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miR-3074-3p promotes myoblast differentiation by targeting *Cav1*

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Muscle fibers are generally formed as multinucleated fibers that are differentiated from myoblasts. Several reports have identified transcription factors and proteins involved in the process of muscle differentiation, but the roles of microRNAs (miRNAs) in myogenesis remain unclear. Here, comparative analysis of the miRNA expression profiles in mouse myoblasts and gastrocnemius (GA) muscle uncovered miR-3074-3p as a novel miRNA showing markedly reduced expression in fully differentiated adult skeletal muscle. Interestingly, elevating miR-3074-3p promoted myogenesis in C2C12 cells, primary myoblasts, and HSMMs, resulting in increased mRNA expression of myogenic makers such as *Myog* and *MyHC*. Using a target prediction program, we identified *Caveolin-1 (Cav1)* as a target mRNA of miR-3074-3p and verified that miR-3074-3p directly interacts with the 3′ untranslated region (UTR) of *Cav1* mRNA. Consistent with the findings in miR-3074-3p-overexpressing myoblasts, knockdown of *Cav1* promoted myogenesis in C2C12 cells and HSMMs. Taken together, our results suggest that miR-3074-3p acts a positive regulator of myogenic differentiation by targeting *Cav1*. [BMB Reports 2020; 53(5): 278-283]

INTRODUCTION

Skeletal muscle is composed of multinucleated myofibers that generate movement through contractile machinery units called sarcomeres. Skeletal muscle also contains multipotent progenitor cells, termed satellite cells, which remain quiescent in resting muscle and are characterized by the expression of paired box protein 7 (Pax7) (1). Upon stimulation such as muscle injury, growth stimulus or exercise, satellite cells are activated and start to proliferate to generate the myogenic precursor cells, called myoblasts, needed for muscle regeneration (2). Thereafter, the expression of Pax7 is downregulated, and the expression of myogenic factors, such as myogenin (Myog) and myogenic regulatory factor 4 (MRF4), are upregulated to exit the cell cycle and induce differentiate to fuse to existing myofibers to form regenerating myofibers. Abnormalities in myogenesis lead to the development of muscle diseases (3-5).

MicroRNAs (miRNAs), the best-studied class of noncoding RNAs, are single-stranded RNAs that act as gene regulators at the post-transcriptional level (6, 7). miRNAs regulate gene expression in a sequence-specific manner by binding to the 3′ untranslated region (UTR) of a target gene mRNA. Such interaction results in either degradation or translational inhibition of the target mRNA, leading to suppression of its corresponding protein. Muscle-specific ablation of Dicer, a key enzyme for the maturation of precursor (pre)miRNAs, revealed that miRNAs are also required for satellite cell function and muscle development (8). miRNAs have emerged as essential and powerful regulators of skeletal muscle regeneration and development that affect many transcriptional pathways (9-13). Expression profiling during myogenic differentiation or skeletal muscle lineage progression has revealed multiple miRNAs with differential expression patterns, and these are likely to act as novel myogenic regulators. Previous reports showed that muscle-specific miRNAs (myomiRs), including miR-1, miR-133 and miR-206, play significant roles during skeletal muscle cell proliferation, differentiation and regeneration (14-18).

miR-3074, which shows conservation with the human orthologue, has been reported to be upregulated in tissue samples of non-small cell lung cancer (NSCLC) patients (19), but its roles in several biological processes, including muscle biology, remain to be elucidated. In this study, we found that miR-3074-3p regulates myogenic differentiation by targeting *Caveolin-1 (Cav1)*, indicating its potential to improve muscle regeneration.

