miR-760 regulates skeletal muscle proliferation in rheumatoid arthritis by targeting Myo18b

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Abstract. MicroRNAs serve an important role in the development of several diseases. Numerous genes regulate the skeletal muscle differentiation of C2C12 myoblasts. The role of miR-760 in rheumatoid arthritis (RA) has not been reported, to the best of our knowledge. Therefore, the aim of the present study was to examine the role of miR-760 in regulating skeletal muscle proliferation in RA. Potential genes functionally involved in the tarsal joint of a collagen-induced RA model were identified using Gene Expression Omnibus. Reverse transcription-quantitative PCR and western blot analyses were performed to determine the mRNA and protein expression levels. The proliferation, cell cycle progression and migration of C2C12 myoblasts were detected using Cell Counting Kit-8, flow cytometry and wound-healing assays, respectively. TargetScan was used to predict the potential target genes of miR-760, and this was verified using a dual-luciferase reporter assay. In the present study, myosin-18b (Myo18b) expression was determined to be downregulated in the RA model. Silencing Myo18b decreased the proliferation, abrogated the cell cycle progression, and reduced the migration and differentiation of C2C12 myoblasts. Expression levels of cyclin-dependent kinase 2, cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, myogenin and myosin heavy chain 6 were all decreased when Myo18b was silenced. Furthermore, overexpression of Myo18b induced opposing effects on C2C12 myoblasts. It was shown that Myo18b was a target gene of miRNA-760. Overexpression of miR-760 decreased proliferation, cell cycle progression, migration and differentiation in C2C12 myoblasts, and decreased the expression of Myo18b. The opposite results were observed when miR-760 was downregulated. In conclusion, miR-760 inhibited proliferation and differentiation by targeting Myo18b in C2C12 myoblasts. The results of the present study may contribute to understanding the mechanisms underlying RA skeletal muscle proliferation, and miR-760/Myo18b may serve as potential targets for treating patients with RA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory response in the synovial tissue of joints, which affects ~1% of population worldwide (1). However, the exact cause remains unclear (2). RA is characterized by a progressive erosion of the articular cartilage and chronic inflammation of the synovial joint (3). It has been reported that oxidative stress, inflammation and lipid peroxidation may affect the progression of RA (4). RA, which is also a common cause of permanent disability, leads to increased medical cost, use of social services and a reduction in the quality of life of patients (5), as the disease may lead to joint stiffness, swelling, pain and muscle wasting. A previous study indicated that muscle wasting was an important cause of RA-associated morbidity and mortality (6). However, the genes regulating the proliferation of skeletal muscle in RA require additional study.

MicroRNAs (miRNAs/miRs), are a type of non-coding RNA (21-24 nucleotides in length) that post-transcriptionally regulate gene expression (7). Aberrant expression of miRNAs has been demonstrated to be involved in several diseases, including various types of cancer, and rheumatic and other autoimmune diseases (8-10). Previous studies have reported that imbalances in miR-146a, miR-155, miR-223 and miR-16 levels existed in the immune cells of patients with RA (11-14). Lai *et al* found that decreased expression of miR-760 was affected by tumor necrosis factor-a in patients with RA (7). In addition, it has been reported that miR-760 regulated the proliferation and apoptosis of a variety of cells, such as human pulmonary artery smooth muscle cells, and human colon cancer and ovarian cancer cells, by targeting specific genes (15-17). However, there are few studies regarding the regulatory mechanisms via which miR-760 affects skeletal

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muscle proliferation in patients with RA, to the best of our knowledge.

Skeletal muscles are primarily composed of different types of fibers (18). Myoblasts are precursor cells, which are hypothesized to serve an important role in injured skeletal muscle (19). Satellite cells mobilize and proliferate as myoblasts when the muscle needs to be repaired and/or remodeled (20). Myosin-18b (Myo18b) is a myosin protein associated with human tumor progression, and loss-of-function mutations of Myo18b have been found in certain patients with nemaline myopathy (21). Notably, according to Berger *et al* (22), a number of myopathies are associated with molecular defects in sarcomeres, and a complete loss of Myo18b function leads to a complete lack of sarcomeric structure, suggesting that Myo18b may serve an important role in sarcomere assembly. In the present study, it was demonstrated that certain miRNAs were differentially expressed in the tarsus joint of a collagen-induced RA mouse model using the Gene Expression Omnibus (GEO) database.

Based on these previous results, factors affecting the proliferation of myoblasts were assessed. Mueller *et al* (23) hypothesized that chronic systemic inflammation may negatively affect myonuclei number and the regenerative potential of satellite cells; however, this hypothesis has not been confirmed. Therefore, additional factors may affect proliferation, differentiation and thus, muscle strength. In the present study, the targeting effects of miR-760 on Myo18b were determined, and it was hypothesized that miR-760 regulated skeletal muscle proliferation in RA by targeting Myo18b.

Materials and methods

Identification of differentially expressed genes. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used on the GEO dataset (24,25), GSE61140 (26), to obtain the gene expression profile in the tarsus joint of a collagen-induced RA mouse model.

Cell culture. Mouse C2C12 myoblasts were obtained from the American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO_2 at 37°C. The cells were passaged using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) when the confluence reached 50%. Subsequently, the cells were moved to a differentiation medium containing DMEM with 2% horse serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin when the cells reached 75-80% confluence, and then myogenic differentiation was induced via the addition of differentiation medium. The media was replaced every 24 h.

Cell transfection. A total of 1x10⁵ C2C12 myoblasts were cultured in 6-well plates, and divided into 4 groups. The first group consisted of: Control cells, untreated cells; negative control (NC) cells, cells transfected with NC siRNA (sense, CAUGUGGUCUGUCGCAUAAUA and antisense, CGGUAC ACCAGACAGCGUAUU); and small interfering (si)Myo18b cells, cells transfected with siMyo18b (sense, 5'-GAGCCA AAGAACAAAUAAAUU-3' and antisense, 3'-UUCUCG

