Original Paper

MiR-27b Promotes Muscle Development by Inhibiting MDFI Expression

Lianjie Hou^a Jian Xu^a Yiren Jiao^a Huaqin Li^a Zhicheng Pan^a Junli Duan^a
Ting Gu^a Chingyuan Hu^b Chong Wang^a

^aNational Engineering Research Center for Breeding Swine Industry, Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou, PRC, ^bDepartment of Human Nutrition, Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, USA

Key Words

MiR-27b • MDFI • Skeletal muscle • Porcine • Satellite cells

Abstract

Background/Aims: Skeletal muscle plays an essential role in the body movement. However, injuries to the skeletal muscle are common. Lifelong maintenance of skeletal muscle function largely depends on preserving the regenerative capacity of muscle. Muscle satellite cells proliferation, differentiation, and myoblast fusion play an important role in muscle regeneration after injury. Therefore, understanding of the mechanisms associated with muscle development during muscle regeneration is essential for devising the alternative treatments for muscle injury in the future. **Methods:** Edu staining, qRT-PCR and western blot were used to evaluate the miR-27b effects on pig muscle satellite cells (PSCs) proliferation and differentiation *in vitro*. Then, we used bioinformatics analysis and dual-luciferase reporter assay to predict and confirm the miR-27b target gene. Finally, we elucidate the target gene function on muscle development *in vitro* and *in vivo* through Edu staining, qRT-PCR, western blot, H&E staining and morphological observation. Result: miR-27b inhibits PSCs proliferation and promotes PSCs differentiation. And the miR-27b target gene, MDFI, promotes PSCs proliferation and inhibits PSCs differentiation *in vitro*. Furthermore, interfering *MDFI* expression promotes mice muscle regeneration after injury. **Conclusion:** our results conclude that miR-27b promotes PSCs myogenesis by targeting *MDFI*. These results expand our understanding of muscle development mechanism in which miRNAs and genes work collaboratively in regulating skeletal muscle development. Furthermore, this finding has implications for obtaining the alternative treatments for patients with the muscle injury.

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Introduction

Skeletal muscle comprises about 45% of the human body mass, and the musculoskeletal system injuries are common. Mechanical muscle damage often accompanied by muscle swelling, atrophy, contracture, and even functional disorders [1, 2]. Understanding of the

Chong Wang

College of Animal Science, South China Agricultural University
Guangzhou, Guangdong 510642 (China)
Tel. 86-20-85285031, Fax 86-20-85280740, E-Mail betty@scau.edu.cn

mechanisms involved in muscle development is critical in identifying treatments for these patients in the future.

Muscle satellite cells, also known as muscle stem cells, reside as quiescent cells beneath the basal lamina that surrounds muscle fibers, and function as myogenic precursors for muscle growth and repair [3, 4]. Satellite cell culture is a useful tool, and it has been used extensively to study muscle development *in vitro* [5]. Many myogenic markers during muscle development were identified in the past several decades. For example, *p21* and *cyclins* are well-established proliferation markers, and *MyoD*, *MyHC*, and *myosin* are the differentiation markers [6-8].

MyoD is one of the earliest markers of myogenesis [9]. High *MyoD* expression in muscle satellite cells represses cell renewal and promotes terminal differentiation [10]. *MyoD* family inhibitor (*MDFI*) functions as a negative regulator of muscle growth by inhibiting muscle hypertrophy [11, 12]. Previous studies on *I-mfa* (inhibitor of *MyoD* family a), the mouse *MDFI* homolog, showed that *I-mfa* interferes the myogenic process by masking the myogenic factor nuclear localization signal and by preventing transcription factors binding to DNA [11]. *I-mfa* knockout mouse showed defects in the formation of vertebrae and ribs, which suggests *I-mfa* also involves in cartilage formation in these tissues [13]. Miyamoto et al. reported that β -catenin interacted with *I-mfa* and that the interaction was enhanced by Wnt3a. They also found that the interaction between β -catenin and *I-mfa* ameliorates the inhibition of *I-mfa* on myogenesis [12]. As far as we know, there is no published data on the effects of miRNA regulating *MDFI* expression in the muscle regeneration process.

MicroRNAs (miRNAs) is small non-coding RNA molecules (containing ~22 nucleotides) found in plants, animals, and some viruses [14]. miRNAs exert their functions in RNA silencing by binding to complementary sequences of its target RNA [15, 16]. For example, in mice, miR-1 and miR-133 are clustered on the same chromosomal loci and transcribed together in a tissue-specific manner during development, and then miR-133 enhances proliferation by targeting serum response factor 3' UTR, whereas miR-1 promotes myogenesis through targeting histone deacetylase 4 [17]. MiR-27b was differently expressed in the same muscle of different pig breeds, the different muscles of a pig, and the different development period of the muscle in the miRNA microarray data [18]. These results suggest miR-27b might play a critical role in pig muscle development. Crist et al. reported that miR-27b binds directly to *Pax3* 3'-UTR; miR-27b delayed satellite cells differentiation and down-regulated the satellite cells entry into the myogenic differentiation program [19]. MiR-27b has also been shown to repress expression of *myostatin*, a myogenic inhibitor, leading to double-muscling phenotype in cattle [20]. We know that *MDFI* is a functional gene and it inhibits myogenesis, and miR-27b promotes muscle development through targeting functional genes. However, there has been no report on whether *MDFI* is the target gene of miR-27b. Therefore, the objective of this study is to explore whether there is a regulatory relationship between miR-27b and *MDFI* during muscle development.

