# Murine miR-483-3p promotes proliferation and suppresses differentiation of C2C12 cells by targeting SRF

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## Abstract

Myogenesis is a complicated process, which is regulated by numerous regulators. MicroRNAs (miRNAs) are conserved non-coding RNAs of ~22 nucleotides, which regulate post-transcriptional gene expression in many physiological and pathophysiological processes. Recent studies have indicated that microRNAs are critical regulators of muscle development. Here, we report miR-483-3p as a new essential regulator of muscle development, mediating myoblast proliferation and myogenic differentiation. miR-483-3p is strongly and almost exclusively expressed in muscle-related tissues such as leg muscle, back muscle, and heart. Its expression is downregulated during mouse development. Overexpression of miR-483-3p in C2C12 cells promotes proliferation and suppresses myogenic differentiation. A dual-luciferase reporter assay demonstrated miR-483-3p direct targets to the 3′-UTR of the SRF gene. Overexpression of miR-483-3p reduced SRF protein levels in C2C12 myoblasts. These results reveal a novel function of miR-483-3p as a positive regulator of C2C12 proliferation and inhibitor of myogenic differentiation via SRF downregulation.


**Keywords:** Mmu-miR-483-3p; SRF; C2C12; proliferation; differentiation

## 1. Introduction

Myogenesis is a complicated process regulated by a number of transcription factors, such as myogenic determination factors Myf5 and MyoD, and differentiation factors myogenin, Myf4, and MEF2 (Buckingham, Bajard et al. 2003). Our recent studys demonstrate myogenesis is regulated by genes such as CRABP2, SMAD7, FNDC5(Yuan, Tang et al. 2013, Hua, Wang et al. 2016, Cai, Xiao et al. 2017). Also we found that myogenesis is related with MicroRNAs(Tang, Yang et al. 2015, Hou, Yang et al. 2016), long intergenic non-coding RNAs(Zhao, Mu et al. 2015, Liang, Yang et al. 2017), chimeric RNAs(Yang, Tang et al. 2016), circular RNAs (Liang, Yang et al. 2017). During skeletal muscle development, cells from the somites commit to the myogenic lineage and progress through proliferation, terminal differentiation, and formation of multinucleated myofibers (Buckingham 2001). Activation of muscle-specific transcription factors such as MyoD and MEF2, resulting in reprogramming of gene expression to govern muscle growth, morphogenesis, differentiation and contractility (Weintraub 1993, Naya and Olson 1999). MicroRNAs (miRNAs) are a class of conserved endogenous non-coding RNAs (~22 nucleotides) that post-transcriptionally inhibit gene expression by reducing the stability and/or translation of target mRNAs (Bartel 2004). They form RNA-induced silencing complexes (RISC), and then bind to the miRNA-binding sites frequently found in the 3′-untranslated region (3′-UTR) of target genes. miRNAs play vital roles in a wide range of physiological and pathological processes such as cell differentiation, proliferation, cancers, heart disease, and aging as also in myogenesis. Mice with the Dicer deletion in muscle die perinatally and presented decreased skeletal muscle mass, increased muscle cell apoptosis and abnormal myofiber morphology (O'Rourke, Georges et al. 2007). The depletion of DGCR8, a factor in the miRNA biogenesis pathway, in striated muscle causes severe dilated cardiomyopathy and heart failure (Rao, Toyama et al. 2009). These results indicate the important roles of miRNAs in myogenesis. To best-understood miRNAs in muscle development we summarized miR-1/miR-133/miR-206, which are specifically expressed in cardiac and skeletal muscles and regulate the fundamental processes of skeletal myogenesis including myoblast/satellite cell proliferation and differentiation (Williams, Liu et al. 2009,



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Carvajal and Rigby 2010, Sun, Ge et al. 2010, Ge and Chen 2011, Tang, Liang et al. 2014) (Chen, Mandel et al. 2006); MicroRNA-148a could promote myogenic differentiation by targeting the ROCK1 gene in C2C12 cells (Zhang, Ying et al. 2012); MicroRNA-155 regulated OLFML3 expression during prenatal muscle development in Pigs(Zhao, Zhang et al. 2012); MicroRNA-21 participates PI3K/Akt/mTOR signaling during skeletal muscle development in pigs(Bai, Liang et al. 2015); MicroRNA-18a has a functional role in muscle physiology by suppressing the expression of Igf1 in a 3'UTR-dependent manner in C2C12(Liu, Wang et al. 2017). All the results state that MicroRNA have very important function in muscle development, more specific study about MicroRNA role in myogenesis are needed.

