1–20 min. The absorbance was measured at 370 nm (reference wavelength 492 nm). A BrdU enzyme-linked immunoassay was used in co-culture experiments with and without inhibitors (atropine and MEK inhibitor) as well as myoblasts exposed to 10^{-6} mol/L or 10^{-8} mol/L ACh for a series of time-points up to 24 h.

Cassette-Based Flow Cytometry. A cassette-based flow cytometer (Orflo Technologies, code: MXZ001) was used in counting the final number of L6 myoblasts after treatment with 10^{-6} mol/L or 10^{-8} mol/L ACh for 48 h. The experimental setup was the same as described for BrdU (10,000 cells/well in a 24-well plate at the start of the experiment). The cells were also counted in a Neubauer chamber. The concentrations used for ACh, 10^{-6} mol/L and 10^{-8} mol/L, were based on previous studies.[20]

Real-Time Quantitative Reverse Transcript Polymerase Chain Reaction. Muscle biopsies were homogenized in lysis reagent (QIAzol; Qiagen, code: 79306) using a hand-held tissue ruptor. The homogenate was placed at the benchtop for 5 min to promote dissociation of nucleoprotein complexes. Chloroform (1:5) was then added to the tube and shaken vigorously for approximately 15 s. The homogenate was centrifuged at 18,600 × *g* at 4°C for 15 min, after which the upper aqueous phase was transferred to a new tube. After the addition of 1.5 vol of 100% ethanol, total RNA was purified using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, code: 74106). Reverse transcription was performed using reverse transcription reagents (TaqMan; Applied Biosystems, code: 4368813) from 1 μg RNA. Real-time quantitative reverse transcript polymerase chain reaction (qRT-PCR) was performed with a gene expression assay (TaqMan; Applied Biosystems) and real-time PCR system (ViiA 7; Applied Biosystems). Thermal-cycling conditions were 50°C for 2 min, 95°C for 20 s, and 40 cycles of 95°C for 1 s and 60°C for 20 s. Data were analyzed with ViiA 7 software (Applied Biosystems). Expression of ribosomal 18s RNA and glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase were used as internal standards or "housekeeping genes." All genes are listed in Table S1 (refer to Supplementary Material online).

Western Blotting. Myoblasts and ASCs were co-cultured in a 6-well plate (Corning, code: 353502) using culture inserts (pore size 0.1 μm, Corning, code: 353102) for 3 days, starting with 50,000 cells each. Myoblasts were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor (Sigma-Aldrich), and total protein was quantified using a protein assay (Bio-Rad Laboratories, code: 500-0006). Samples were run on pre-cast polyacrylamide gels (Bio-Rad Laboratories) for 60 min at 150 V. The gels were transferred to polyvinylidene fluoride membranes and run for 60 min at 100 V. Membranes were blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich, code: A2058) in Tween-20 with Tris-buffered saline (TBST; 10 mmol/L Tris base, 100 nmol/L NaCl, 0.1% Tween-20) for 60 min at room temperature, and stained with phosphorylated extracellular signal–regulated kinase (p-ERK1/2; 1/2,000; Cell Signaling, code: 4370) or β-actin (Cell Signaling, code: 4970) overnight at 4°C. Membranes were washed with TBST for 3 × 15 min, after which horseradish peroxidase–conjugated secondary antibodies (1/2,000; Cell Signaling, code: 7074) were added for 60 min at room temperature. Finally, membranes were incubated in enhanced chemiluminescence solution (GE Healthcare, code: RPN2232) for 60 s and analyzed in a dual-mode imaging system (Odyssey Fc; Li-COR Biosciences).

Immunocytochemistry. The uASCs and dASCs were seeded in 8-well chamber slides at a density of 15,000 cells/well and cultured for 24 h. Cells were fixed in 2% paraformaldehyde, washed 3 times in PBS, and blocked in normal serum (1/20) for 15 min. Cells were then incubated with primary antibody (choline acetyltransferase; 1/100; Millipore, code: AB144P) for 60 min at 37°C. After further washing and blocking, secondary antibodies were added for 30 min at 37°C. Before mounting the slides they were washed a final time in PBS. Mounting medium containing 4′6-diamidino-2-phenylindole-dihydrochloride (DAPI; Vector Laboratories, code: H-1200) was used. Slides were analyzed using a Axioskop 2 Plus microscope (Zeiss) equipped with a camera (Model DP70, Olympus). The investigators who assessed the results were blinded to the intervention.