RESULTS

A mimic of miR-3074 promotes myogenic differentiation and fusion in myoblasts

We previously reported the expression of miRNAs in mouse

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muscle and mouse myoblasts using RNA-seq (20, 21). To identify miRNAs regulating myogenic differentiation, we compared the expression of pre-miRNAs between primary myoblasts and gastrocnemius (GA) muscle tissues isolated from 6-month-old mice. Among the 85 pre-miRNAs that showed significant changes ($P < 0.001$) between myoblasts and GA muscle tissues (Fig. 1A), 47 pre-miRNAs were significantly downregulated and 38 pre-miRNAs were upregulated in GA muscle tissues (Supplementary Tables 1 and 2). miR-3074 was one of the miRNAs with higher abundance in myoblasts, indicating its important role in the maintenance of homeostasis in myoblast cells. To investigate the role of miR-3074, we overexpressed a mimic (M) of miR-3074 in C2C12 cells and then induced myogenic differentiation. After 48 hr, we analyzed the expression levels of myogenic markers, such as *Myog* and *MyHC*, by qRT-PCR (Fig. 1B). miR-3074 significantly upregulated the myogenic markers. In addition, miR-3074 promoted myogenesis in primary myoblasts, resulting in increased expression of myogenic markers, such as *Myog* and *MyHC* (Fig. 1C). Consistently, the proportion of myotubes with more than two nuclei was significantly increased in primary myoblasts transfected with M-miR-3074 relative to those transfected with control mimic (M-Ctrl) (Fig. 1D). Since we found no significant change showing a similar absorbance in colorimetric assay using CCK-8 (Fig. 1E), these results suggested that miR-3074-3p promoted myogenesis in a cell cycle-independent manner.

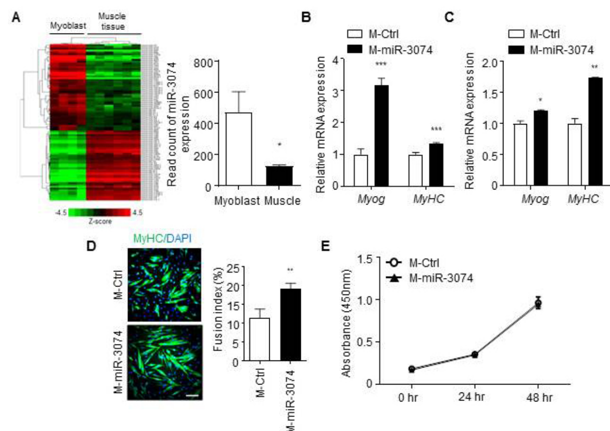

Fig. 1. Elevating miR-3074 promoted myoblast differentiation. (A) Differentially expressed pre-miRNAs between primary myoblasts ($n = 4$) and gastrocnemius (GA) muscle tissues ($n = 6$) isolated from 6-month-old mice. The read count of pre-miR-3074 was significantly decreased in GA muscle tissues. (B) Relative mRNA expression of *Myog* and *MyHC* in C2C12 cells transfected with 100 nM miR-3074 mimic (M) or mimic control (Ctrl). (C) Relative mRNA expression of *Myog* and *MyHC* in primary myoblasts transfected with 30 nM M-miR-3074 or M-Ctrl. (D) Representative immunofluorescence images of differentiated primary myoblasts transfected with 30 nM M-miR-3074 or M-Ctrl. *Green*, MyHC; *Blue*, DAPI. Scale bar, 100 μm. The fusion index was assessed by dividing the number of nuclei in MyHC-positive myotubes with ≥2 nuclei by the total number of nuclei analyzed. (E) CCK-8 assay in cellular proliferation of C2C12 cells transfected with 30 nM M-miR-3074 or M-Ctrl during 48 hr. The data are presented as the mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

miR-3074-3p regulates Cav1 expression by targeting its 3′ UTR

Since pre-miRNA is generally processed into two mature miRNAs (22, 23), we overexpressed either miR-3074-3p or miR-3074-5p in C2C12 cells and induced myogenic differentiation. Although the expression of both mature miRNA strands decreased during myogenesis, only miR-3074-3p was able to promote myogenesis in C2C12 cells (data not shown). To identify potential targets of miR-3074-3p, we used the target prediction algorithm TargetScan (www.targetscan.org). One of the identified potential targets was Cav1 (Fig. 2A), a structural protein component of caveolae, which has been shown to control satellite cell activation during muscle repair (24). To verify whether miR-3074-3p can regulate *Cav1* expression, we performed a luciferase reporter assay using a construct containing the luciferase-3′ UTR of *Cav1* and M-miR-3074-3p. M-miR-3074-3p reduced luciferase activity, which was effectively abolished by deletion of the miR-3074-3p site on the 3′ UTR (Fig. 2B). To further confirm that Cav1 is regulated by miR-3074-3p, we transfected M-miR-3074-3p into primary myoblasts and C2C12 cells and analyzed the expression of Cav1. As anticipated, Cav1 protein content was decreased in M-miR-3074-3p-overexpressing cells (Fig. 2C). Since Cav3 protein content was not altered by elevating miR-3074-3p, miR-3074-3p may not affect the switching or compensation between Caveolin isoforms. Consistent with our RNA-seq data, the expression of