The focus of our work is miRNA-483-3p. The precursor of miRNA-483 is 73 nt and can be cleaved into 2 mature miRNAs, miR-483-5p and miR-483-3p. The human and mouse miR-483 genes are located in the second intron of IGF2 (insulin-like growth factor 2) (Zhang, Zhang et al. 2009). miR-483 has been associated with many cancers and is overexpressed in Wilms', colon, breast and liver cancers(Veronese, Lupini et al. 2010). miR-483 exhibits coregulation with IGF2 expression and can modulate the pro-apoptotic complex BBC3/PUMA (P53-upregulated modulator of apoptosis), which protects cancer cells from apoptosis (Veronese, Lupini et al. 2010). In pancreatic cancer, miR-483-3p significantly represses DPC4/Smad4 and promotes cell proliferation and colony formation (Hao, Zhang et al. 2011). In wounded epithelial cells, miR-483-3p inhibits keratinocyte migration and proliferation and down-regulates MK2, MKI67, and YAP1, thus controlling keratinocyte growth arrest at the final steps of re-epithelialization (Bertero, Gastaldi et al. 2011). The function of miR-483 in myogenesis has not been reported.

SRF (serum response factor) is a transcriptional regulator of proliferation, migration, differentiation, angiogenesis, and apoptosis (Modak and Chai 2010). Due to its critical role in mesoderm-derived tissues, most SRF studies have focused on muscle structure and function, cardiovascular development and maintenance, and smooth muscle generation and repair. In mice lacking SRF expression in skeletal muscle, normal formation of muscle fibers is followed by a failure to undergo hypertrophic growth after birth (Li, Czubryt et al. 2005). SRF binds to SREs in the promoters of the α-actin and myosin light chain (MyLC) genes,



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which are expressed in cardiac, skeletal, and smooth muscle.

MiR-483 regulates cell proliferation in various cancers; thus, we question whether it plays a role in myoblast proliferation and myogenic differentiation just as its co-expressed counterpart IGF is essential for muscle development and growth. Using a bioinformatics approach, we predicted that SRF was a potential target gene of miR-483-3p. We proposed that miR-483-3p functioned in myoblast proliferation and myogenesis via SRF targeting. In this study, we found that miR-483-3p is preferentially expressed in muscle-related tissues such as leg muscle, back muscle, and heart. miR-483-3p can promote myoblast proliferation while inhibit C2C12 myogenic differentiation by negatively regulating the expression of C2C12 differentiation marker Myh1 and myogenin. The reduction of myotube formation was also observed in miR-483-3p-overexpressing differentiating C2C12 cells. The dual-luciferase assay confirmed that SRF is a direct target of miR-483-3p and its protein level is down-regulated in miR-483-3p overexpressed C2C1 cell. Our study therefore indicated that miR-483-3p promotes myoblast proliferation and suppresses myogenic differentiation by targeting the SRF gene.

## 2. Materials and Methods

### *2.1 Ethics Statement*

The procedure followed in the care and euthanasia of the study animals was in accordance with the Guide for the Care and Use of Laboratory animals published by US National Institutes of Health (revised 1996) and protocols approved by The Hubei Province for Biological Studies Animal Care and Use Committee. All dissections were performed with the mice under isoflurane anesthesia, and were followed by euthanasia with an overdose of sodium pentobarbital. All efforts were made to minimize suffering.

### *2.2 Tissue samples preparation and cell culture*

BALB/c mice were purchased from the Wuhan Center for Disease Control. Ten different tissues, including heart, liver, spleen, lung, kidney, stomach, back muscles, leg muscle, abdominal fat, and small intestine of three 6-week-old male mice were collected and treated as our previous report(Zhou, Yang et al. 2017). We also took the leg muscles of postnatal 2 days, 2 weeks, 4 weeks, 6 weeks, and adult mice to analyze miR-483-3p gene expression



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pattern.

The mouse skeletal myoblast cell line C2C12 and baby hamster kidney (BHK-21) cells were purchased from the China Center for Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) supplemented with 10% fetal bovine serum (FBS, Invitrogen Life Technologies), 100 units/mL penicillin-100 g/mL streptomycin (Invitrogen Life Technologies) and cultured in a 5% $CO_2$-humidified incubator at 37°C. For proliferation, C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS. For myotube differentiation, C2C12 myoblasts at approximately 80% confluence were switched to differentiation DMEM supplemented with 2% horse serum (Recchiuti, Krishnamoorthy et al.) (HS, Sigma).

*2.3 RNA isolation, RT-PCR, and Q-PCR*

Total RNA was extracted from 20 mg of each sample using the Tissue RNA Extraction Kit (Tiangen). Total RNA from proliferating and differentiating C2C12 cells was isolated using Trizol reagent (Invitrogen). The miRNA was reverse-transcribed in a stem-loop assay with mmu-miR-483-3p primer, while the mRNA was reverse-transcribed with oligo18T and random primer. Briefly, 2 μg total RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Fermentas Scientific Inc., Cat. No. K1621); the cDNA was diluted 5-fold and 1 l was used in a 20- l Q-PCR reaction system. The quantity of endogenous mature miRNA and mRNA was detected by SYBR I-based quantitative PCR using the miRNA- and gene-specific primers. The Q-PCR reaction was performed as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s in a Lightcycler 480 (Roche). All reactions were run in triplicate. miRNA expression was normalized to U6 RNA while the internal control gene for myogenin and Myh1 expression was β-Tubulin(Niu, Yang et al. 2016). Relative expression was calculated by the comparative ΔΔCT method, and values were expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). Primers are listed in Table 1.