GUUUCUUGUUUAUUU-5'). The second group consisted of: Control cells; mock cells, cells transfected with pcDNA3.1 (+) (GenomeDitech Co., Ltd.) only; and Myo18b cells, cells transfected with Myo18b-pcDNA3.1 (+) (GenomeDitech Co., Ltd.). The third group consisted of: NC cells; siMyo18b cells; mock cells and Myo18b cells. The fourth group consisted: Mimics control cells, cells transfected with mimics control (forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3'); miR-760 mimics cells, cells transfected with miR-760 mimics (forward, 5'-CGGCUC UGGGUCUGUGGGGA-3' and reverse, 5'-UCCCACAGA CCCAGAGCCG-3'); inhibitors control cells, cells transfected with inhibitors control (5'-CAGUACUUUUGUGUAGUA CAA-3'); miR-760 inhibitors cells, cells transfected with miR-760 inhibitors (5'-TCCCCACAGACCCAGAGCCG). The siMyo18b, NC siRNA, miR-760 mimics, miR-760 inhibitors, mimic control and inhibitor control were purchased from Shanghai GenePharma Co., Ltd.; transfection with siRNAs (50 nM), mimics (50 nM) and inhibitors (50 nM) transfection was performed using Lipofectamine® 2000 kit (Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were cultured in DMEM at 37°C with 5% CO_2 for another 48 h prior to subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. RT-qPCR analysis was performed to determine the expression levels of Myo18b, miR-760, cyclin-dependent kinase 2 (CDK2), cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, myogenin (MyOG) and myosin heavy chain IId/x (MyH6) in C2C12 myoblasts. To determine the expression of Myo18b during the myogenic differentiation of C2C12 myoblasts, cells were harvested after 0, 1, 3, 5 or 7 days. Relative expression levels of Myo18b, miR-760, CDK2, cyclin D1, MMP-2, MMP-9, MyOG and MyH6 in the C2C12 myoblasts were detected after 48 h of transfection with control, NC, siMyo18b, mock, Myo18b, mimics control, miR-760 mimics, inhibitor control and miR-760 inhibitor. Total RNA was extracted from C2C12 myoblasts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNAs were reverse transcribed to cDNAs using 1 μg RNA and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), the reverse transcription reaction conditions were set to 37°C for 30 min, and reverse transcriptase inactivation was conducted at 85°C for 5 min. Subsequently, the cDNA products were used for qPCR using a Light Cycler 480 RT-PCR (Roche Diagnostics) and SYBR Green Master mix (Roche Diagnostics) in a final volume of 20 μl/well. The thermocycling conditions for miR-760 were: 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. Relative expression of miR-760 was normalized to U6 expression levels. SYBR Green on the 7500 qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to determine the relative expression levels of Myo18b, CDK2, cyclin D1, MMP-2, MMP-9, MyOG and MyH6. The thermocycling conditions were as follows: Initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. GAPDH was used as an internal reference gene, and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (27). The sequences of the primers used are listed in Table I.

Table I. Sequences of primers used for quantitative PCR.

Gene	Forward, 5'-3'	Reverse, 5'-3'
Myo18b	GGAAGCAGTTAGCTGTCGC	TTGACTGGTCGTCCTGAGAGA
U6	GCTTCGGCAGCACATATACTAA	AACGCTTCACGAATTTGCGT
miR-760	GGCTCTGGGTCTGTGGG	GAACATGTCTGCGTATCTC
CDK2	TCATGGATGCCTCTGCTCTCAC	TGAAGGACACGGTGAGAATGGC
Cyclin D1	TGTTCGTGGCCTCTAAGATGAAG	GGAAGTGTTCGATGAAATCGTG
MMP-2	CAAGGATGGACTCCTGGCACAT	TACTCGCCATCAGCGTTCCCAT
MMP-9	GCTGACTACGATAAGGACGGCA	CCCTCAGAGAATCGCCAGTACT
MyOG	CCATCCAGTACATTGAGCGCCT	CTGTGGGAGTTGCATTCACTGG
MyH6	GCTGGAAGATGAGTGCTCAGAG	CCAGCCATCTCCTCTGTTAGGT
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

Myo18b, myosin-18b; miR, microRNA; CDK2, cyclin-dependent kinase 2; MMP, matrix metalloproteinase; MyOG, myogenin; MyH6, myosin heavy chain IId/x.

Western blot analysis. Following transfection, western blotting was used to determine the protein expression levels of Myo18b, MyOG and MyH6 in C2C12 myoblasts in the control, NC, siMyo18b, mock, Myo18b, mimics control, miR-760 mimics, inhibitor control and miR-760 inhibitor groups. PBS was used to wash C2C12 myoblasts twice. Total proteins were extracted from C2C12 myoblasts using a protein lysis buffer (pH 7.4, 150 mmol/l NaCl, 20 mmol/l Tris, 1 mmol/l EGTA, 1 mmol/l EDTA, 1 mmol/l β-glycerolphosphate, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 μg/ml leupeptin, 1 mmol/l Na_3VO_4 and 1 mmol/l phenylmethyanesulfonyl fluoride). Following lysis, the lysates were incubated on ice for 30 min. The supernatant was collected by centrifuged at 10,000 x g for 30 min at 4˚C. A bicinchoninic acid assay kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to measure the protein concentration. Equivalent quantities of proteins (30 μg) were resolved on a 12% gel using SDS-capillary gel electrophoresis and transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.), which were probed with the following primary antibodies after blocking in 5% non-fat milk at room temperature for 2 h: Myo18b (cat. no. 36810002; 1:1,000; Novus Biologicals, LLC); GAPDH (cat. no. ab181602; 1:10,000; Abcam); MyOG (cat. no. ab77232; 1:1,000; Abcam); or MyH6 (cat. no. ab185967; 1:1,000; Abcam). Membranes were incubated with primary antibodies overnight at 4˚C, and subsequently with the goat anti-rabbit immunoglobulin G (HRP) secondary antibody (cat. no. ab205718; 1:3,000; Abcam) at room temperature for 2 h. An enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) was used to visualize the signal, and Quantity One software (version 4.62; Bio-Rad Laboratories) was used to perform densitometry analysis.

Cell proliferation assay. Cells were transfected as aforementioned. The C2C12 myoblasts were divided into 4 groups as follows: NC group, siMyo18b group, mock group and Myo18b group. To determine proliferation, $2x10^3$ C2C12 myoblasts were seeded into 96-well plates and cultured in DMEM with 5% CO_2 at 37˚C. Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) reagent (10 μl/well) was added

at 24 and 48 h, and the cells were further incubated for 3 h at 37˚C. Subsequently, the absorbance at 450 nm was detected using a plate reader (Thermo LabSystems; Thermo Fisher Scientific, Inc.).

Cell cycle assay. In the cell cycle assay, cells were collected and fixed in 70% ethanol overnight at 4˚C. Subsequently, the cells were stained in PBS containing propidium iodide and RNase for 30 min at 4˚C in the dark. Cell cycle distribution was determined using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software (version 5.1; BD Biosciences).

Wound-healing assay. Following transfection, cells were seeded in a 6-well plate with complete medium (DMEM with 10% FBS). Then, cells were starved in serum-free complete medium for 6-8 h, and a 200 μl pipette tip was used to create a straight wound in the cell monolayers when the cells had reached ~100% confluence. The cells were washed once with PBS. The scratch was observed and imaged under a fluorescence microscope (magnification, x100) at 0 and 24 h, and the wound width was measured. All experiments were performed in triplicate.