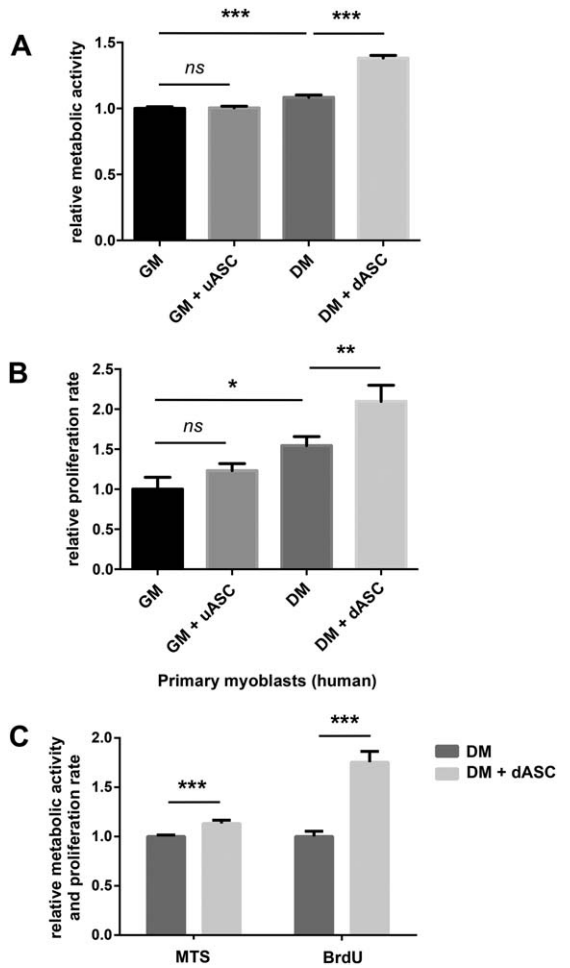

FIGURE 1. Differentiated ASCs increase the proliferation rate in L6 myoblasts and human primary myoblasts. L6 myoblasts were co-cultured with ASCs for 72 h (1:1 ratio). **(A)** MTS cell proliferation assay. Myoblasts co-cultured with undifferentiated adipose-derived stem cells (uASCs) were compared with myoblasts cultured in normal growth medium (GM). Myoblasts co-cultured with differentiated adipose-derived stem cells (dASCs) were compared with myoblasts cultured in differentiating medium (DM), that is, growth medium supplemented with growth factors. **(B)** BrdU cell proliferation assay. Experimental setup as in **(A)**. Results show that dASCs significantly increased the proliferation rate in myoblasts. **(C)** The experiments were repeated using primary myoblasts isolated from human muscles. Results were similar to those seen in L6 myoblasts. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. $P < 0.05$ considered statistically significant. *ns*, not significant.