*2.4 Real-time cell proliferation detection*

C2C12 cells were seeded into dedicated 16-well E-Plates in an xCELLigence/RT - CES instrument (Roche) and cultured for 20 h to 20–30% confluence. Cells in 6 wells were transfected with 20 pmol negative control oligonucleotide duplex mimic (miR-NC) and



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mmu-miR-483-3p mimic. The E-Plate was kept in a $CO_2$ incubator to ensure normal cell growth. Proliferation rate was monitored continuously by software installed on the computer connected to the instrument. The proliferation curve was generated by this software.

*2.5 Cell proliferation assay using Cell Counting Kit 8*

C2C12 cells were seeded into 96-well plates and cultured for 20 h to 20–30% confluence. Cells in 10 wells were transfected with 20 pmol NC or mmu-miR-483-3p mimics. After transfection for 30 h and growth to 80–90% confluence, 20 l of the CCK-8 reagent (Beyotime) was added to each well and the cells were cultured in the $CO_2$ incubator for another 1 h. The OD of each well at 450 nm was recorded in a microplate reader.

*2.6 Luciferase assay*

The 3′-UTR of the SRF gene containing the mmu-miR-483 target sequence was cloned into the psiCHECK™-2 dual luciferase reporter plasmid (Promega, Cat. No. C8021) at the 3′-end of the coding sequence of Renilla luciferase to produce psi-WT-SRF-3′-UTR. The mutant construct (psi-MT-SRF-3′-UTR, GGAGTGA in SRF 3′-UTR was mutated to GAGGTGA of the mmu-miR-483-3p targeting site) was produced by mutagenic PCR. The accuracy of the plasmid inserts was determined by complete sequencing.

BHK cells were cultured in 24-well plates for 24 h before transfection with the reporter constructs; 200 ng wild-type or mutant SRF 3′-UTR reporter plasmids and 400 ng mmu-miR-483–3p or negative control miRNA mimics were added to each well. After 48 h transfection, luciferase activity was determined with a dual luciferase reporter assay system (Promega cat. E1980). The Renilla luciferase activity was normalized to firefly luciferase. Luciferase activity was repeated in 3 independent replicates.

*2.7 Western blotting*

C2C12 cells were seeded in 6-well plates and grown to 70–80% confluence before exchanging the medium with differentiation medium, and the cells were transfected with the mmu-miR-483-3p and negative control miRNA mimics. After transfection for 36 h, the medium was exchanged with fresh differentiation medium, and the mmu-miR-483-3p and negative control miRNA mimics were transfected into the cells again. After 36 h for the secondary transfection, cells were washed twice with ice-cold PBS, scraped into 350 l PBS,



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192 and centrifuged at 5000 rpm for 5 min before lysis with 100 l protein extraction reagent
193 (Thermo Scientific cat No: 78503). Total protein was quantified by the BCA assay. Protein
194 (100 g) and loading buffer was separated by 12% SDS-PAGE for 120 min at 90 V and
195 immediately transferred to 0.22- m polyvinylidene fluoride membranes for 35 min at 250
196 mA. The membranes was blocked with TBS containing 0.1% Tween-20 and 5% bovine
197 serum albumin (BSA) for 2 h at room temperature, then immunoblotted with primary
198 antibodies at 4°C overnight. The primary antibody for SRF was rabbit-anti-mouse
199 mono-antibody from CST (#5147) diluted 1:500. Goat anti-rabbit secondary antibody
200 conjugated with horseradish peroxidase (HRP) was added and incubated at 37°C for 90 min,
201 followed by detection with enhanced chemiluminescence (ECL, Pierce Biotechnology Inc.,
202 Cat. no. 34094).

203 *2.8 Immunofluorescence*

204 Cells were plated in 6-well plates and culture for 24 h to 70–80% confluence; the
205 medium was exchanged for DM and mmu-miR-483-3p and negative control miRNA mimics
206 were transfected into the cells every 36 h for 7 d. Cells were switched to fresh differentiation
207 medium 6 h after each transfection. After 7 d differentiation, the medium was removed and
208 cells were washed 3 times with PBS (5 min per wash at 37°C), then fixed with 4%
209 paraformaldehyde in PBS for 20 min at room temperature. Cells were treated with PBS
210 containing 0.2% Triton X-100 and rinsed 3 times for 5 min prior to incubation with primary
211 antibody overnight at 4°C. The primary antibodies were mouse monoclonal anti-MYH1/2/3
212 (1:500; Santa Cruz, #53092). Cells were rinsed 3 times (5 min each) in PBS followed by a
213 1-h incubation at 37°C with the secondary antibody 3 more PBS washes. The secondary
214 antibody was Alexa 488 goat anti-mouse IgG antibody (1:500; Invitrogen, #A-10680).
215 Antibodies were diluted in PBS containing 5% BSA. After immunostaining, cells were
216 observed and photographed under a fluorescence microscope (Olympus). And the
217 fluorescence ratio in the picture was calculated by Photoshop 7.0.