Dual-luciferase reporter assay. The putative binding sites of miR-760 in the Myo18b-3'-untranslated region (3'-UTR) were analyzed using TargetScan 7.2 ((http://www.targetscan.org/vert_72/). The site of interaction between miR-760 and target gene Myo18b-3'-UTR was verified using a dual-luciferase reporter assay. After amplifying and purifying Myo18b-3'-UTR and Myo18b-3'-UTR mutant (mut), both PCR products were cloned into the pGL3 vector (Promega Corporation) to generate Myo18b-3'-UTR plamids and Myo18b-3'-UTR mut plamids. The C2C12 myoblasts ($2x10^3$ cells/ml) were co-transfected with Myo18b-3'-UTR/Myo18b-3'-UTR mut (0.5 μg) and miR-760 mimics/control (50 nM) using Lipofectamine 2000, according to the manufacturer's protocol. Relative luciferase activity was measured using a Dual-Luciferase Reporter assay kit (Promega Corporation) 48 h of transfection on a
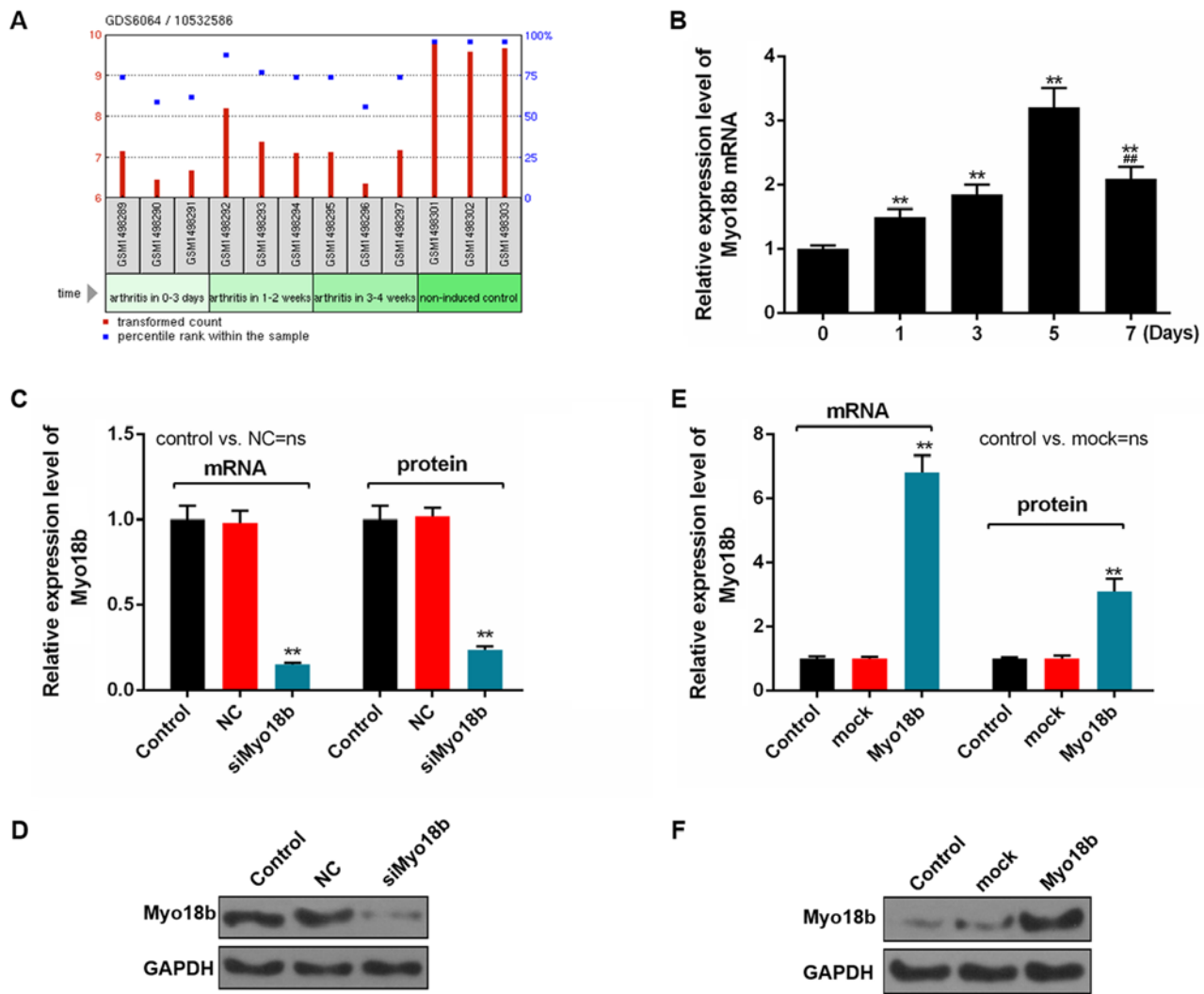
Figure 1. Myo18b regulates the differentiation of C2C12 myoblasts. (A) Expression of Myo18b was decreased in the tarsus joint of a collagen-induced rheumatoid arthritis model based on bioinformatics analysis. The combination of numbers letters represent database ID. (B) Relative expression levels of Myo18b increased as the myoblasts differentiated, and peaked at day 5. **P<0.01 vs. day 0; ##P<0.01 vs. day 5. (C) The mRNA and protein expression levels of Myo18b were decreased following transfection with siMyo18b. **P<0.01 vs. NC. (D) Representative western blot showing the protein expression levels of Myo18b in cells transfected with NC and siMyo18b. (E) The mRNA and protein expression levels of Myo18b were increased following transfection with Myo18b overexpression vector. **P<0.01 vs. Mock. (F) Representative western blot of the protein expression levels of Myo18b in cells transfected with mock and Myo18b overexpression vector. Myo18b, myosin-18b; si, small interfering; NC, negative control; ns, not significant.

fluorescence spectrophotometer (Infinite M200; Tecan Group, Ltd.) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. All data analysis was performed using SPSS version 20.0 (IBM Corp.) or GraphPad Prism version 7 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation. The aforementioned experiments were independently repeated three times. A one-way ANOVA followed by a post-hoc Tukey's test was used to analyze differences among the experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Myo18b expression is downregulated in the tarsus joint of collagen-induced RA. The gene expression profile in the tarsal joint of a mouse model of RA was first determined, and the

data suggested that expression of Myo18b was lower in the tarsus joint of mice with collagen-induced RA, compared with non-induced controls (Fig. 1A).

Myo18b expression increases during the myogenic differentiation of C2C12 cells. To determine the expression of Myo18b during the myogenic differentiation of C2C12 myoblasts, cells were harvested and the expression levels of Myo18b were determined in cells after 0, 1, 3, 5 and 7 days. The RT-qPCR analysis showed that the expression levels of Myo18b increased significantly on days 1, 3, 5 and 7 compared with 0 days (P<0.01), and Myo18b level decreased significantly on day 7 compared with 5 days (P<0.01; Fig. 1B). Additionally, the expression of Myo18b on day 7 was significantly upregulated compared with day 0 (P<0.01; Fig. 1B).