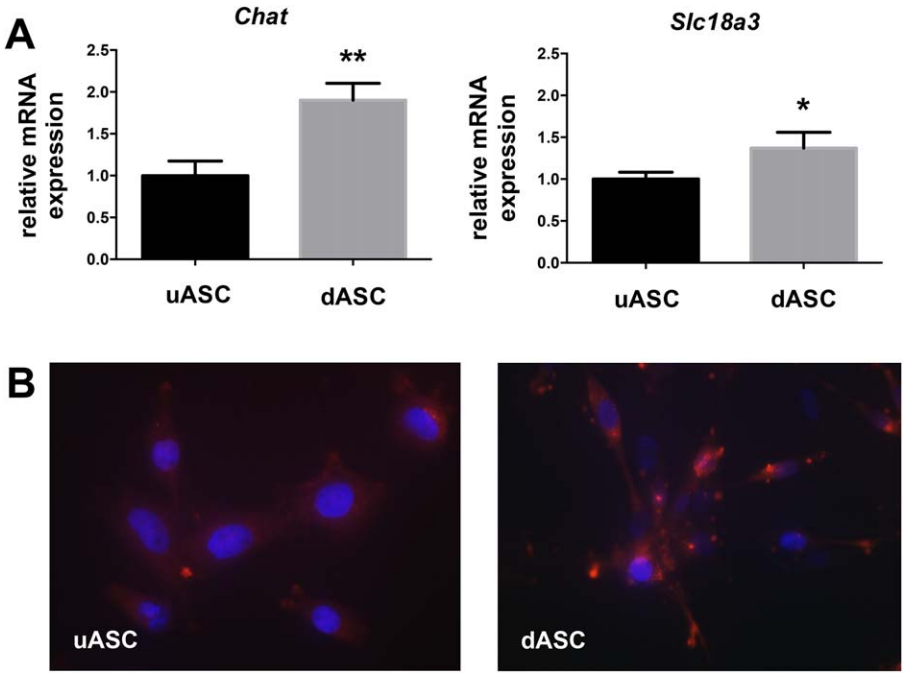

FIGURE 2. ASCs express the machinery for production and secretion of ACh. **(A)** Real-time qRT-PCR comparison of the expression of *Chat* and *Slc18a3*. Compared with uASCs, the dASCs had a significantly higher expression of *Chat* and *Slc18a3*. Slc18a3, vesicular acetylcholine transporter. **(B)** Immunocytochemistry of uASCs and dASCs using antibody against choline acetyltransferase (ChAT; red). DAPI was used to stain the nuclei (blue). $^{*}P < 0.05$ and $^{**}P < 0.01$. $P < 0.05$ considered statistically significant.

Statistical Analysis. All experiments were performed with a minimum of 3 biological repeats using ≥3 technical replicates per sample group. Analyses of *ex-vivo* tissue were from 6 rats. Results are presented as mean ± standard deviation. Statistical analysis was carried out using 1-way analysis of variance with *post-hoc* test (Bonferroni correction) for multiple comparisons, and the Student *t*-test for unpaired samples. The intensity of bands from Western blots was quantified using ImageJ sofware (NIH). $P < 0.05$ was considered statistically significant.

RESULTS

Schwann Cell–like Differentiated Adipose-Derived Stem Cells Increase Proliferation of L6 Myoblasts. L6 myoblasts were co-cultured with dASCs for 72 h. The dASCs significantly increased the metabolic activity (Fig. 1A) and proliferation rate (Fig. 1B) of the myoblasts, whereas the uASCs had no effect. Similar results were obtained when the experiments were repeated using primary myoblasts derived from human tissue (Fig. 1C).

Differentiated ASCs Express Molecules Associated with ACh Production. The gene expression of *Chat* and *Slc18a3*, the vesicular ACh transporter, was analyzed in ASCs (Fig. 2A). Cells were immunostained with an antibody targeting the enzyme choline acetyltransferase (ChAT), which is responsible for the synthesis of ACh (Fig. 2B). These cytological stainings revealed increased amounts of ChAT in dASCs when compared with uASCs.

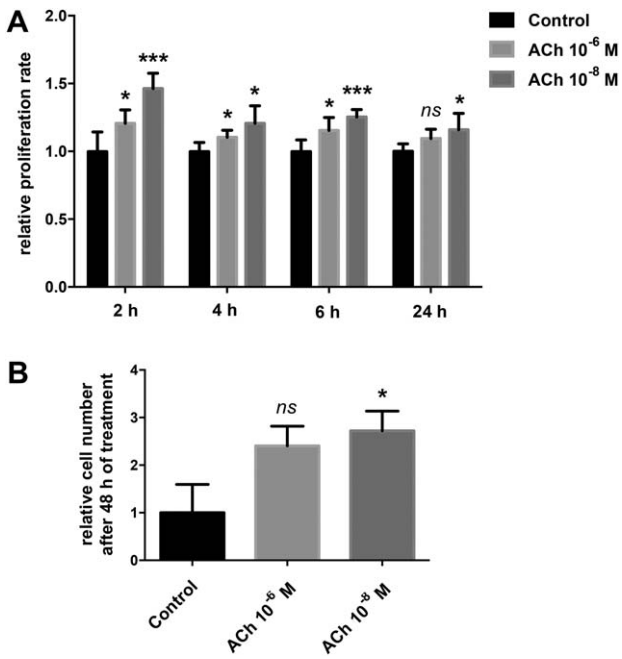

FIGURE 3. ACh induces proliferation of L6 myoblasts. L6 myoblasts were treated with 2 different concentrations of ACh for 2, 4, 6, 24, and 48 h. **(A)** Myoblasts labeled with BrdU. Both concentrations of acetylcholine had a significant effect on the proliferation rate; however, at 24 h, a concentration of 10^{-8} mol/L ACh did not have a significant effect when compared with control. Each time-point was analyzed separately (Student *t*-test). **(B)** After 48 h of stimulation the cells were counted. Myoblast cultures treated with 10^{-8} mol/L ACh showed significantly greater numbers of cells compared with control. $^{*}P < 0.05$ and $^{***}P < 0.001$. $P < 0.05$ considered statistically significant. *ns*, not significant.