Materials and Methods

All animals used in this study were with the approval of the College of Animal Science, South China Agricultural University. All experiments were conducted following 'the instructive notions with respect to caring for laboratory animals' issued by the Ministry of Science and Technology of the People's Republic of China.

Isolation and culture of porcine muscle satellite cells

Three one-day-old male Landrace piglets (approximately 1.5 kg) were anesthetized using sodium pentobarbital, and muscles from legs were used for PSCs isolation. The method used for the PSCs isolation has been described previously [5, 8]. Briefly, the hind deltoid muscles were dissected and rinsed with phosphate buffered saline (PBS) containing 0.5% Penicillin-Streptomycin Solution (GIBCO) to remove blood vessels and other connective tissues. The muscle tissues were sliced into 1 mm³ pieces and digested for 2 h in PBS containing 0.1% collagenase II (GIBCO, Grand Island, NY, USA). Then the mixture was passed

through a 100µm cell strainer to remove large tissue residues. The flow-through mixture was washed three times with PBS and passed through a 70 µm cell strainer. The flow-through cells were plated into 75 cm² flasks containing DMEM/F12 medium with 20% fetal bovine serum (FBS, GIBCO) and 0.5% chicken embryo extract. Three hours later, the unattached cells were transferred to another new flask. On the next day, cells in the flasks were treated with 0.25% trypsin for 5 min, washed with PBS and re-plated in a new flask. After 60 to 90 min the upper suspension was transferred to a new flask to collect satellite cells. Cell purity was examined by PAX7 immunofluorescence assay (Fig. 9). The purified satellite cells were then plated into 6-well cell culture plate at a density of 2.5×10^4 cells/cm². The PSCs were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C under a humidified 5% CO₂ atmosphere. When the PSCs reached approximately 95% confluency, cells were allowed to differentiate in DMEM/F12 with 2% horse serum, 100 U/mL penicillin, and 100 µg/ml streptomycin (GIBCO BRL) for 7 days. After differentiation, the cells were washed twice with cold phosphate-buffered saline (PBS) before protein extraction or total RNA extraction.

PSCs transfection

PSCs were seeded in 6-well plates at the density of 2×10^5 cells per well. PSCs transfected with pCDNA3.1-MDFI, siRNA-MDFI, miR-27b inhibitor, miR-27b mimics or control by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. We used qRT-PCR to detect transfection efficiency after each transfection. We transfected the PSCs with miR-27b mimic or inhibitor when the cells reach approximately 60% confluency. From previous studies conducted in our laboratory, the miR-27b mimic or inhibitor could remain active for 10-14 days. The medium was replaced with new growth medium 6 hours later, and cells were maintained in the growth medium for an additional 12 h before myogenic differentiation induction. MiR-27b mimics and inhibitor were purchased from GENEWIZ (Suzhou, China), and the control is the scramble sequence negative control provided by GENEWIZ.

RNA extraction and PCR analysis

Methods used for the RNA extraction and PCR analysis have been described previously [21]. In brief, the thermal cycling was performed as follows: 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The relative expression of mRNAs and microRNA were normalized with *β-actin* or *U6* levels using the $\Delta\Delta C_t$ method [22]. *U6* is a widely used normalizer in the miRNA studies [23, 24]; many previous miRNA-27b studies also used *U6* as the normalizer [25, 26]. $\Delta\Delta C_t$ is defined as the ratio of the relative mRNA level of the target gene between the experimental group and the control group. Primers were designed using Primer Premier 5 according to the pig genes sequence obtained from NCBI. Primers used for PCR are shown in Table 1.

Edu labeling

The transfected PSCs were incubated in the growth medium for twenty-four hours and then were used for Edu labeling by Cell-Light™ Edu Apollo®488 *In vitro* Imaging Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The Edu labeled PSCs were washed with PBS, then observed and recorded using a Nikon TE2000-U inverted microscope (Nikon Instruments, Tokyo, Japan). The Edu positive PSCs were counted using Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD, USA).