Myo18b regulates proliferation and cell cycle progression in C2C12 myoblasts. The efficiency of siMyo18b and Myo18b
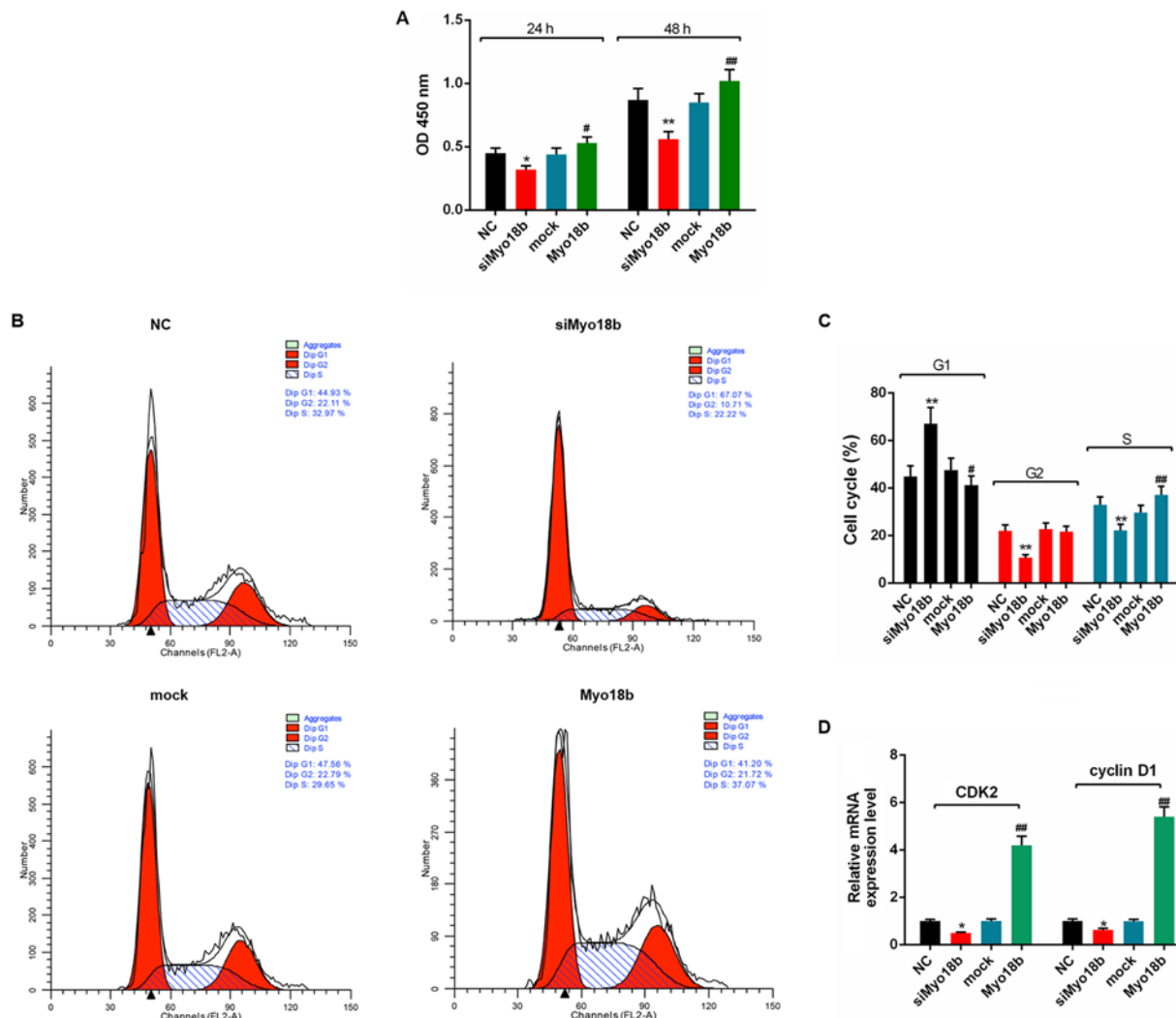
Figure 2. Myo18b regulates the proliferation and cell cycle progression of C2C12 myoblasts. (A) Myo18b silencing decreased proliferation of C2C12 myoblasts; Myo18b overexpression had the opposite effect. *P<0.05, **P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. Mock. (B) Flow cytometry analysis of the effects of Myo18B expression on cell cycle progression. (C) Quantification of the flow cytometry analysis presented in panel (B). **P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. Mock. (D) Relative mRNA expression levels of CDK2 and cyclin D1 in cells transfected with either siMyo18b or Myo18b overexpression vector. *P<0.05 vs. NC; ##P<0.01 vs. Mock. Myo18b, myosin-18b; OD, optical density; si, small interfering; NC, negative control; CDK2, cyclin dependent kinase 2.

transfection was determined using RT-qPCR and western blot analyses. The results showed that the expression of Myo18b in the cells transfected with siMyo18b was significantly decreased compared with the control or NC groups (P<0.01; Fig. 1C and D). However, Myo18b expression in the Myo18b group was significantly increased compared with the control or mock groups (P<0.01; Fig. 1E and F).

Subsequently, the effects of Myo18b on the growth of C2C12 myoblasts were assessed. The CCK-8 assay results showed that the cell viability in the siMyo18b group was significantly reduced compared with the NC group at 24 (P<0.05) and 48 h (P<0.01) after transfection with siMyo18b, whereas the cell viability in the Myo18b group was significantly increased compared with the mock group at 24 (P<0.05) and 48 h (P<0.01) after transfection with Myo18b (Fig. 2A). Furthermore, flow cytometry analysis showed that the number of C2C12 myoblasts in the G1 phase was significantly increased in the siMyo18b group (P<0.01), and the number of cells in G2 and S phase were significantly decreased (P<0.01) compared with the NC group (Fig. 2B and C). The number of C2C12 myoblasts in the G1

phase were significantly decreased when cells were transfected with Myo18b (P<0.05), whereas the number of cells in the S phase were significantly increased (P<0.01), compared with the mock group (Fig. 2B and C). RT-qPCR analysis showed that the expression levels of CDK2 and cyclin D1 in C2C12 myoblasts transfected with siMyo18b were significantly decreased (P<0.05), and the expression levels in C2C12 myoblasts transfected with Myo18b were significantly increased (P<0.01; Fig. 2D), compared with the respective controls.

Myo18b regulates the migration of C2C12 myoblasts. The results of the wound-healing assay showed that the migratory rate of C2C12 myoblasts transfected with siMyo18b was significantly decreased compared with the NC group (P<0.01). The migratory rate of C2C12 myoblasts transfected with Myo18b was significantly increased compared with the mock cells (P<0.01; Fig. 3).

Myo18b regulates differentiation of C2C12 myoblasts. The results of RT-qPCR and western blotting analysis results
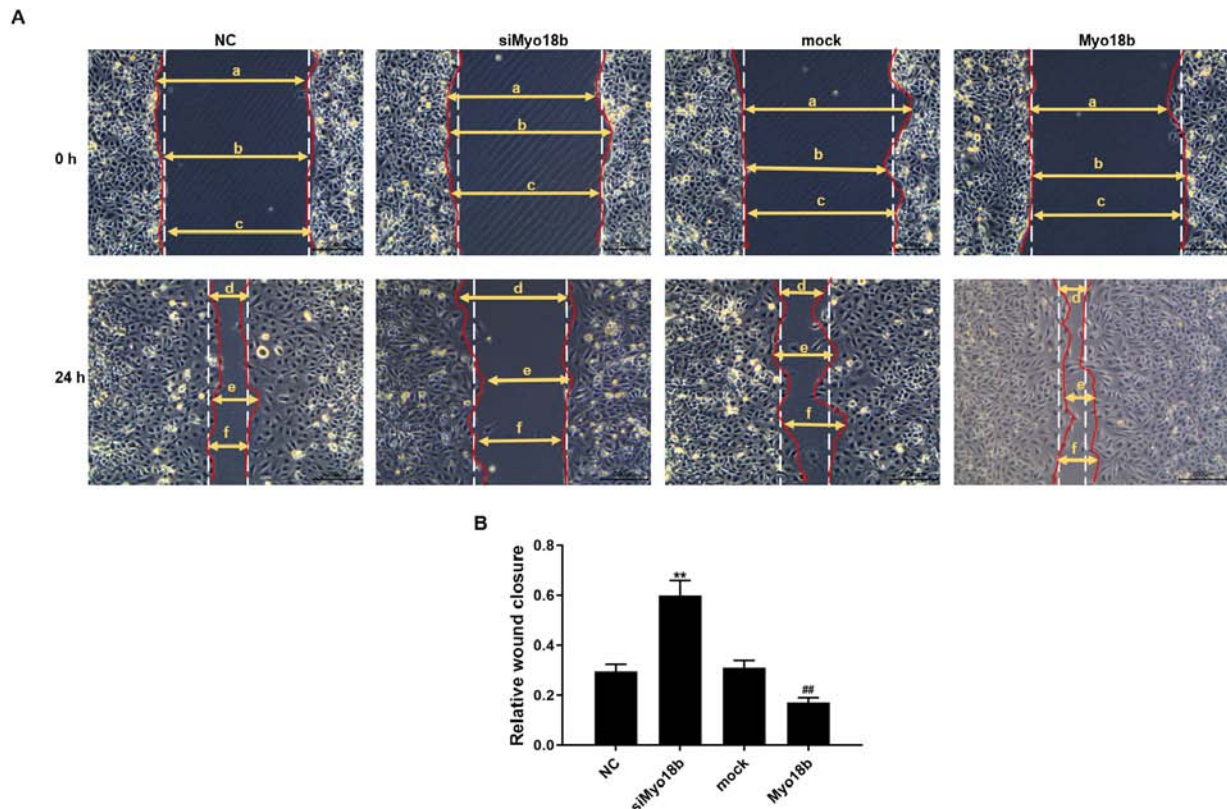
Figure 3. Myo18b regulates migration of C2C12 myoblasts. (A) Representative wound-healing images of cells transfected with either siMyo18b or Myo18b overexpression vector. Relative wound closure was calculated by dividing the sum of d, e and f by the sum of a, b and c. (B) Relative wound closure of C2C12 myoblasts transfected with siMyo18b was significantly increased compared with the NC group, and the relative wound closure of C2C12 myoblasts transfected with Myo18b was significantly decreased compared with the mock group. Scale bar, 200 μm. Magnification, x100. **P<0.01 vs. NC; ##P<0.01 vs. mock. Myo18b, myosin-18b; si, small interfering; NC, negative control.