218 *2.9 Statistical analysis*

219 All data are presented as the means ± SD of at least 3 independent experiments.
220 Statistical significance was determined by the Student's t-tests using Microsoft Excel. P values



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<0.05 were considered significant, while P values <0.01 were considered highly significant.

## 3. Results

### *3.1 mmu-miR-483-3p expression in mouse tissues and during muscle development*

In order to understand mmu-miR-483-3p function in muscle development, we employed RT-qPCR to quantify mmu-miR-483-3p expression in mouse tissues at various developmental stages, and during mouse skeletal myoblast C2C12 differentiation. mmu-miR-483-3p was strongly expressed in muscle-related tissues such as leg muscle and back muscle and was rarely expressed in other tissues (Fig. 1A), suggesting a role of mmu-miR-483-3p in muscle tissues development. Expression of mmu-miR-483-3p in proliferating myoblasts was relatively low, increased significantly at the beginning of differentiation, then decreased gradually during the differentiation process (Fig. 1B). mmu-miR-483-3p expression reached a maximum in 2-d postnatal mouse leg muscle and gradually decreased to adulthood, at which the expression level was nearly 1/15 of the level observed on the second postnatal day (Fig. 1C).

### *3.2 mmu-miR-483-3p promotes C2C12 cell proliferation and suppresses expression of myogenin and Myh1*

To explore the function of mmu-miR-483-3p in muscle development, we determined its role in cell proliferation and differentiation. We transfected C1C12 cells with mmu-miR-483-3p mimics and a negative control then recorded real-time cell proliferation on an xCELLigence system. After transfection, cell proliferation was recorded for 80 h and the cell index was calculated. As shown in Fig. 2A, cells transfected with mmu-miR-483-3p had a higher cell index than those transfected with NC mimics, suggesting that mmu-miR-483-3p transfected C2C12 cells has a higher proliferation rate than that of NC cells.

In order to confirm the positive function of mmu-miR-483-3p in C2C12 cell growth, CCK8 assay was further applied to measure the proliferation rate of C2C12. We transfected



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mmu-miR-483-3p or NC mimics into C2C12 cells; 30 h after transfection, CCK8 was added to the medium and absorbance was detected on a microplate reader. The absorbance was significantly higher in cells transfected with mmu-miR-483-3p than that in the NC group (Fig. 2B). This data was consistent with the data from the xCELLigence system, which strongly supported our conclusion that mmu-miR-483-3p promotes C2C12 myoblast proliferation.

During muscle development, the differentiation of myoblast into myotubes is inevitable. In order to determine whether mmu-miR-438-3p participates in this transition, we transfected C2C12 cells with mmu-miR-483-3p mimics or NC mimics and then induced the cells to initiate differentiation while we monitored the expression of differentiation marker genes. As shown in Fig. 2C and Fig. 2D, when DM was added for 24 h, the expression of myogenin and Myh1 showed up but there was no difference in marker gene expression between the mmu-miR-483-3p and NC groups. Following the extension of the differentiation time the expression of both markers was dramatically elevated indicating that the cells were undergoing differentiation. We also observed that at 38 h, 48 h, and 64 h, the expression of myogenin and Myh1 in cells transfected with mmu-miR-483-3p mimics was significantly lower than that in NC mimics. We conclude that mmu-miR-483-3p serves as a negative regulator during C2C12 differentiation.

*3.3 mmu-miR-483-3p inhibits C2C12 cell differentiation*

C2C12 is a special cell line with the ability to differentiate into myotubes, making it a good model to mimic muscle development and elaborate the in vitro mechanism of myoblast cell differentiation. We used DM to stimulate myoblast differentiation and mmu-miR-483-3p or NC mimics were transfected into cells every 36 h to maintain high expression. With 7 d of treatment, the Myh protein distribution in both groups was detected by immunofluorescence to compare differences in myotube formation ability. As shown in Fig. 3, miR-483-3p expression caused a 16.39% reduction of fluorescence ratio of the scope ($P<0.05$). The differences in cell revealed that mmu-miR-483-3p has negative effects on cell fusion and myotube formation. These results strongly suggested mmu-miR-483-3p is a negative regulator of myotube formation and thus suppresses C2C12 cell differentiation.

*3.4 mmu-miR-483-3p directly targets SRF*



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With a basic understanding of mmu-miR-483-3p function, we next explored its target genes. Analyses with TargetScan revealed potential mmu-miR-483-3p target sequences around the 3–UTR of the transcriptional factor SRF, which is essential in muscle development (Fig. 4A). To validate the predicted miRNA-binding sequences for mmu-miR-483-3p, the 3–UTR of SRF containing the miRNA-binding sequences was cloned into the psiCHECK™-2 dual luciferase reporter plasmid (psiCHECK$^{TM}$2-SRF-3'-UTR). Luciferase activity was significantly attenuated in cells transfected with mmu-miR-483-3p mimic. In contrast, luciferase in mutant SRF 3′-UTR (psiCHECK$^{TM}$2-SRF-3′-UTR-mut) targeting sequences was resistant to inhibition by the mmu-miR-483-3p mimic (Fig. 4B). Therefore, mmu-miR-483-3p most likely regulates SRF expression by targeting the miRNA-binding sequences in the 3–UTR region of SRF mRNA. Subsequently, we assessed whether endogenous SRF is down-regulated by miR-483–3p. western blotting results indicated that Endogenous SRF protein levels significantly declined in the mmu-miR-483-3p group versus the NC control (Fig. 4C). In a reciprocal experiment, inhibition of endogenous miR-483–3p using antisense inhibitors of miR-483–3p caused a longer persistence of endogenous SRF proteins during differentiation. These results demonstrated that SRF is directly regulated by miR-483–3p.