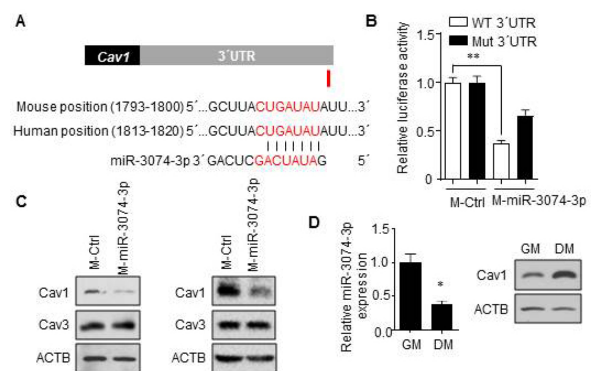

Fig. 2. miR-3074-3p inhibited *Cav1* expression by directly binding to the *Cav1* 3′ UTR. (A) The miR-3074-3p binding site (*red*) in the mouse *Cav1* 3′ UTR (position 1793-1800) is conserved in the human *CAV1* 3′ UTR (position 1813-1820). (B) Effects of miR-3074-3p on the activity of luciferase reporters bearing wild-type (WT) or a deletion mutant (Mut) of its binding site for *Cav1* 3′ UTR. $**P < 0.01$. (C) Immunoblots of Cav1 and Cav3 in (*left*) primary myoblasts and (*right*) C2C12 cells transfected with M-miR-3074-3p or M-Ctrl. (D) (*left*) Relative expression of miR-3074-3p and (*right*) protein content of Cav1 in C2C12 cells cultured in growth medium (GM) or differentiation medium (DM). ACTB was used as a loading control. $*P < 0.05$.

miR-3074-3p was significantly decreased after induction of differentiation in C2C12 cells. Consistent with the decreased expression of miR-3074-3p, the protein content of Cav1 was increased in differentiating C2C12 cells (Fig. 2D). Collectively, these findings suggest that miR-3074-3p regulates the expression of Cav1 protein by directly binding to its 3′ UTR.

Caveolin-1 negatively regulates myogenic differentiation in C2C12 cells

To further evaluate whether *Cav1* is involved in myogenesis, we inhibited *Cav1* expression using small interfering RNA (siCav1) in C2C12 cells and then induced muscle differentiation. Consistent with the results obtained using M-miR-3074-3p, knockdown of *Cav1* significantly promoted myogenesis, resulting in increased expression of myogenic marker genes (Fig. 3A) and proteins (Fig. 3B). In addition, inhibition of *Cav1* expression significantly promoted myotube formation (Fig. 3C). In contrast, overexpression of CAV1 using adenovirus expressing CAV1 (Ad-CAV1) inhibited myogenesis, resulting in significantly downregulated myogenic markers (Fig. 3D). These results strongly suggest that *Cav1* negatively regulates myogenesis.

miR-3074-3p promotes myogenesis of HSMMs

Since the miR-3074-3p seed sequence is conserved between the human and mouse *Cav1* 3′ UTR (Fig. 2A), we hypothesized that miR-3074-3p might also be able to regulate human CAV1 expression levels. We thus analyzed CAV1 protein levels and differentiation in HSMMs transfected with miR-3074-3p mimic (M). Consistent with the results in mouse myoblasts, M-miR-3074-3p downregulated the CAV1 protein levels (Fig. 4A) and upregulated the expression levels of myogenic markers such as *MYOG* and *MYH1* (Fig. 4B). Consistently, knockdown of *CAV1* significantly upregulated the expression levels of *MYOG* and *MYH1* (Fig. 4C). Taken together, these findings suggest that miR-3074-3p promotes myogenesis in HSMMs via regulating the expression of *CAV1*.