showed that siMyo18b downregulated the mRNA expression levels of MMP-2 and MMP-9, whereas Myo18b overexpression increased the expression of these genes in C2C12 myoblasts (Fig. 4A). Furthermore, the mRNA and protein expression levels of MyOG and MyH6 were downregulated in the siMyo18b group compared with the NC group, and increased in cells overexpressing Myo18b compared with the mock group (P<0.01; Fig. 4B-D). These results suggested that siMyo18b decreased differentiation in C2C12 myoblasts and Myo18b overexpression induced opposing effects.

Myo18b is a target gene of miR-760 in C2C12 myoblast. A binding site between miR-760 and Myo18b-3'-UTR was identified using TargetScan (Fig. 5A). A dual-luciferase reporter assay revealed that miR-760 mimics significantly decreased the luciferase activity when the reporter gene contained the Myo18b-3'-UTR in C2C12 myoblasts (P<0.01). However, no decrease in luciferase activity was observed when the reporter gene contained the Myo18b-3'-UTR mut (P>0.05; Fig. 5B).

Upregulation of miR-760 decreases proliferation and cell cycle progression of C2C12 myoblasts. The expression levels of miR-760 in C2C12 myoblasts were determined using RT-qPCR analysis following transfection. The results showed that the expression of miR-760 in C2C12 myoblasts

was significantly increased in cells transfected with miR-760 mimics compared with cells transfected with the control mimics (P<0.01). After transfection with miR-760 inhibitor, the miR-760 expression level was decreased, compared with cells in the inhibitor control group (P<0.01; Fig. 5C).

A CCK-8 assay showed there were no significant differences observed in terms of the cell viability at 24 h among the groups (P>0.05), and that the cell viability at 48 h was decreased significantly in the cells transfected with miR-760 mimics, compared with the mimics control group (P<0.05) after 48 h. Cell viability at 48 h was increased significantly in the cells transfected with miR-760 inhibitor compared with the inhibitors control group (P<0.05; Fig. 5D). Furthermore, flow cytometry analysis showed that the percentage of C2C12 myoblasts transfected with miR-760 mimics in G1 was significantly increased (P<0.01), whereas the percentages of cells in G2 and S were significantly decreased (P<0.01) compared with the mimics control group (Fig. 6A and B). The percentage of cells transfected with miR-760 inhibitors in G1 were decreased (P<0.05), and the percentage of cells in S phase were significantly increased (P<0.01) compared with the inhibitors control group (Fig. 6A and B). RT-qPCR analysis revealed that the mRNA expression levels of cyclin D1 were significantly decreased in the miR-760 mimics group compared with the mimics control group (P<0.01), while the CDK2 level was
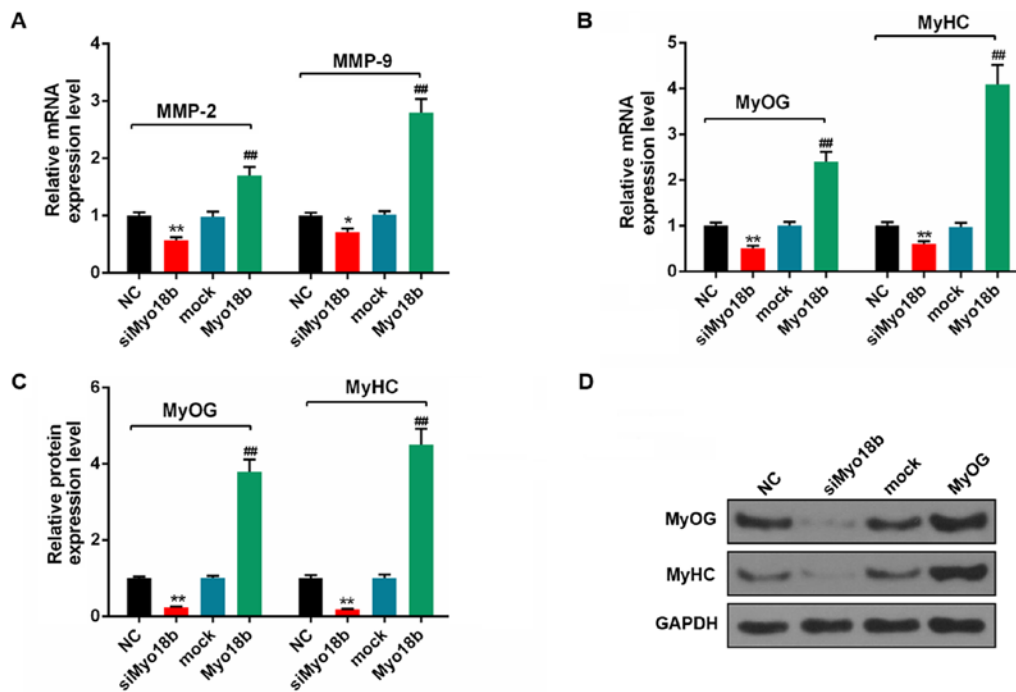
Figure 4. Myo18b regulates differentiation of C2C12 myoblasts. (A) Myo18b silencing decreased the mRNA expression levels of MMP-2 and MMP-9 in C2C12 myoblasts. Overexpression of Myo18b increased the mRNA expression levels of MMP-2 and MMP-9 in C2C12 myoblasts. *P<0.05, **P<0.01 vs. NC; ##P<0.01 vs. Mock. (B) Myo18b silencing decreased the mRNA expression levels of MyOG and MyH6 in C2C12 myoblasts. Overexpressing Myo18b increased the mRNA expression levels of MyOG and MyH6 in C2C12 myoblasts. **P<0.01 vs. NC; ##P<0.01 vs. Mock. (C) Myo18b silencing decreased the protein expression levels of MyOG and MyH6 in C2C12 myoblasts. Overexpressing Myo18b increased the protein expression levels of MyOG and MyH6 in C2C12 myoblasts. **P<0.01 vs. NC; ##P<0.01 vs. Mock. (D) Representative blots of MyOG and MyH6 protein in C2C12 myoblasts when Myo18b was either silenced or overexpressed. Myo18b, myosin-18b; MMP, matrix metalloproteinase; NC, negative control; si, small interfering; MyOG, myogenin; MyH6, myosin heavy chain IId/x.
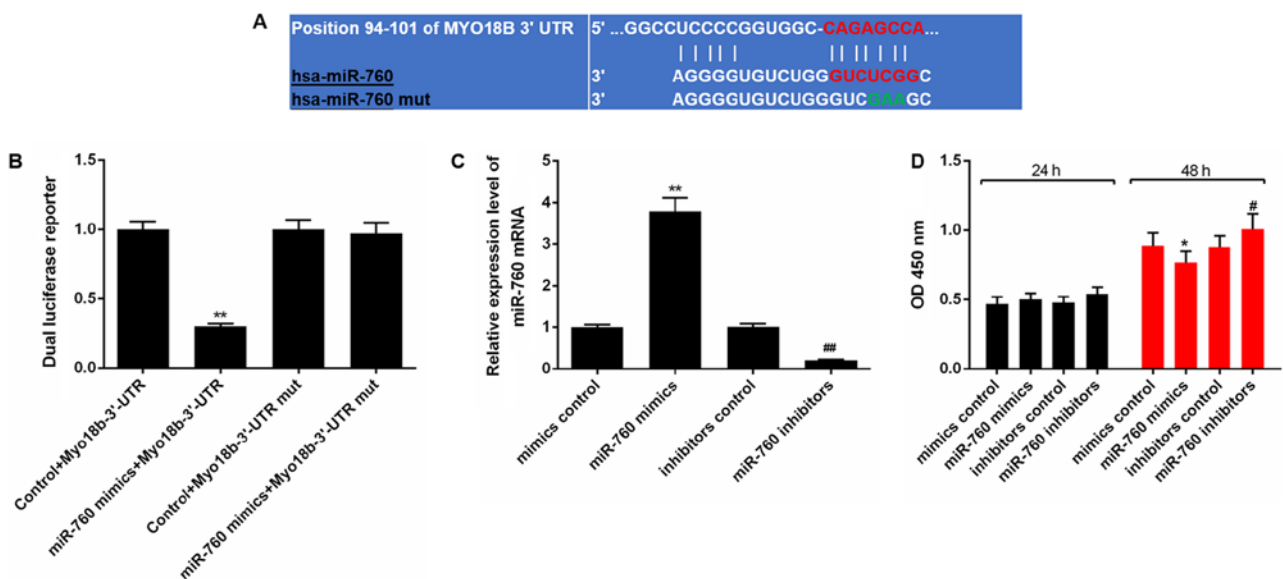