## 4. Discussion

Muscle development and myogenesis is a complicated process coordinated by networks including signal factors, transcription factors, and microRNAs. Recent observations have revealed the importance of microRNAs in mammalian muscle development regulation (Ge and Chen 2011). The well-known myogenic miRNAs are the miR-1/miR-206 and miR-133a/miR-133b families (Williams, Liu et al. 2009). miR-1, miR-133, and miR-206 expression is most dramatically increased during myoblast differentiation.

We identified miR-483-3p as a novel miRNA that plays a positive role in C2C12 myoblast proliferation and a negative role in myogenic differentiation. miR-483-3p is highly expressed in adult muscle-related tissues in mouse such as leg muscle, back muscle, and heart and its expression is downregulated during development from 2 d postnatal to adult stage in the leg muscle. Expression of miR-483-3p was sharply up-regulated in the early stage of



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C2C12 differentiation at day 1 and day 2 and rapidly decreased to day 4. This expression pattern was very similar for miR-1 and miR-133 (Chen, Mandel et al. 2006). miR-483 coding genes located in the second intron of IGF2 (insulin-like growth factor 2) (Zhang, Zhang et al. 2009), and its expression exhibited co-regulation with IGF2 expression in cancers (Veronese, Lupini et al. 2010). The expression pattern of miR-483-3p may be influenced by IGF2 expression in mouse muscle tissues and during muscle different development.

Research on the molecular mechanisms that regulate cellular proliferation and differentiation of myoblasts is a central theme of muscle development. Our previous data showed that miR-483-3p overexpression affects C2C12 proliferation and differentiation. CCK-8 and real-time cell proliferation detection assays revealed that C2C12 cells with elevated miR-483-3p grew faster than normal cells. Recent studies on miR-483-3p have revealed its function in regulating cell proliferation. In wounded epithelium, overexpression of miR-483-3p inhibits keratinocyte migration and proliferation (Bertero, Gastaldi et al. 2011). However, ectopic expression of miR-483-3p in pancreatic cancer cell lines significantly promoted cell proliferation and colony formation in vitro (Hao, Zhang et al. 2011). Therefore, miR-483-3p has a dual role in cell proliferation which significantly differs in epithelial to pancreatic cancer cells and seems to depend on cell type. We also detected the expression of C2C12 differentiation markers Myh1 and myogenin to verify function in cell differentiation. These genes were downregulated in miR-438-3p-overexpressing cells, indicating its negative regulation of C2C12 myogenic differentiation. These results were confirmed by immunofluorescence with antibody to Myh1/2/3 after miR-483-3p transfection during cell differentiation, which showed a decline in the rate of cell fusion and myotube formation.

The identification of targets is critical for the functional characterization of miRNAs. The functions and targets of miR-483-3p have been reported in several cancer cell lines, in this research our findings revealed the role of miR-483-3p in myogenic suppression and identified SRF as target of miR-483-3p. The dual-luciferase system showed that miR-483-3p directly bind to the 3′-UTR of SRF and regulate its expression in a posttranscriptional manner. The suppression role of miR-483-3p on SRF was abolished by mutating the seed region of the predicted miR-483-3p binding site in SRF, indicating that miR-483-3p may be a major



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335 miRNA regulator of SRF expression via post-transcriptional regulation.

336 SRF is a well-known MADS-box transcription factor critical for muscle differentiation.
337 Lacking skeletal muscle SRF expression, muscle fibers formed, but failed to undergo
338 hypertrophic growth after birth in mice (Li, Czubryt et al. 2005). SRF, also a critical
339 transcription factor in smooth muscle cells (SMCs), controls SMC differentiation and
340 proliferation (Horita, Simpson et al. 2011). SRF regulates MyoD expression by directly
341 binding to the divergent serum response factor (SRF)-binding CArG element which functions
342 as an enhancer and is required for MyoD expression during myoblast growth and muscle
343 regeneration (L'Honore, Rana et al. 2007). A research using C2C12 cell line indicated that
344 neuregulin1 signaling targets SRF and CREB and activates the muscle spindle-specific gene
345 Egr3 through a composite SRF–CREB-binding site (Herndon, Ankenbruck et al. 2013). It is
346 interesting that SRF can regulate miRNA expression and yet be regulated by miRNAs, as
347 demonstrated by our work in muscle development. In vascular smooth muscle cells, the
348 SRF/myocardin complex binds CArG sequences to activate miR-143/145 transcription
349 (Boucher, Peterson et al. 2011). miR-1 and miR-133 are positively regulated by SRF to
350 promote mesoderm formation from ES cells (Ivey, Muth et al. 2008). In skeletal muscle,
351 miR-133 enhances myoblast proliferation by repressing SRF (Chen, Mandel et al. 2006). Our
352 results suggest SRF is regulated by miR-483-3p; thus, miR-483-3p is another important SRF
353 regulator. It is consistent with miR-483-3p targeted SRF to decreased the migration and tube
354 formation while increased the apoptosis of EPCs (Kong, Hu et al. 2016).