Western blot analysis

The method used for the Western blot analysis has been described previously [21]. In brief, homogenized tissues or

Table 1. Primers Used for PCR Analyses

Gene (Pig)	Forward sequence (5'-3')	Reverse sequence (5'-3')
MDFI	CCTGGACAGCACTGACCTCG	TGGTACTGCCCTTGCTCTTCT
MyoD	TGCGTATTCTCAACCCCTTC	AGTATGCAAGGGTGGAGTGG
Myogenin	AGGCTACGAGCGGACTGA	GCAGGGTGCTCCTCTTCA
Myosin	AGACGAGGAAGAGCACG	CTCAATGTAGCCGATGG
MyHC	AAGGCATCATCAAGGACACTC	TGCGGCAGGTTGGCTCT
CCNB	CGGGATCCATGGCGCTC	CCGCTCGAGTTACACCT
CCND	TGTTTGCAAGCAGGACTTTG	ACGTCAGCTCCACACTCTT
CCNE	TGGGCTTACGACGATGATG	ACGGAACCTGCTGACGGCTGT
P21	AGGACCATGTGGACCTGTTG	CGGCGTTTGGAGTGGTAGAA
PCNA	TTCGGTGCCTTCTCTCC	CCTTCCTTTCGTGAGC
β-actin	ACGGGACATCAAGGAGAAGC	GGCACTGTGTTGGCGTAGAG
Gene (Mice)	Forward sequence (5'-3')	Reverse sequence (5'-3')
MDFI	GAGCGGTCAAGTCCCTTCT	CAGGGTGAGTGGATCTTGCTAC
MyoD	CGCCTGAGCAAAGTGAATGAG	GCGGTCCAGGTGCGTAGAA
Myogenin	CCGTGGGCATGTAAGGTG	AGGCGCTCAATGTACTGGAT
CCND	CGGCAGTCAAGGGAATGGT	TGAGGAGCAGAAGTGCAGAAG
P21	CGAGTTCACCGTCAGCA	TCTTCAAGACAACCCATCA
β-actin	TGCTGTCCCTGTATGCCTCT	CTTTGATGTACGCACGATTT

cells were lysed in RIPA buffer containing protease inhibitors (1 μ M PMSF). Thirty-microgram protein lysates were separated with SDS-PAGE and then electroblotted to polyvinylidene fluoride. Electrophoresis supplies were purchased from Bio-Rad (California, USA). The membranes were blocked with 6% skim milk buffer for 2 h at room temperature and incubated with different antibodies overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The protein quantification was using Image J. The antibodies used in this study are listed in Table 2.

Table 2. Antibody validation profile

Primary antibody	Clone	Company	Catalog No.	Dilution
CCNB	Monoclonal	abcam	ab212977	1:2000
CCND	Polyclonal	Bioss	bs-0623R	1:700
CCNE	Polyclonal	abcam	ab33911	1:2000
MDFI	Monoclonal	Bioss	bs-18743R	1:600
MyoD	Monoclonal	abcam	ab16148	1:2000
Myogenin	Monoclonal	abcam	ab1835	1:2000
Myosin	Monoclonal	CST	#8824	1:3000
β -Actin	Monoclonal	Bioss	bsm-33036M	1:1000
Secondary antibody	Conjugate Used	Company	Catalog No.	Dilution
Goat Anti-mouse IgG	HRP	Bioss	bs-0296G	1:3000
Goat Anti-rabbit IgG	HRP	Bioss	bs-0295G	1:3000

Target gene prediction

The target gene prediction was conducted using the software mirTargets 1.2 in conjunction with TargetScan, MicroCosm, Pictar and miRDB databases.

Luciferase Reporter Assay

MDFI 3' UTR sequence and *MDFI*-3'UTR-mut sequence were amplified and inserted into pmirGLO Vector (Ambion, Carlsbad, CA, USA), respectively. For the luciferase reporter assay, HEK 293T cells were co-transfected with pmirGLO-*MDFI*-3'UTR plus either miR-27b mimics or Control for 48 hours. Either pmirGLO or pmirGLO-*MDFI*-3'UTR-mut was used as a control for pmirGLO-*MDFI*-3'UTR. The activities of firefly and Renilla luciferases were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), and firefly luciferase activity was normalized to that of Renilla luciferase.

The *MDFI* 3'UTR sequence, the marked sequence is the seed sequence

The wild-type *MDFI* 3' UTR:

CCCCACCCCTGGGCCC	CCCCCACCCAGGGCCCTCCC	GGACCCAGCCAGTGACAG
GGGTTGGGGGGTGCTGTCC	TCCTCGCTGGTTTCCAAGGAC	AACAGCACGGAGGACCTGGG
CAGGGGTGGGATTGGAGGAC	CAGTCCTGGATCAGCTGCCA	GGGCTGCGGAACACTGTGAA
AGTTGGGGGGGGCGGGGCAGG	GACACGGGGGGAGGCTTGGA	CGAGGGGCTGTGTCTACCTCT
CACTGCCCCCTGTGCCTGCCTC	CCATCACCCCCACCACCACA	CCAGGCTTAGAGGACAGCAA
AATGTGAAAAGAGACACCCC	ACCCGACCTCAGCCAA	GCACCTCCCCAGGCTCCTCCC
TGACCCGACTGGGACCCAGA	GGGCGGGGCTGGGCTTGGGCT	CGGGACAGGGTGGGATGGGAT
GGGGTGGGGTGGGTGGGAACC	CGTATAGAAAAGACTGGAGGA	GTTCCCATCGTGGCGCAGCGG
AAACGAACCCAACTAGTACCC	ACCCGTGAGGATGTGGGTTTCG	ATCCTTGGCCTTGCTCAGTGGG
TTAAGGATCTGGCATTGCTGT	GGCTGTGGTGCAGGCCGGCA	GCTGCCGCTCTGATTCAACCC
CTAGCCTGGGAACCTTCCATATG	CTGCAAAATGCAGCCCTATAAAA	AAAGCAAAAAAAAAAAAAAAAAA
AAAAAACTGGAAGGGGAGAGGG	AGGGAATGACTGGGGGGAGGGT	CTGCTCTACTTGTGTGTGTA
TAAAGATAAGTGTCATCTCA		

The mutated *MDFI* 3' UTR:

CCCCACCCCTGGGCCCC	CCCCACCCAGGGCCCTCCCGAC	CCCAGCCAGTGACAGGGGTTGG
GGGGTGCCTGTCTCTCGCTGG	TTTCCAAGGACAACAGCACGGAG	GACCTGGGCAGGGGTGGGATTGG
AGGACCAGTCTGGATCAGCTGCC	AGGGCTGCGGAACACTAGGAAAGT	TGGGGGGGCGGGGAGGGACACGG
GGGGAGGCTTGGACGAGGGGCTGT	GTCTACCTCTCACTGCCCCCTGTGCC	TGCCCTCCATCACCCACCACCAC
CACCAGGCTTAGAGGACAGCAAAA	TGTGAAAAGAGACACCCACCCGAC	CTCAGCCAAGCACCTCCCCAGGCTC
CTCCCTGACCCGACTGGGACCCAG	AGGGCGGGGCTGGGCTTGGGCTCGG	GACAGGGTGGGATGGGATGGGGTG
GGGTGGGTGGGAACCCGTATAGAA	AAGACTGGAGGAGTTCCATCGTGG	CGCAGCGGAAACGAACCCAACTAGT
ACCCACCCGTGAGGATGTGGGTTCG	ATCCTTGGCCTTGCTCAGTGGGTTA	AGGATCTGGCATTGCTGTGGCTGTG
GTGCAGGCCGGCAGCTGCCGCTCT	GATTCAACCCCTAGCCTGGGAACT	TCCATATGCTGCAAAATGCAGCCCT
ATAAAAAAGCAAAAAAAAAAAAAA	AAAAAAAAAACTGGAAGGGGAG	AGGGAGGGAATGACTGGGGGGAGG
GTCTGCTCTACTTGTGTGTGTA	TAAAGATAAGTGTCATCTCA	

Tissue sampling and H&E

Mice were purchased from Guangdong Medical Lab Animal Center. Lentivirus containing siRNA-*MDFI* or siRNA-Control were purchased from Shanghai JiKai Gene Chemical Technology Co., LTD. Twelve mice were divided into two groups with six mice in each group. Mice were injected with LV- siRNA-Control or LV- siRNA-*MDFI*. Gastrocnemius muscles were dissected from each mouse one week after injection for extracting total RNA and proteins. Mice gastrocnemius muscles were stained with Hematoxylin and Eosin (H&E), and the cross-section area of individual myofibers was photographed using Nikon TE2000-U inverted microscope (Nikon Corporation, Tokyo, Japan) and measured using Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (S.E.M.), and at least three independent individuals or replicates were used per group. The assumptions of normality of data and homogeneity of variances between the groups was analyzed by SPSS. Significant differences between treatment groups were determined by one-way ANOVA (SPSS 18.0, Chicago, IL, USA). Significance was achieved when $p < 0.05$. * is $p < 0.05$ and ** is $p < 0.01$.

Results

MiR-27b inhibits PSCs proliferation

MiR-27b mimics and inhibitor were transfected into PSCs for 36h. MiR-27b mimics reduced ($p < 0.05$) the percentage of Edu positive cells and miR-27b inhibitor increased ($p < 0.05$) the percentage of Edu positive cells (Fig. 1A, 1B). The qRT-PCR result showed miR-27b mimics decreased ($p < 0.05$) mRNA level of *CCNB*, *CCND*, *CCNE*, and *PCNA* and increased ($p < 0.05$) *p21* mRNA level (Fig. 1C). Western blot confirmed that miR-27b mimics reduced *CCNB* ($p = 0.062$), *CCND* ($p < 0.05$), and *CCNE* ($p < 0.05$) protein level, and increased *p21* ($p < 0.01$) protein level (Fig. 1D). Consistent with these results, after transfection with miR-27b inhibitor, mRNA level of *CCNB*, *CCND*, *CCNE*, and *PCNA* were increased ($p < 0.05$) and *p21* mRNA level was decreased ($p < 0.01$, Fig. 1C). Western blot confirmed that miR-27b inhibitor increased *CCNB* ($p < 0.01$), *CCND* ($p < 0.01$), and *CCNE* ($p < 0.05$) protein level, and decreased *p21* ($p < 0.01$) protein level (Fig. 1D). The relative protein levels obtained using WB bands gray scanning were presented in Fig. 8A. These results show miR-27b inhibits PSCs proliferation.