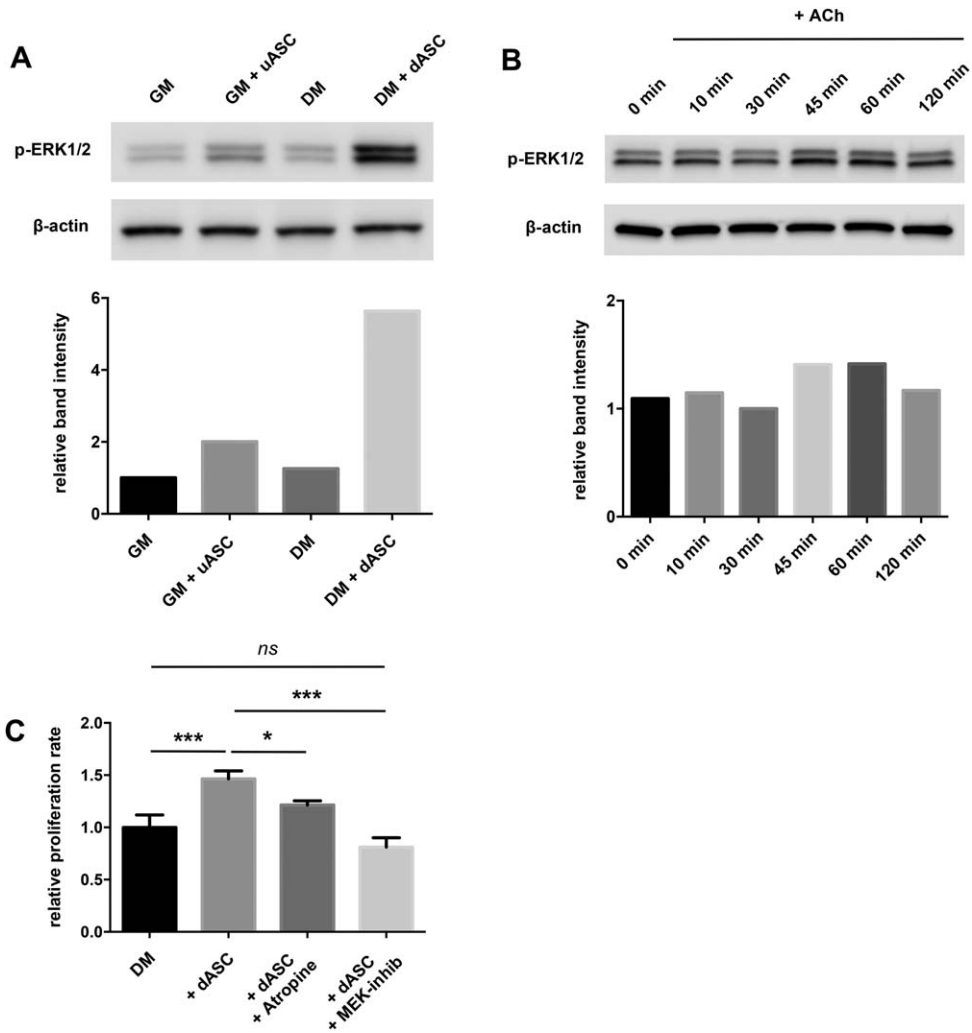

FIGURE 4. Atropine decreases the proliferation rate in myoblast/dASC co-cultures. **(A)** Western blot analysis of extracts from myoblasts co-cultured with ASCs shows an activation of p-ERK1/2. **(B)** L6 myoblasts were exposed to ACh for 0, 10, 30, 45, 60, and 120 min, after which total protein was extracted and subjected to gel electrophoresis. Activation of p-ERK1/2 was apparent after 45–60 min. **(C)** Myoblasts co-cultured with dASCs in differentiating medium (DM) in the presence of atropine or MEK inhibitor. Blocking the muscarinic ACh receptors (+dASC+atropine) as well as blocking MEK (+dASC+MEK inhibitor) significantly reduced the proliferation rate compared with L6 myoblasts co-cultured with dASCs in the absence of these molecules (+dASC). $^{*}P < 0.05$ and $^{***}P < 0.001$. $P < 0.05$ considered statistically significant. *ns*, not significant.