355 In conclusion, we suggest miR-483-3p is an important regulator in C2C12 myoblast
356 proliferation and myogenic differentiation. It was strongly expressed in muscle-related tissues
357 and was downregulated during postnatal development. SRF is a potential target of
358 miR-483-3p, as demonstrated by dual-luciferase reporter assay and western blotting. Our data
359 revealed a novel microRNA-mediated regulatory mechanism in which miR-483-3p positively
360 regulates myoblast proliferation and suppresses myogenic differentiation via SRF
361 downregulation. Our research provides a new mechanism for the novel function of
362 miR-483-3p in regulating muscle development.



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## Author contributions

Jing Huang and Ying Liu wrote the manuscript and performed part of experiments;

Jing Huang and Liangliang Fu performed the experiments;

Hegang Li and Bingkun Xie contributed towards the study materials;

Guojian Ma conceived and designed the experiments and performed the experiments.

## Conflict of interest

None of the authors has any conflict of interest to declare.

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**Table 1:** Primer sequences

| Primer name | Sequence (5' to 3') | Size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| RT-miR-483-3P | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAGACGGG | | |
| Q-miR-483-3P | CTGGTAGGTCACTCCTCCC | 64 | 61 |
| | TCAACTGGTGTCGTGGAGTCGGC | | |
| Q-U6 | GTGCTCGCTTCGGCAGCACATAT | 107 | 61 |
| | AAAATATGGAACGCTTCACGAA | | |
| Q-myogenin | CAATGCACTGGAGTTCGGT | 134 | 60 |
| | CTGGGAAGGCAACAGACAT | | |
| Q-Myh1 | CGCAAGAATGTTCTCAGGCT | 110 | 60 |
| | GCCAGGTTGACATTGGATTG | | |
| Q-tubulin | GACTATGGACTCCGTTCGCTC | 264 | 60 |
| | TATTCTTCCCGGATCTTGCTG | | |
| Q-SRF | AGGATGGAGTTCGGGAGGTAG | 296 | 60 |
| | GCTGGCTCTGACACAGGGTAA | | |
| SRF-3'UTR | TGTGGGAGATTCTGGGATTGC | 956 | 62 |
| | GAGGAGTGAAACAGGAGCAGAGA | | |



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**Figure legends**

**Figure 1. mmu-miR-483-3p expression in mouse tissues and during development**

(A) The tissue distribution of mmu-miR-483-3p was assessed by real-time PCR. The values of mmu-miR-483-3p in the small intestine were normalized to 1.

(B) Expression of mmu-miR-483-3p during C2C12 myoblast differentiation. RNA of C2C12 myoblasts cultured in growth medium (0 d) and differentiation medium for (1 d), (2 d) or (4 d) was isolated, and mmu-miR-483-3p expression was analyzed by real-time PCR. The value of mmu-miR-483-3p expression at 0 d was set to 1.

(C) The developmental profile of mmu-miR-483-3p expression in leg muscle during the postnatal period. Expression of mmu-miR-483-3p at adulthood was set to 1.

Error bars indicate the SD (n = 3) of relative expression levels of mmu-miR-483-3p to U6, determined by real-time quantitative PCR. Results are averaged from 3 independent experiments.

**Figure 2. mmu-miR-483-3p promotes C2C12 cell proliferation and suppresses C2C12 myogenic differentiation**

(A) Proliferation curves of cells transfected with miR-483-3p or negative control (NC). Cell proliferation was monitored in real-time by an xCELLigence system after seeding in a special plate. Twenty-four hours after transfection, the cell index of cells treated with miR-483-3p mimics was higher than that of cells transfected with NC. Error bars indicate standard deviation from 6 independent experiments.

(B) The effect of miR-483-3p mimics on the growth of C2C12 cells. The value of relative absorbance indicated the numbers of living cells. Data are represented as the mean ± SD of duplicate samples. Similar results were obtained from 3 independent experiments. (** $p <$ 0.01)

(C) (D) Expression of C2C12 differentiation marker genes was significantly repressed by miR-483-3p. Expression of myogenin (C) and Myh1 (D) genes in transfected cells were analyzed by real-time PCR. Bars represent the mean ± SD (n = 3). The values were normalized to endogenous tubulin mRNA and the value of 24-h transfected miR-483-3p



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mimics was set to 1. (* $p < 0.05$)

Figure 3. Immunofluorescence images of Myh1/2/3 (myosin heavy chains encoded by Myh1, 2 and 3) distribution in C2C12 cells

C2C12 cells transfected with miR-483-3p (a, c) and NC (b, d) were randomly selected for imaging (original magnification 40× (A), 100× (B)).