MiR-27b promotes PSCs differentiation

PSCs were transfected with miR-27b mimics or inhibitor and changed to the differentiation medium for seven days. We measured the differentiation of PSCs by the anti-MyHC immunofluorescent assay. Overexpressing miR-27b increased the MyHC positive cells while inhibiting miR-27b lowered the MyHC positive cells (Fig. 2A). Overexpression of miR-27b elevated the mRNA level of *MyoD* ($p < 0.05$), *Myogenin* ($p < 0.05$), *Myosin* ($p < 0.05$) and *MyHC* ($p < 0.01$) (Fig. 2B). Western blot result showed overexpression of miR-27b also elevated protein level of *MyoD* ($p < 0.05$), *Myogenin* ($p < 0.01$), and *Myosin* ($p < 0.05$) (Fig. 2C). The relative protein levels obtained using WB bands gray scanning were presented in Fig. 8B. Consistent with these results, miR-27b inhibitor decreased the myogenic-related gene expression (Fig. 2B, 2C). These results show miR-27b promotes PSCs differentiation.

MDFI is a direct target gene of miR-27b

To further investigate the potential mechanism by which miR-27b regulates the PSCs myogenesis, we need to identify the target gene of miR-27b. Through the sequence alignment we found that the miR-27b seed sequence is highly conserved (Fig. 3A), the miR-27b complementary seed sequence in the *MDFI* 3'UTR is also highly conserved (Fig. 3B), so there may be a potential binding site in *MDFI* UTR for miR-27b (Fig. 3C).

To verify the targeted relationship between miR-27b and *MDFI*, we first measured the expression of *MDFI* through qRT-PCR and Western blot in the PSCs transfected with miR-27b

Fig. 1. MiR-27b inhibits PSCs proliferation. A. MiR-27b mimics lowered the percentage of Edu positive PSCs, and miR-27b inhibitor increased the percentage of Edu positive PSCs. Representative images of the immunofluorescent staining for proliferating PSCs are shown. Proliferating PSCs were labeled with Edu fluorescent dye (red). B. The quantitative data of proliferating PSCs in Fig. A. C. qRT-PCR confirmed miR-27b is negatively correlated with proliferation-related genes in PSCs transfected with miR-27b mimics and inhibitor. D. Western blot result showed the protein level corresponds to the mRNA result. *, $p < 0.05$; **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 200 $\times$. The scale bar on the photomicrographs represents 50 μ m.

Fig. 2. MiR-27b promotes PSCs differentiation. We measured the expression of myogenic marker genes after PSCs were transfected with miR-27b mimics or inhibitor. A. MiR-27b mimics increased the percentage of MyHC positive PSCs, and miR-27b inhibitor decreased the percentage of MyHC positive PSCs. Representative images of the immunofluorescent staining for differentiated PSCs are shown. MyHC: red, a molecular marker of myogenesis; DAPI: blue, cell nuclei; Merge: the PSCs fused into primary myotubes are shown in the insets. B. The qRT-PCR confirmed miR-27b increased ($p < 0.05$) mRNA level of myogenic marker genes after differentiation induction. C. We used western blot to measure protein level of myogenic marker genes after differentiation induction. Western blot showed the protein result corresponds to the mRNA result after differentiation induction. *, $p < 0.05$; **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 200 $\times$. The scale bar on the photomicrographs represents 50 μ m.

mimics or inhibitor. The results showed overexpressing miR-27b decreased MDFI expression while inhibiting miR-27b increased the *MDFI* expression (Fig. 3D and 3E). The MDFI protein levels obtained using WB bands gray scanning were presented in Fig. 8C. Subsequently, we constructed the pmirGLO-MDFI 3'UTR recombinant vector containing the binding site of miR-27b. We found relative luciferase activity was decreased ($p < 0.01$; Fig. 3F) when HEK-293T cells were co-transfected with miR-27b mimics and pmirGLO-MDFI-3'UTR. However, miR-27b mimics does not affect mutated pmirGLO-MDFI-3'UTR relative luciferase activity (Fig. 3F). This result indicates that *MDFI* is the direct target gene of miR-27b.