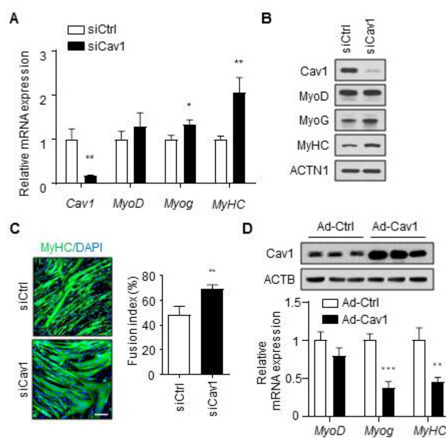

Fig. 3. Cav1 inhibited myogenic differentiation in C2C12 cells. (A) Relative mRNA expression of *MyoD*, *Myog*, and *MyHC* and (B) immunoblots of the indicated proteins in C2C12 cells transfected with 100 nM siCav1 or siCtrl. ACTN1 was used as a loading control. $^*P < 0.05$, $^{**}P < 0.01$. (C) Representative images of differentiated C2C12 cells transfected with 100 nM siCav1 or siCtrl. *Green*, MyHC; *Blue*, DAPI; Scale bar, 100 μm. The fusion index was assessed by dividing the number of nuclei in MyHC-positive myotubes with ≥2 nuclei by the total number of nuclei analyzed. $^{**}P < 0.01$. (D) (*top*) Immunoblots of Cav1 and (*bottom*) relative mRNA expression of *MyoD*, *MyoG* and *MyHC* in C2C12 cells infected with Ad-Cav1 or Ad-Ctrl.

DISCUSSION

Myogenesis is tightly regulated by myogenic regulatory factors (MRFs), such as MyoD, Myogenin, MRF4 and Myf5 (25-27). In addition to MRFs, several miRNAs that are differentially expressed during myogenic differentiation have been reported to regulate myogenesis by binding the 3′ UTR of target genes. These miRNAs have emerged as key players in skeletal muscle myogenesis by participating in multiple gene regulation processes (28). miR-146b, one of the miRNAs upregulated during satellite cell activation and myoblast differentiation, has been reported to promote myogenic differentiation by targeting *Smad4*, *Notch1*, and *Hmga2* (29). miR-26a is also upregulated during myogenesis and promotes myogenesis by targeting *Ezh2*, a known suppressor of skeletal muscle cell differentiation (30). In this paper, we report for the first time that miR-3074-3p, which is differentially expressed during myogenesis, is a positive myogenic regulator targeting *Cav1*. Although a few reports have described miRNA profiling of differentiating C2C12 cells and their function to date (31, 32), our study is the first to analyze miRNA profiles using myoblasts and muscle tissues freshly isolated

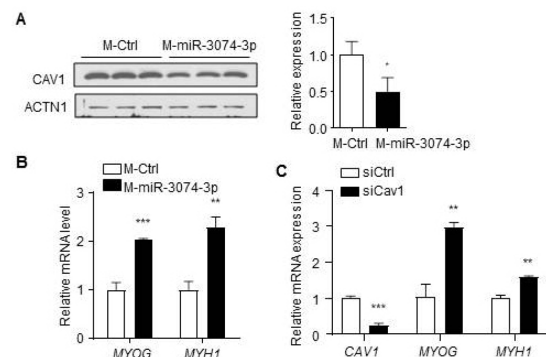

Fig. 4. miR-3074-3p regulated the myogenesis of HSMMs by targeting *CAV1*. (A) (*left*) Immunoblots of CAV1 in HSMMs transfected with 30 nM M-miR-3074-3p or M-Ctrl and (*right*) quantification of CAV1. $^*P < 0.05$. (B) Relative mRNA expression of *MYOG* and *MYH1* in HSMMs transfected with 30 nM M-miR-3074-3p or M-Ctrl. (C) Relative mRNA expression of *MYOG* and *MYH1* in HSMMs transfected with 30 nM siCav1 or siCtrl. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

from mouse hindlimb to exclude artifacts originating from cell lines. Our profiling results revealed that the expression patterns of miRNAs, such as miR-1, miR-133, miR-31, miR-206, and miR-486, were similar in myogenesis, as described previously (13, 31-33). Notably, we identified a novel miRNA, miR-3074-3p, in our analysis and demonstrated its function in muscle differentiation. We believe that our profiling data will be valuable for studying the roles of miRNAs in muscle differentiation and complement previous profiling data using cell lines.