Figure 5. Myo18b is a target gene of miR-760 in C2C12 myoblasts. (A) Binding site between miR-760 and Myo18b predicted using the TargetScan website. (B) Dual-luciferase assay to demonstrate the ability of miR-760 to bind directly to Myo18b-3'-UTR. **P<0.01 vs. Control + Myo18b-3'UTR. (C) miR-760 expression levels were increased following transfection with miR-760 mimics, and transfection with miR-760 inhibitors decreased miR-760 levels. **P<0.01 vs. mimics control; ##P<0.01 vs. inhibitors control. (D) Overexpression of miR-760 decreased the proliferation of C2C12 myoblasts, and silencing miR-760 increased proliferation after 48 h. *P<0.05 vs. mimics control; #P<0.05 vs. inhibitors control. Myo18b, myosin-18b; mut, mutant; UTR, untranslated region; miR, microRNA.

slightly reduced but not significant. The expression levels of CDK2 and cyclin D1 in miR-760 inhibitor group were

significantly higher compared with the mimics control group (P<0.01; Fig. 6C).
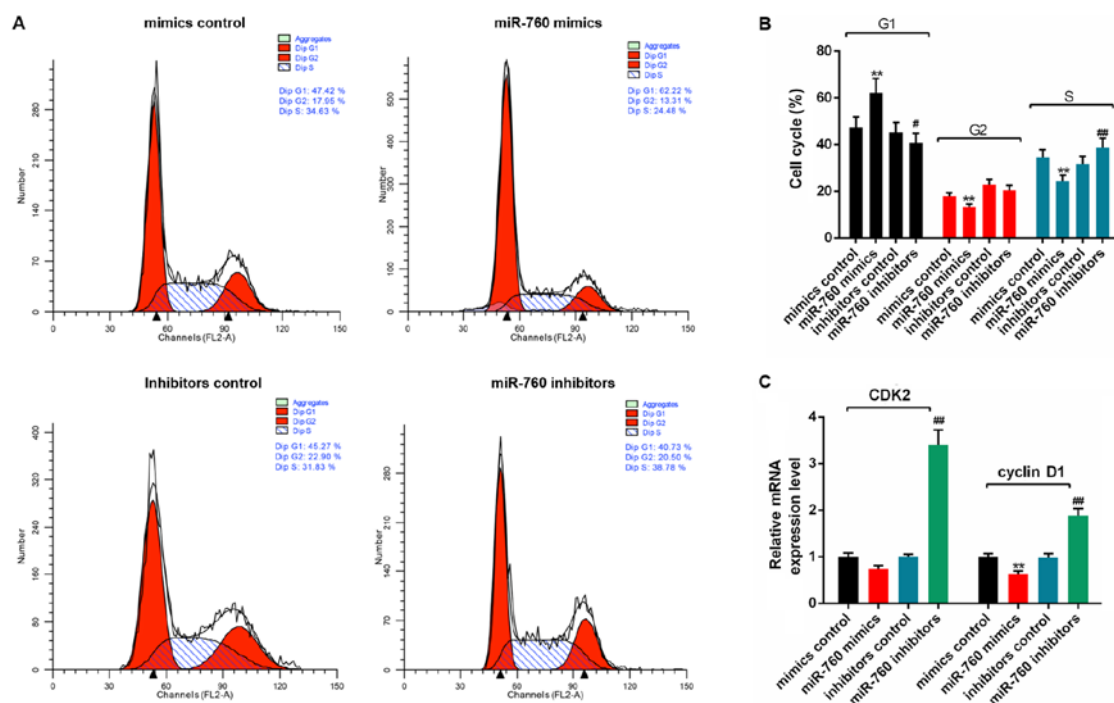
Figure 6. miR-760 regulates cell cycle progression in C2C12 myoblasts. (A) Representative histograms of flow cytometry analysis of the proportion of cells in different stages of the cell cycle. (B) miR-760 overexpression decreased cell cycle progression in C2C12 myoblasts and silencing miR-760 increased cell cycle progression. **P<0.01 vs. mimics control; #P<0.05, ##P<0.01 vs. inhibitors control. (C) miR-760 overexpression decreased the mRNA expression levels of cyclin D1. Silencing miR-760 increased the mRNA expression levels of CDK2 and cyclin D1. **P<0.01 vs. mimics control; ##P<0.01 vs. inhibitors control. miR, microRNA; CDK12, cyclin dependent kinase 2; Dip, diploid.