MDFI promotes PSCs proliferation

Since *MDFI* is the direct target gene of miR-27b, we then explored the role of *MDFI* in PSCs development. We overexpressed and inhibited *MDFI* expression in transfected PSCs with pcDNA3.1(+)-*MDFI* recombinant vector and siRNA-*MDFI*, respectively. Overexpressing *MDFI*

Fig. 3. MDFI is a direct target gene of miR-27b. A. The sequence of mature miR-27b of different species. B. The alignment result of MDFI 3'UTR sequence from different species. Red: the complete complementary sequence of the MDFI 3'UTR. C. The complementary pairing of ssc-miR-27b with the targeted gene MDFI 3'UTR. D. MDFI protein level was negatively correlated with miR-27b level. E. qRT-PCR result confirmed MDFI mRNA level was negatively correlated with miR-27b level. F. Luciferase reporters were transfected into HEK-293T cells with either miR-27b mimics or control, respectively. Luciferase activity was determined 24 h after transfection. **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group.

increased ($p < 0.01$) the percentage of Edu positive cells and siRNA-MDFI decreased ($p < 0.05$) the percentage of Edu positive cells (Fig. 4A, 4B). The qRT-PCR result showed pcDNA3.1(+)-MDFI recombinant vector increased the mRNA level of *CCNB* ($p < 0.05$), *CCND* ($p < 0.01$), *CCNE* ($p < 0.01$) and *PCNA* ($p < 0.01$) and decreased ($p < 0.05$) *p21* mRNA level (Fig. 4C). Western blot result confirmed that pcDNA3.1(+)-MDFI recombinant vector increased protein level of *CCNB* ($p < 0.05$), *CCND* ($p < 0.05$), and *CCNE* ($p < 0.05$), and decreased *p21* ($p < 0.01$) protein level (Fig. 4D). After PSCs were transfected with siRNA-MDFI, we found siRNA-MDFI decreased ($p < 0.05$) the mRNA level of *CCNB*, *CCND*, *CCNE*, and *PCNA* and increased ($p < 0.01$) *p21* mRNA level (Fig. 4C). Western blot result confirmed that siRNA-MDFI decreased protein level of *CCNB* ($p < 0.05$), *CCND* ($p = 0.062$), and *CCNE* ($p < 0.05$) and increased *p21* ($p < 0.01$) protein level (Fig. 4D). The relative protein levels obtained using WB bands gray scanning were presented in Fig. 8D. From the MDFI overexpression and inhibition results, we conclude that MDFI promotes PSCs proliferation.

MDFI inhibits PSCs differentiation

We then determined the role of MDFI in PSCs differentiation. PSCs were transfected with either pcDNA3.1(+)-MDFI recombinant vector or siRNA-MDFI and then cultured in the differentiation medium for seven days. We used the anti-MyHC immunofluorescent assay to measure the differentiation of PSCs. Overexpressing MDFI decreased the MyHC positive cells while inhibiting MDFI increased the MyHC positive cells (Fig. 5A). Then the expression of *MyoD*, *Myogenin*, *Myosin*, and *MyHC* was measured. Overexpression of MDFI lowered the mRNA level of *MyoD* ($p < 0.05$), *Myogenin* ($p < 0.05$), *Myosin* ($p < 0.05$) and *MyHC* ($p < 0.01$) (Fig. 5B). Western blot result showed overexpression of MDFI lowered the protein level of *MyoD* ($p < 0.01$), *Myogenin* ($p < 0.05$), and *Myosin* ($p < 0.01$) (Fig. 5C). The relative protein levels obtained using WB bands gray scanning were presented in Fig. 8E. Consistent with these results, inhibiting MDFI increased the myogenic-related gene expression (Fig. 2B, 2C). These results show MDFI inhibits PSCs differentiation.

MiR-27b promotes PSCs myogenesis by targeting MDFI.

Since miR-27b inhibits PSCs proliferation, promotes PSCs differentiation and MDFI is a direct target gene of miR-27b, we co-overexpressed miR-27b and MDFI to verify whether MDFI attenuated the miR-27b effect on PSCs development. Through the Edu immunofluorescent staining, we found MDFI weakens the miR-27b inhibition effect on PSCs proliferation. The MyHC immunofluorescent staining results shown MDFI reduced the miR-27b promotion

Fig. 4. MDFI promotes PSCs proliferation. A. Overexpressing MDFI increased the percentage of Edu positive PSCs and interfering MDFI decreased the percentage of Edu positive PSCs. Representative images of the immunofluorescent staining for proliferating PSCs are shown. Proliferating PSCs were labeled with Edu fluorescent dye (red). B. The quantitative data of proliferating PSCs number in Fig. A. C. qRT-PCR confirmed MDFI is positively correlated with proliferation-related genes in PSCs transfected with pcDNA3.1(+)-MDFI recombinant vector and siRNA-MDFI respectively. D. Western blot result showed the protein level corresponds to the mRNA result. *, $p < 0.05$; **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 200 $\times$. The scale bar on the photomicrographs represents 50 μ m.