(C) The fluorescence ratio in the picture (A) was calculated by Photoshop 7.0. Data are represented as the mean ± SD of duplicate samples. Similar results were obtained from 3 independent experiments. (** $p < 0.01$)

**Figure 4. miR-483–3p directly targets SRF gene.**

(A) Putative binding site of miR-483-3p in SRF 3′-UTR (TargetScan Database). The mutated sites are indicated in underlined italics.

(B) Wild-type (psi-SRF-3′-UTR) and mutated (psi-SRF-3′-UTR-mut) 3′-UTR sequences of SRF cloned downstream of the Renilla luciferase gene in psiCheck2 were transfected into BHK-21 cells in the presence of NC or miR-483-3p mimics. miR-483-3p mimics downregulated the activity of the psi-SRF-3′-UTR reporter. Mutation in the 3′-UTR of SRF abolished this effect. The Rluc/Luc ratio was normalized to 1 in the NC group. Data are means ± SD from 3 independent experiments. (** $p< 0.01$)

(C) Western blotting of SRF protein in C2C12 cells transfected with mmu-miR-483-3p or mmu-miR-483-3p inhibitor and controls. Similar results were obtained in 3 independent experiments.



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Figure 1.

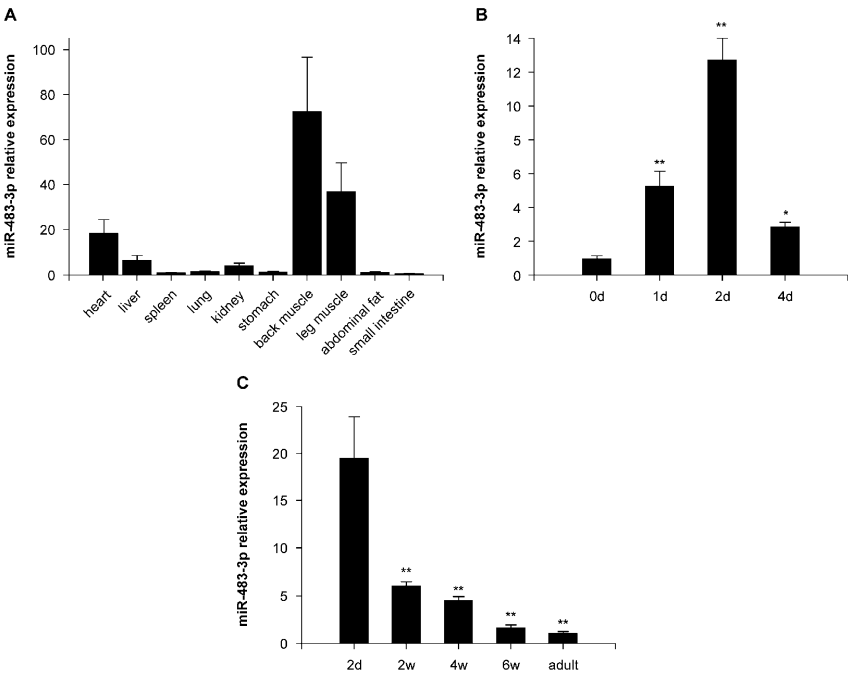


Figure 2

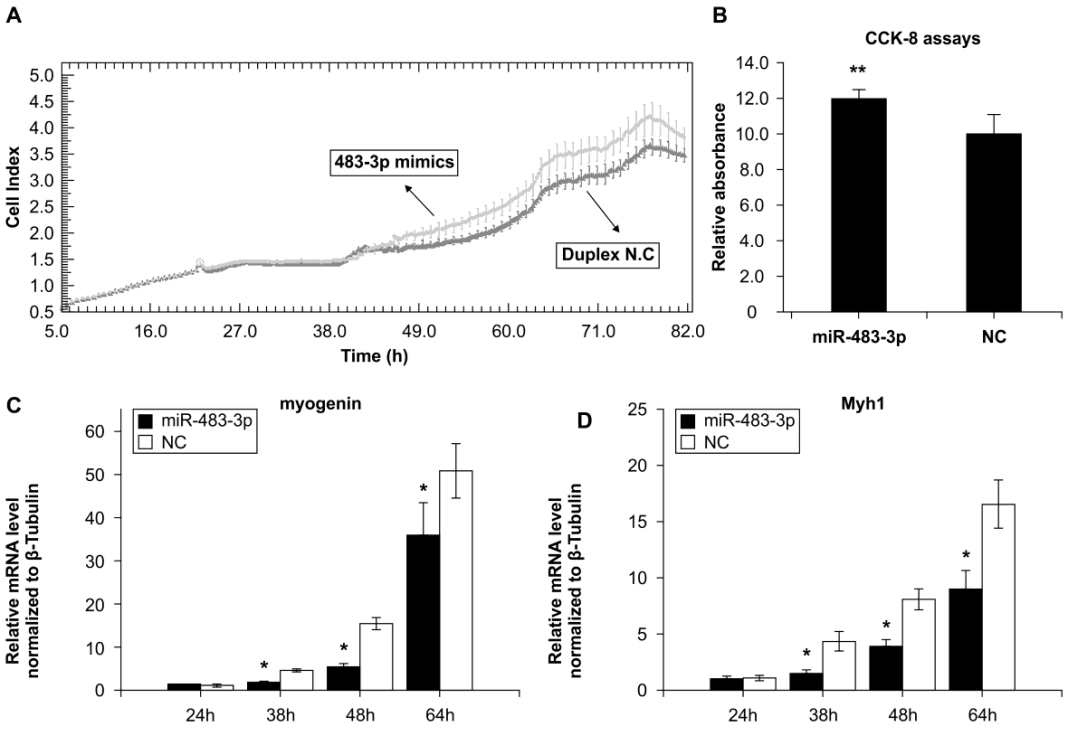




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Figure 3

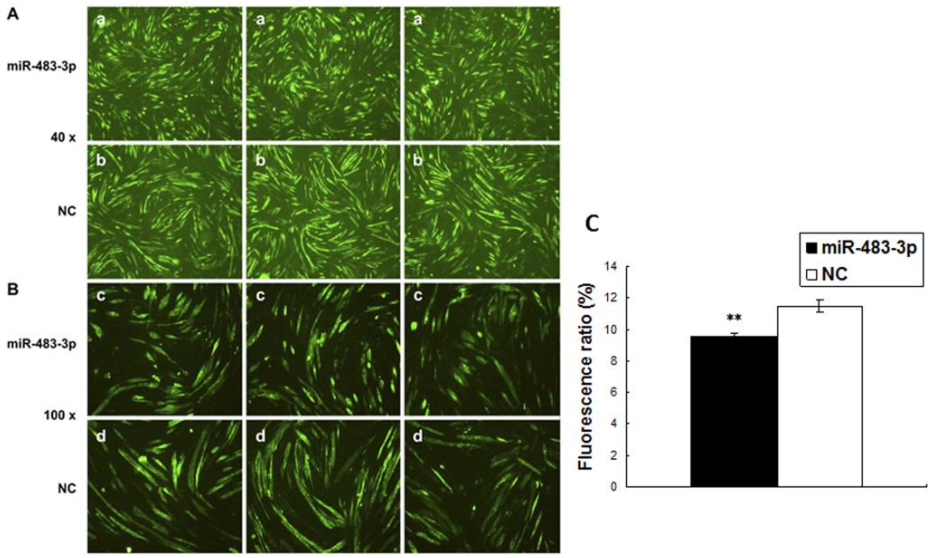
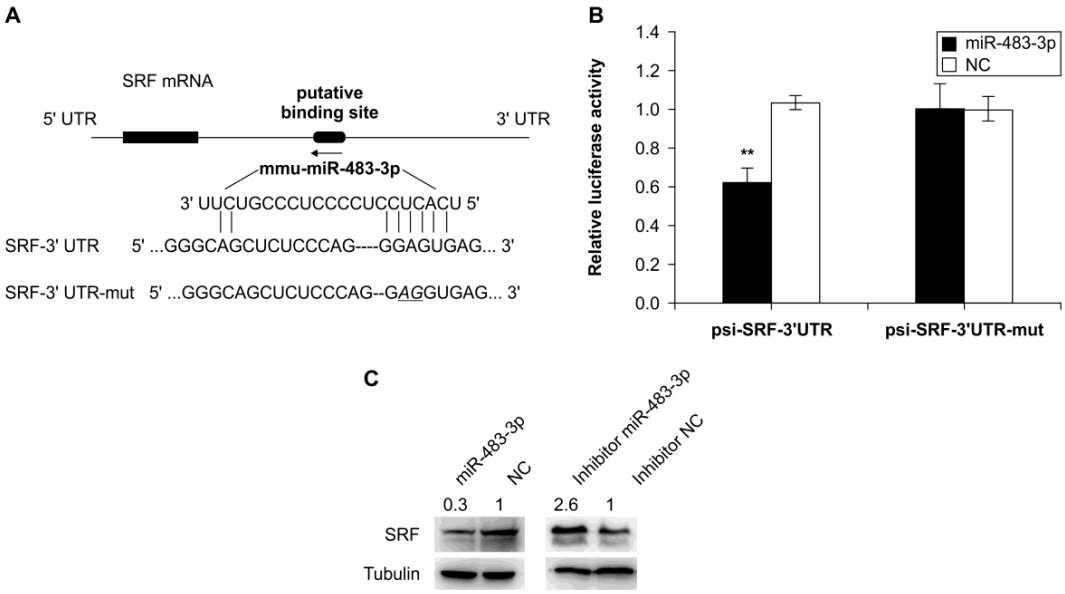



Figure 4



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