ACh Induces Proliferation in L6 Myoblasts. L6 myoblasts cultured in the presence of ACh showed enhanced proliferation (Fig. 3A). The effect was most pronounced after 2 h using a concentration of 10^{-8} mol/L ACh, but was significant at all timepoints. For the higher concentration of ACh (10^{-6} mol/L), the effect was not significant after 24 h when compared with control. The experiment was repeated for 48 h and the number of live cells measured. A concentration of 10^{-8} mol/L ACh almost doubled the quantity of viable cells (Fig. 3B). The higher concentration, however, did not have a significant effect.

Atropine Decreases Proliferation Rate in Myoblast/dASC Co-Cultures. Western blot analysis indicated enhanced expression of p-ERK1/2 in myoblasts co-cultured with dASCs (Fig. 4A). Next, we determined whether ACh also activated p-ERK1/2 in L6 myoblasts. ACh administration revealed an activation of p-ERK1/2 and its effect peaked at approximately 45–60 min after exposure (Fig. 4B). The dASC-induced proliferation in our co-cultures was significantly reduced by either blocking the muscarinic ACh receptors with atropine or by incubation with a MEK inhibitor (Fig. 4C).

Muscarinic ACh Receptors Are Upregulated in Denervated Muscle. The expression profile of muscarinic ACh receptors was investigated in denervated muscles. In healthy, that is, non-denervated, contralateral muscle, the M_3 and M_2 receptor subtypes were most commonly expressed (Fig. 5A). In denervated muscle, the M_3 subtype was significantly upregulated compared with the contralateral leg (Fig. 5B).

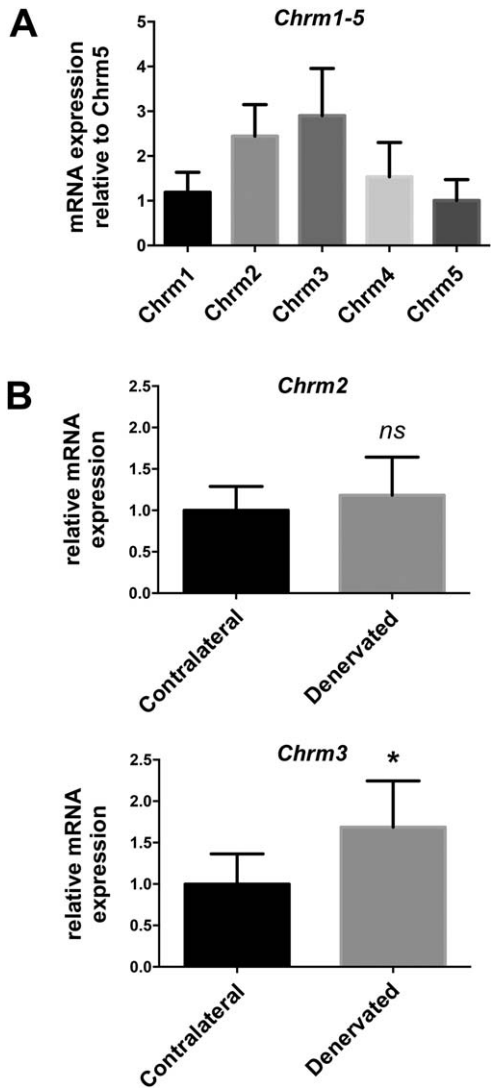

FIGURE 5. Muscarinic ACh receptors are upregulated in denervated muscle. The expression profile of muscarinic ACh receptors in rat gastrocnemius muscle. **(A)** Results showing expression of M_1–M_5 in non-denervated muscle. Data presented as relative to M_5, which had the lowest expression. **(B)** Compared with the non-denervated, contralateral muscle, the expression of M_3 was upregulated. $^*P < 0.05$. $P < 0.05$ considered statistically significant. *ns*, not significant.