Caveolins, principal components of caveolae, are required for small invagination of the plasma membrane in most cell types (34). Caveolae acts as a signaling platform that recruits numerous signaling molecules, such as insulin receptor, mitogen-activated protein kinase and epidermal growth factor receptor. *Cav1*, one of the caveolin genes, is most prominently expressed in endothelial cells, adipocytes and smooth muscle cells. Cav1 forms a homo-oligomer or a hetero-oligomer with Cav2 and functions as a scaffolding protein that binds to signaling molecules through its caveolin scaffolding domain, thereby activating or inhibiting signaling pathways (35). However, the role of Cav1 in skeletal muscle is largely unknown. A previous report showed that Cav1 was downregulated in differentiated myotubes treated with dexamethasone, resulting in the suppression of insulin signaling and the subsequent upregulation of *MAFbx/Atrogin1* and *MuRF1* (36). During muscle regeneration, HGF is released from injured fibers and downregulates Cav1 expression in satellite cells. D. Volonte et al. suggested that Cav1 might be not only a structural protein component of caveolae but also a key player in muscle regeneration. These previous reports suggested possibility that Cav1 may play different roles in myoblasts and myotubes. Although the downregulation of Cav1 has been shown to activate ERK and promote muscle regeneration by stimulating satellite cell proliferation (24), in the present study, miR-3074-3p had no significant effect on cellular proliferation. We therefore concluded that miR-3074-3p promotes myogenesis in a cell cycle-independent manner. Future investigation into the precise mechanism underlying Cav1-dependent regulation in myogenesis may provide valuable insight for the development of therapeutics to combat muscle diseases.

In the present study, we found that conserved miR-3074-3p regulated Cav1 expression in C2C12 cells and HSMMs, thereby enhancing myogenesis. Collectively, our results suggest that miR-3074-3p may act as a positive regulator of myogenesis via fine-tuning Cav1 expression and may have the potential to promote muscle regeneration.

MATERIALS AND METHODS

miRNA sequencing and analysis

Small RNA-enriched total RNA from mouse myoblast (n = 4) and gastrocnemius muscle (n = 6) samples was extracted using the *mir*Vana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol, and miRNA libraries were prepared following the Illumina library preparation protocol (Illumina Inc., San Diego, CA, USA) as previously described (20). Mirbase was used to filter known miRNAs, and *Rattus norvegicus* was used as a related species. Experiments with mice were performed according to established protocols approved by the Animal Care and Use Committee of KRIBB.

Cell culture

Primary myoblasts were isolated from hind limb muscles as described previously (37). Differentiation of primary myoblasts was induced by culturing cells in differentiation medium comprising DMEM (Gibco) supplemented with antibiotics and 5% horse serum (Gibco). C2C12 cells (ATCC) were cultured in DMEM (Gibco) with amphotericin B-penicillin-streptomycin and 10% FBS (Gibco). Human Skeletal Muscle Myoblasts (Lonza Walkersville, Inc.) were cultured in SkBM-2 (Lonza) supplemented with growth factors (Lonza). At 24-48 hr after transfection, differentiation was initiated by changing the medium to differentiation medium containing DMEM/F12 (Gibco), antibiotics, and 2% horse serum (Gibco). To overexpress Caveolin-1 in C2C12 cells, cells were infected with adenovirus expressing Caveolin-1 (Ad-Cav1) or control (Ad-Ctrl) by 30 MOI. After 24 hr of infection, myoblast differentiation was induced by changing the growth medium to differentiation medium.

Cell proliferation assay

C2C12 cells in 24-well plates at 1×10^4/well were transfected with 30 nM M-miR-3074-3p or M-Ctrl. After 24 or 48 hr, the cultured medium was transfered into 96-well plates, then CCK-8 (Dojindo) was added into each wellthe cells. After incubation for 1 hr, the absorbance was measured at 450 nm using SpectraMax M2e microplate reader (Molecular Devices).