Fig. 5. MDFI inhibits PSCs differentiation. A. Overexpressing MDFI decreased the percentage of MyHC positive PSCs and interfering MDFI increased the percentage of Edu positive PSCs. Representative images of the immunofluorescent staining for differentiated PSCs are shown. MyHC: red, a molecular marker of myogenesis; DAPI: blue, cell nuclei; Merge: the PSCs fused into primary myotubes are shown in the insets. B. qRT-PCR result confirmed MDFI decreased the mRNA level of myogenic marker genes after differentiation induction. C. We used western blot to measure the protein level of myogenic marker genes after differentiation induction. Western blot result showed the protein level corresponds to the mRNA result after differentiation induction. *, $p < 0.05$; **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 200 $\times$. The scale bar on the photomicrographs represents 50 μ m.

effect on PSCs differentiation (Fig. 6A, 6B), so we conclude that miR-27b promotes PSCs myogenesis by targeting *MDFI*.

Interfering MDFI promotes the muscle development in vivo

The miRNA is known to exert their function through inhibiting the expression of their target genes. Results from our *in vitro* study have identified MDFI is the target gene of miR-27b. To our knowledge, there is no published report about the effects of MDFI on skeleton muscle regeneration after injury. Therefore, we decided to explore the role of MDFI in PSCs development and muscle regeneration after injury. We first damaged the gastrocnemius muscle of Kunming mouse by cardiotoxin (CTX) injection. Then we infected the muscle

Fig. 6. MiR-27b promotes PSCs myogenesis by targeting MDFI. A. MiR-27b function on PSCs proliferation and differentiation requires MDFI. Representative images of the immunofluorescent staining for proliferating PSCs and differentiated PSCs are shown. B. The quantitative data of proliferating PSCs number in Fig. A. *, $p < 0.05$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 200 $\times$. The scale bar on the photomicrographs represents 50 μ m.

Fig. 7. Interfering MDFI promotes the muscle development *in vivo*. A. H&E staining of hind limb muscle one week after injection with lentivirus packaged siRNA (LV-Si-MDFI). B. Total fibers number in one field after interfering hind limb muscle MDFI. C. The average area of muscle fiber cross-sections was increased after interfering MDFI. D. The average diameter of myofiber was increased after interfering MDFI. E. qRT-PCR detection of MDFI, the mRNA level of myogenic-related genes. F. Western blot determination of the protein level of myogenic-related genes. *, $p < 0.05$; **, $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. Magnification 20 $\times$. The scale bar on the photomicrographs represents 50 μ m.

with the lentivirus packaged siRNA LV-Si-MDFI to elucidate the *MDFI* effect on muscle regeneration. We harvested the gastrocnemius muscles one week after the injection and measured the morphological characteristics of gastrocnemius muscles and the expression of related genes. Inhibition of *MDFI* increased the diameter of single muscle fibers (Fig. 7A, 7C, 7D). But the total number of fibers in one field was no difference between the two groups (Fig. 7B). Inhibiting *MDFI* increased ($p < 0.01$) the mRNA level of *MyoD* and *Myogenin*, decreased the mRNA level of *MDFI* ($p < 0.01$) and *CCND1* ($p < 0.05$), Fig. 7E). Western blot result showed interfering *MDFI* lowered the protein level of *MDFI* ($p < 0.01$) and *CCND1* ($p < 0.01$), increased the protein level of *MyoD* ($p < 0.05$), and *P21* ($p < 0.01$) (Fig. 7F). The relative protein levels obtained using WB bands gray scanning were presented in Fig. 8F. These results demonstrate that inhibition of *MDFI* *in vivo* promoted the muscle regeneration after injury.

Discussion

Skeletal muscle comprises 45% of the human body mass, and musculoskeletal system injuries are common [27]. Muscle injuries in sports account for 10%–55% of all muscle injuries. Over 90% of these injuries are either contusions or strains [28, 29]. Traumatic injuries from motor vehicle accidents contribute to an increasing need for better treatment options for muscle regeneration [30]. After an injury, muscle satellite cells are activated, re-enter the cell cycle to proliferate and subsequently differentiate into myoblasts. The proliferation and differentiation of muscle satellite cells is the basis of muscle regeneration [31, 32]. Therefore, understanding of the mechanisms associated with muscle development is essential for devising the alternative treatments for muscle regeneration.

The development of muscle satellite cells is affected by many factors, including myogenic regulatory factors (such as *MyoD*, *Myf5*, *Myogenin* and *MRF4*), miRNAs, the muscle satellite cells microenvironment and age [33, 34]. Pig is a useful medical model for humans due to its similarity in size and physiology. In our study, we used pig muscle satellite cell as the model to explore the regulatory relationship between the *MDFI* and miR-27b *in vitro*. Since *in vitro* study may not represent the true physiological state in living organisms, we also conducted the *MDFI* in animals. The muscle regeneration model after Cardiotoxin damage has been used in mice [35, 36], we chose to conduct the *in vivo* study in mice. Since our laboratory has explored the effects of different concentrations of CTX on the degree of muscle damage in Kunming mice and has established a complete CTX damaging the mouse muscle protocol we chose this particular model for this study.