DISCUSSION

The results of this study show that dASCs, but not uASCs, increased the proliferation rate in myoblasts, suggesting that this may be one mechanism by which dASCs reduce muscle atrophy *in vivo*, as shown by Schaakxs *et al.*[12] Our co-culture system did not involve direct contact between the myoblasts and stem cells, indicating that secreted molecules mediated the proliferation, which is consistent with previous studies showing the ASC secretome to be rich in angiogenic and neurotrophic factors.[21]

Non-neuronal production of ACh has been discussed widely in recent years, and has been detected in practically all living cells.[22] Herein we have investigated the expression of ChAT and vesicular ACh transporter, 2 important ACh-production markers, in ASCs and found evidence of endogenous cholinergic signaling. In particular, dASCs showed positive anti-ChAT immunoreactivity as well as increased expression of *Chat* and *Slc18a3* mRNA. Based on this finding, we hypothesized that dASCs could activate muscarinic receptors on L6 myoblasts through paracrine secretion of ACh. Indeed, the inclusion of atropine in our co-culture experiments significantly reduced the proliferation of myoblasts, although not to the same extent as in control. This suggests that there are other alternative mechanisms induced by dASCs that can cause proliferation in myoblasts. In this study we limited our investigation to muscarinic ACh receptors and did not investigate the expression or possible role of the nicotinic ACh receptors. However, other studies have shown that the proliferative effects are mediated via muscarinic ACh receptors, not least the M_3 receptor subtype.[23,24]

We have also shown that myoblasts exposed to dASCs are more active in terms of proliferation and that the effect is not dependent on the growth factors used in the differentiating medium, but rather due to molecules secreted by the stem cells themselves. The inclusion of a MEK inhibitor strengthened the conclusion that factors secreted by dASCs, such as ACh, mediate p-ERK1/2 signaling *in vitro*. One could expect the mechanism *in vivo* to be the same, as mitogenic signaling in all types of muscle involves activation of ERK.[16,18,25] Furthermore, ERK is required for terminal differentiation of skeletal myoblasts,[26] which may be of importance in myogenic regeneration processes. Myogenic differentiation is known to correlate with increased proliferation. However, after 72 h of co-culture we did not observe any difference in the gene expression of MyoD, myogenin, or Pax3. Also, macroscopically there was no formation of myotubes. This does not rule out the possibility that dASCs harbor properties that induce myogenic differentiation in the longer term.

Exogenous administration of ACh revealed a time-dependent phosphorylation of p-ERK1/2. The effect of ACh seemed to be highest after 45–60 min of exposure, with gradual degradation over time. This was not surprising considering the high turnover rate of ACh and the fact that the drug was administered only once. Hypothetically, when myoblasts were co-cultured with dASCs, they were constantly exposed to ACh, which may explain the strong phosphorylation of ERK1/2 seen after 48 h. Interestingly, the lower concentration of ACh (10^{-8} mol/L) had a greater response in our experiments. This could be due to the fact that long-term stimulation with high concentrations of ACh reduces the sensitivity of agonists and

downregulates several muscarinic receptors, including the M_2 and M_3 subtypes.[27,28]

In an attempt to link our results to the regenerative effects of dASCs in denervated muscle, we examined the expression of muscarinic receptor subtypes M_1–M_5 in denervated rat muscle. In the contralateral leg the expression varied, with a dominance of the M_2 and M_3 subtype. After comparison with the expression in the denervated leg, we found the M_3 subtype to be upregulated. This is of interest because the M_3 subtype has been shown to induce intracellular calcium mobilization and mediate cell proliferation in human colon cancer.[23] Also, M_3 receptor antagonists have been reported to reduce cell growth in small cell lung carcinoma cells, which were shown to use ACh as an auto- and paracrine growth factor.[24] Upregulation of the M_3 receptor in denervated muscle could therefore be a sign of ACh deprivation and disturbed organ homeostasis, especially because the opposite, namely downregulation, is seen after long-term stimulation with ACh. It should also be taken into account that unilateral interventions can produce bilateral effects, including gene alterations.[29,30] In other words, the observed differences in muscarinic ACh receptor expression between the two legs could actually be even greater.

Adipose stem cells provide a new option for muscle regenerative therapies. These cells can easily be obtained in large quantities and they represent a safer alternative to embryonic stem cells. Possibly the easiest and safest method for protecting and recovering denervated muscle is through isolating the neurotrophic and myogenic factors released from dASCs. Our study suggests that ACh may be one such factor and also that exogenous administration of ACh could help to maintain muscle homeostasis after peripheral nerve injury. Overall, our findings provide further evidence in support of the proposed role of ASCs in regenerative medicine.

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