Transfection and luciferase assay

siRNAs or miRNA mimics were transfected into primary myoblasts, C2C12 myoblasts, or HSMMs using RNAiMAX (Invitrogen) according to the manufacturer's protocol. miRNA mimics were purchased from mirVana (MC18636, Invitrogen) or AccuTarget (MI0014181, Bioneer). siRNAs were purchased from AccuOligo (1334902, Bioneer). Mimics of miRNA and siRNA (30-100 nM each) were transfected into primary myoblasts, C2C12 or HSMMs using RNAiMAX (Invitrogen) according to the manufacturer's recommended protocols.

For the luciferase assays, the wild-type 3′ UTR fragment of *Cav1* mRNA and the deletion mutant 3′ UTR fragment of *Cav1* mRNA were cloned into pmirGLO (Promega). Each of the vectors was cotransfected with 30 nM M-miR-3074-3p or M-Ctrl into 293T cells by using Lipofectamine 2000 (Invitrogen). After 48 hr of transfection, cell lysates were used for the luciferase assay with the Dual-Luciferase Reporter Assay System (Promega) and Victor X3 (Perkin Elmer).

Quantitative RT-PCR and miRNA expression analysis

RNA preparation and cDNA synthesis were performed according

to standard protocols. Quantitative RT-PCR was performed using StepOnePlus[TM] (Applied Biosystems). Data were normalized to *36B4* or *GAPDH* mRNA levels in each reaction. The sequences of the PCR primers are as follows: Mouse *36B4* Forward: AGATTCGGGATATGCTGTTGG, Mouse *36B4* Reverse: AAA GCCTGGAAGAAGGAGGTC; Mouse *Cav1* Forward: ACGAT GTCTGGGGGCAAATAC, Mouse *Cav1* Reverse: TCATATCTC TTTCTGCGTGC; *MyoD* Forward: ACTACAGTGGCGACTCA GATGC, *MyoD* Reverse: CCGCTGTAATCCATCATGCCATC; *Myogenin* Forward: CTACAGGCCTTGCTCAGCTC, *Myogenin* Reverse: ACGATGGACGTAAGGGAGTG; *MyHC* Forward: AAAAGGCCATCACTGACGC, *MyHC* Reverse: CAGCTCTCT GATCCGTGTCTC; Human *GAPDH* Forward: GAGTCAACG GATTTGGTCGT, Human *GAPDH* Reverse: TTGATTTTGGAG GGATCTCG; Human *MYOG* Forward: GGGGAAAACTACCT GCCTGTC, Human *MYOG* Reverse: AGGCGCTCGATGTACT GGAT; Human *MYH1* Forward: CCCTACAAGTCCTTGCCA GTG, Human *MYH1* Reverse: CTTCCCTGCGCCAGATTCTC. Human *CAV1* primers were purchased from Bioneer. For the analysis of mature miRNA expression, assays were performed using the TaqMan MicroRNA Assay kit according to the manufacturer's protocol (Applied Biosystems). *U6* snRNA served as the endogenous control for normalization.

Immunoblot analysis

Mouse tissues and isolated myoblasts were homogenized in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM $MgCl_2$) containing protease and phosphatase inhibitors. Lysates were centrifuged at 15,000×g for 20 min at 4°C, and the resulting supernatants were subjected to SDS-PAGE followed by immunoblot analysis. Antibodies used for immunoblotting included those specific for ACTB (β-actin; Abcam), ACTN1 (Alpha-actinin 1; Santa Cruz Biotechnology), MYOG (Myogenin; Santa Cruz Biotechnology), MyHC (Myosin heavy chain; Santa Cruz Biotechnology), and CAV1 (Caveolin1; Cell Signaling Technology). ACTB and ACTN1 served as the endogenous controls for normalization.

Immunofluorescence staining

For immunostaining, differentiated C2C12 myotubes or primary myotubes were fixed in 4% paraformaldehyde and incubated with 0.3% Triton X-100 to enhance permeability. Fixed samples were blocked with 3% bovine serum albumin in PBS and treated with anti-MyHC (Santa Cruz Biotechnology), followed by washing in PBS and incubation with Alexa Fluor 488 (Invitrogen) secondary antibodies. The fusion index was determined by dividing the number of nuclei in MyHC-positive myotubes with ≥2 nuclei by the total number of nuclei analyzed (38).

Statistical analysis

Quantitative data are presented as the mean ± SD unless indicated otherwise. Differences between means were evaluated using Student's unpaired t test. P values < 0.05 were considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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