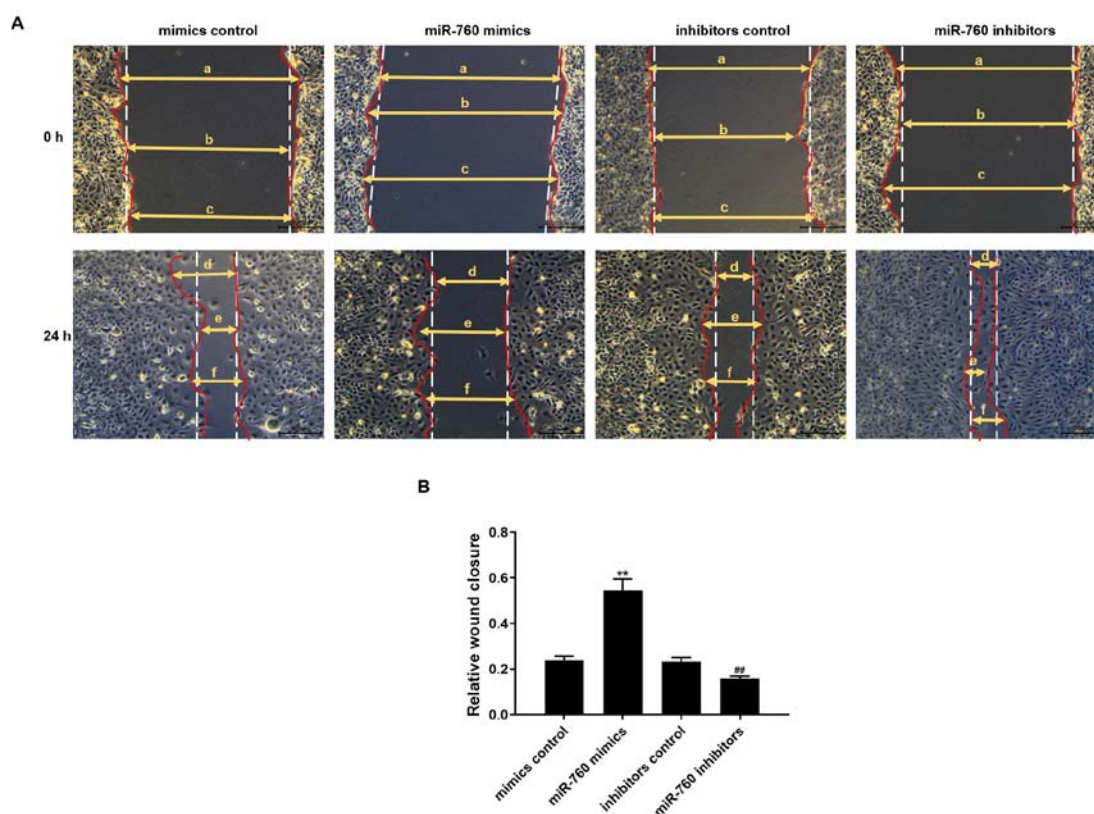

Figure 7. miR-760 regulates the migration of C2C12 myoblasts. (A) Representative wound-healing images of cells transfected with either miR-760 mimics or miR-760 inhibitors. Relative wound closure was calculated by dividing the sum of d, e and f by the sum of a, b and c. (B) Relative wound closure of C2C12 myoblasts transfected with miR-760 mimics was significantly increased compared with the mimics control. Cells transfected with the miR-760 inhibitors exhibited a significantly reduced wound closure compared with inhibitors control. Scale bar, 200 μm. Magnification, x100.**P<0.01 vs. mimics control; ##P<0.01 vs. inhibitors control. miR, microRNA.
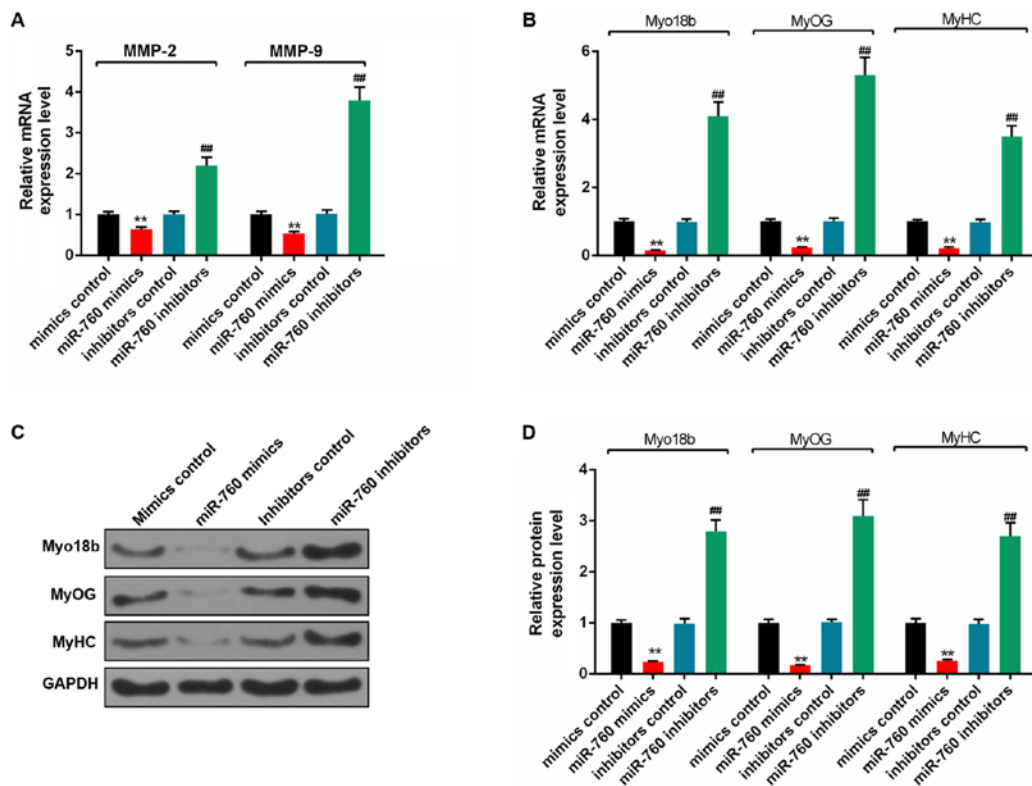
Figure 8. miR-760 regulates the differentiation of C2C12 myoblasts. (A) miR-760 overexpression decreased the mRNA expression levels of MMP-2 and MMP-9 in C2C12 myoblasts. **$P<0.01$ vs. mimics control; ##$P<0.01$ vs. inhibitors control. (B) Overexpression of miR-760 decreased the mRNA expression levels of Myo18b, MyOG and MyH6 in C2C12 myoblasts. **$P<0.01$ vs. mimics control; ##$P<0.01$ vs. inhibitors control (C) Representative western blot showing the protein expression levels of Myo18b, MyOG and MyHC in cells transfected with miR-760 mimics and miR-760 inhibitors. (D) miR-760 overexpression decreased the protein expression levels of Myo18b, MyOG and MyH6 protein in C2C12 myoblasts. Transfection of miR-760 inhibitors increased the protein expression levels of these proteins. **$P<0.01$ vs. mimics control; ##$P<0.01$ vs. inhibitors control. miR, microRNA; Myo18b, myosin-18b; MMP, matrix metalloproteinase; MyOG, myogenin; MyH6, myosin heavy chain IId/x.