For any experiment, a negative control should be included to allow for control of parameters such as intra-individual variability, variability among animal strains, tools utilized. Since previous research conducted by our laboratory on miR-34c has shown the LV-Si-Control does not affect the muscle development [37], we did not include a negative control in this experiment. However, the difference between si-MDFI and control mice was significant, and there were six mice in each group, so we believe this result should still be valid.

MyoD, a myogenic transcription factor, has been extensively studied and it plays an essential role in muscle development. However, *MDFI*, the inhibitor of *MyoD*, we can find only three *MDFI*-related papers. The role of *MDFI* in regulating muscle development remains to be elucidated.

After activation, muscle satellite cells re-enter the cell cycle to proliferate. After several rounds of replication, muscle satellite cells exit the cell cycle and differentiate into myoblasts. Finally, the myoblasts fuse to the existing muscles. *P21* and cyclins are the key factors regulating the cell cycle. Since *CCND* is the first cyclin produced in the cell cycle, we measured *CCND* expression as the PSCs proliferation marker. *CCND* binds to the existing cyclin-dependent kinases (CDK) 4, forming the active *CCND*-CDK4 complex. Then, *CCND*-CDK4, in turn, phosphorylates the retinoblastoma susceptibility protein (Rb). Rb dissociates from the E2F/DP1/Rb complex, activating E2F. E2F promotes expression of various genes such as *CCNE* and *DNA polymerase*. Therefore, we also measured *CCNE* expression as the PSCs

Fig. 8. WB bands gray scanning quantitative results. A for Figure 1D; B for Figure 2C; C for Figure 3D; D for Figure 4D; E for Figure 5C; F for Figure 7F.

Fig. 9. The isolated satellite cells were identified by PAX7 immunofluorescence assay.

proliferation marker. CCNE binds to CDK2, forming the CCNE-CDK2 complex, which pushes the cell from G₁ to S phase. CCNB-CDK1 complex activation causes breakdown and prophase initiation. Subsequently, CCNB-CDK1 deactivation causes the cell to exit mitosis [6, 38, 39]. The P21 protein is a cell cycle regulator, and it binds to cyclin-CDKs complexes and inhibits cyclin-CDKs activities [7, 40]. Thus, *CCNB* and *P21* have also been used as the PSCs proliferation markers in our study. *MyoD* is also involved in removing cells from the cell cycle through increasing the p21 transcription. *MyoD* is inhibited by CDKs, CDKs, in turn, are inhibited by p21. Thus *MyoD* enhances its own activity in a feed-forward manner [41, 42]. In our study, through the mRNA, protein and cell number results, we established that *MyoD* expression is increased and *p21* expression is decreased by interfering *MDFI*, and finally, resulted in elevated PSCs proliferation. *MDFI* can identify the basic helix loop helix (bHLH) structure on the *MyoD* [11, 13]. Since many myogenic transcription factors contain the bHLH structure, future studies will include the identification of additional transcription factors regulated by the *MDFI*.

The miRNAs are critical regulators of many biological processes through modulating expression of genes in the post-transcriptional level. Proliferation and differentiation are mutually exclusive during muscle regeneration, and miRNAs are critically involved in balancing these two processes [43]. Many miRNAs, such as miR-1, miR-133, miR-29, miR-214, miR-206, miR-486, miR-208b, and miR-499 were involved in the regulation of skeletal myogenesis by binding to its target genes [44, 45]. For example, in mice, miR-1 and miR-133 are clustered on the same chromosomal loci and transcribed together in a tissue-specific manner during development, but miR-133 enhances proliferation by repressing serum response factor, whereas miR-1 promotes myogenesis through repressing histone deacetylase 4 [17]. Additionally, our previous study found miR-34c form a reciprocal regulatory loop with its target gene Notch 1 [37]. These reports support the claim miRNAs are critically involved in muscle regeneration.

miR-27b has been shown to inhibit cardiac cell growth and induce cardiac hypertrophy by targeting *PPAR γ* [46]. MiR-27b interference was reported to increase *Pax3* expression leading to muscle stem cell proliferation, delaying the onset of differentiation; miR-27b mimics ensures rapid and robust entry into the myogenic differentiation program [19]. In our study, through the mRNA, protein and cell count results, we ascertained that miR-27b decreased *MDFI* expression, which leads to inhibition of PSCs proliferation and promotion of PSCs differentiation. In conclusion, our study demonstrated that miR-27b inhibits PSCs proliferation and promotes PSCs differentiation *in vitro*. The mechanism is miR-27b inhibits the expression of its target gene, *MDFI*. Through interfering *MDFI* expression *in vivo*, our results showed that inhibiting *MDFI* expression promotes mice muscle regeneration after injury. These results expand our understanding of muscle development mechanism in which miRNAs and genes participate in controls skeletal muscle development. Furthermore, this finding has implications for obtaining the alternative treatments for muscle regeneration after injury in the future.

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Disclosure Statement

The authors declare to have no conflict of interests.

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