miR-760 decreases the migration of C2C12 myoblasts. A wound healing assay was used to determine the effects of miR-760 on the migratory capacity of C2C12 myoblasts following transfection with a miR-760 mimic or inhibitor. The results showed that the migratory abilities of C2C12 myoblasts transfected with miR-760 mimics for 24 h were significantly reduced compared with the mimics control group ($P<0.01$). The migratory capacity in cells transfected with miR-760 inhibitors at 24 h was significantly increased compared with the inhibitors control group ($P<0.01$; Fig. 7).

miR-760 decreases differentiation of C2C12 myoblasts. RT-qPCR and western blot analyses showed that the expression levels of MMP-2, MMP-9, Myo18b, MyOG and MyH6 in C2C12 myoblasts transfected with miR-760 mimics were significantly decreased compared with the mimics control group ($P<0.01$; Fig. 8A and B). The expression levels of these genes were significantly increased in cells transfected with miR-760 inhibitor, compared with the inhibitor control group ($P<0.01$; Fig. 8C and D).

Discussion

RA is a chronic autoimmune disease that causes progressive articular destruction, functional loss of joints and related comorbidities of bone, vasculature and metabolism, and thus physiologically affects patients (28). RA results from complex interactions between genes and the environment (29). Studies have shown that reducing inflammation, inhibiting angiogenesis and inducing apoptosis of fibroblast-like synoviocytes may effectively improve RA (30-32). Skeletal muscle atrophy has been observed in patients with RA, and Myo18b is an important component involved in the rapid formation of skeletal muscle (33,34). miRNAs have been reported as important regulators of skeletal muscle development and differentiation (35). Previously, expression of miR-760 was demonstrated to be altered in several diseases (36,37). However, the role of miR-760 in regulating the proliferation of RA skeletal muscle remains unclear. The aims of the present study were to examine the regulatory effects of miR-760 during skeletal muscle proliferation in RA, and to determine the underlying mechanism.

RA is associated with muscle wasting, which impedes prognosis. Therefore, promoting skeletal muscle proliferation has significant clinical value for patients with RA (38,39). Certain genes and multiple proteolytic systems are involved in muscle atrophy (40). Furthermore, the release of cytokines during chronic inflammation may promote satellite cell activation and myogenesis that help promote skeletal muscle proliferation (41). According to Malfatti *et al* (42), Myo18b encodes an unconventional myosin protein which is primarily expressed in skeletal and cardiac muscles that are associated with nemaline myopathy and cardiomyopathy. Similarly, Alazami *et al* (43) showed that a rare syndrome, presenting

Klippel-Feil anomaly and myopathy, may have been caused by a mutation in Myo18b. In the present study, it was demonstrated that Myo18b was downregulated in the tarsus joint of RA; thus, it was suggested that miR-760 may have regulated skeletal muscle proliferation in RA by targeting Myo18b. To confirm a link between miR-760 and Myo18b, a dual-luciferase reporter assay was performed, and the results showed that Myo18b was a direct target gene of miR-760.

Myoblast fusion is a crucial step in skeletal muscle differentiation, and the regulation of genes such as cyclin D1 and MyOG may affect the proliferation and differentiation of myoblasts (44,45). CDKs are proline-directed serine/threonine-protein kinases, which are primarily involved in cell cycle regulation, and intracellular and extracellular signal fusion (46). CDKs can bind to cyclins to form heterodimers and promote the progression of the cell cycle. As a type of protein kinase, CDK2 is involved in the regulation of the G1/S phases of the cell cycle in patients with RA (47). In the present study, it was demonstrated that Myo18b expression was increased as C2C12 myoblasts differentiated at 1, 3 and 5 days, but decreased on day 7. Based on this result, it may be the case that the rate of apoptosis increases after day 5. In a previous study, miRNAs were found to regulate cell proliferation, cell cycle progression and migration by altering the expressions of various factors, such as MALAT1 (48). In the present study, the results showed that miR-760 overexpression and Myo18b silencing significantly decreased the expression levels of CDK2 and cyclin D1 in C2C12 myoblasts. Additionally, it was demonstrated that miR-760 overexpression and Myo18b silencing decreased the proliferation and migration of C2C12 myoblasts. These results suggested that miR-760 may inhibit the proliferation and migration of C2C12 myoblasts by suppressing Myo18b and thus slowing down skeletal muscle formation. Furthermore, MMP-2 and MMP-9 are the primary MMPs responsible for the degradation of type IV collagen, primarily in the basement membrane close to the muscular layer (49,50). MMPs are associated with increased motility, differentiation and regeneration of skeletal muscle. Zhai *et al* found that salicin decreased the levels of inflammatory factors and inhibited the expression of MMP-1/-3 in interleukin-1β-induced RA-fibroblast-like synoviocytes, thereby improving RA (30). Therefore, regulation of MMPs may prove to be an effective method of treating patients with RA.

Skeletal muscles are composed of heterogeneous types of fibers based on MyHC expression (51). In patients with RA, varying degrees of muscle mass decline and reduction in cross-sectional area have been reported, and a slight but significant decrease in MyHC content has also been observed (52). MyOG, a key transcription factor in myogenesis, plays an important role in the regulation of myoblast differentiation and is critical for the terminal differentiation of differentiated myoblasts (45). In addition, a previous study suggested that upregulation of MyOG expression was required for myoblasts to fuse into new or existing myotubes, and that the knockdown of MTMR7 promoting C2C12 myoblast early differentiation was associated with the upregulation of MyOG (53). In the present study, the results showed that miR-760 inhibited MMP-2, MMP-9, MyOG and MyH6 expression levels in C2C12 myoblasts by downregulating Myo18b. Based on these results, it was hypothesized that miR-760 may affect skeletal muscle proliferation and differentiation by regulating cell cycle

and myocyte differentiation, and such a phenomenon may be associated with the regulation or the expression of Myo18b, MyOG and MyHC.

In conclusion, it was revealed that Myo18b was a target gene of miR-760. Overexpression of miR-760 downregulated the expression levels of CDK2, cyclin D1, MMP-2, MMP-9, MyOG and MyH6. miR-760 decreased proliferation, cell cycle progression, migration and differentiation in C2C12 myoblasts by targeting Myo18b. The results of the present study may improve understanding of the molecular mechanisms underlying the proliferation of skeletal muscle in patients with RA. There are certain limitations to the present study. For example, all experiments were performed *in vitro* and thus, whether the results are replicated *in vivo* remains to be determined. Additionally, miR-760 targets other genes involved in the proliferation and differentiation of C2C12 myoblasts; thus, the effects miR-760 on the various cellular behaviors may have been mediated via other proteins.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the author on reasonable request.

Authors' contributions

XT conceived and designed the study. JW, SZ, HW, GF, JZho, JZha, GJ and CX acquired analyzed and interpreted the data. XT drafted the article and revised for important intellectual content. All the authors approved publication of the final version and agree to be held accountable for all aspects of the work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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