MicroRNA-148a Promotes Myogenic Differentiation by Targeting the ROCK1 Gene*

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Background: miRNAs participate in the regulation of skeletal muscle development.

Results: miR-148a promotes myogenic differentiation and down-regulates ROCK1 gene at the translational level.

Conclusion: miR-148a plays a positive role in skeletal muscle development via ROCK1 down-regulation.

Significance: Our findings reveal a novel mechanism in which miR-148a controls myogenesis through the RhoA/ROCK pathway.

MicroRNAs are evolutionarily conserved small RNAs that post-transcriptionally regulate gene expression and have emerged as critical regulators of skeletal muscle development. Here, we identified miR-148a as a novel myogenic microRNA that mediated myogenic differentiation. The expression levels of miR-148a increased during C2C12 myoblast differentiation. Overexpression of miR-148a significantly promoted myogenic differentiation of both C2C12 myoblasts and primary muscle cells. Blocking the function of miR-148a with a 2′-O-methylated antisense oligonucleotide inhibitor repressed C2C12 myoblast differentiation. Using a bioinformatics approach, we identified Rho-associated coiled-coil containing protein kinase 1 (ROCK1), a known inhibitor of myogenesis, as a target of miR-148a. A dual-luciferase reporter assay was used to demonstrate that miR-148a directly targeted the 3′-UTR of ROCK1. In addition, the overexpression of miR-148a decreased the protein expression of ROCK1 in C2C12 myoblasts and primary muscle cells. Furthermore, ROCK1 inhibition with specific siRNA led to accelerated myogenic differentiation progression, underscoring a negative regulatory function of ROCK1 in myogenesis. Therefore, our results revealed a novel mechanism in which miR-148a positively regulates myogenic differentiation via ROCK1 down-regulation.

The skeletal muscle development is a multistep pathway, in which mesodermal precursor cells are selected to form myoblasts that are withdrawn from the normal cell cycle and subsequently differentiate into myotubes (1, 2). The entire process is orchestrated by the myogenic transcription factors MyoD, Myf5, myogenin, MRF4, and Mef2. These transcription factors coordinate the expression of genes involved in muscle growth, morphogenesis, differentiation, and contractility (3). In addition, increasing evidence indicates that miRNAs play an important role in skeletal muscle development (4, 5).

MicroRNAs (miRNAs)§ represent an abundant class of short noncoding RNAs that post-transcriptionally regulate mammalian genes by translational repression or degradation of the transcript (6). Dicer is essential for processing of pre-miRNAs into the mature form (7). The harmful consequences of Dicer deletion in mouse embryonic skeletal muscle demonstrate that miRNA processing plays an essential role in muscle development (8–10). The transcripts miR-1, miR-133, and miR-206 have been identified as muscle-specific miRNAs. They are regulated by the myogenic transcription factors and are required for skeletal muscle formation (11–14). The miR-1 and miR-206 promote myogenesis by targeting histone deacetylase 4 (HDAC4) and DNA polymerase α (Pola1), respectively, whereas miR-133 inhibits myoblast differentiation and enhances proliferation by repressing serum response factor (SRF) (15, 16). Additional miRNAs have been reported to participate in skeletal myogenesis and include miR-181 (17), miR-26 (18), miR-24 (19), miR-378 (20), miR-27b (21), miR-221/222 (22), miR-486 (23), miR-29 (24), miR-208b/miR-499 (25), and miR-214 (26). Our microRNA transcriptome profiling analysis, using a Solexa deep sequencing approach revealed that miR-148a is differently expressed during swine fetal skeletal muscle development (data not shown). This result suggested a potential role of miR-148a in skeletal muscle development. The miR-148a has been reported to play important roles in cancer cell proliferation, apoptosis and metastasis in some tumor types including cholangiocarcinoma, prostate, esophageal, and colon cancers (27–29). However, until now, the function of miR-148a in myogenesis has not been reported. The direct targets of miR-
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148a reported thus far include TGF2, DNMT3, PXR, CAND1, MSK1, and p27 (30–34). RhoA/ROCK signaling has been reported to play a critical role in muscle development. Rho kinases (ROCKs), as the direct downstream effectors of RhoA, participate in a wide range of fundamental cellular processes, such as transduction, vesicle trafficking, and cytoskeletal organization. Currently, two members of the ROCK family (ROCK1 and ROCK2) have been identified (35–40). RhoA-dependent activation of SRF results in the expression of muscle-specific genes, but its role as a positive or negative regulator is controversial (41–47). Recent studies have demonstrated that ROCK1 acts as a negative regulator of myogenesis. It acts independently of RhoA to prevent cell fusion and myotube formation (48, 49). Overexpression of constitutively active ROCK1 impairs the differentiation of both avian and mouse myoblasts. Conversely, the inhibition of ROCK1 via Y27632 and siRNA leads to accelerated progression in the lineage and enhanced cell fusion (50). The inactivation of ROCK1 has been shown to be necessary for Forkhead transcription factor (FKHR) nuclear translocation and myoblast fusion (51). However, ROCK2 is up-regulated during skeletal muscle myogenesis and it has been reported to positively control myogenic differentiation (52).

Using a bioinformatics approach, we predicted that Rho-associated coiled-coil containing protein kinase 1 (ROCK1) was a potential target gene of miR-148a. We hypothesized that miR-148a participated in myogenesis via the down-regulation of ROCK1. In this study, we found that miR-148a was up-regulated during C2C12 myoblast differentiation. Loss- and gain-of-function analyses revealed that miR-148a functioned as a positive regulator of myogenesis. Subsequent experiments confirmed that ROCK1 was a target of miR-148a and was down-regulated by miR-148a at the translational level. We also confirmed the results with anti-ROCK1 siRNA to show that the reduction of ROCK1 promoted the differentiation of both C2C12 myoblast and primary muscle cells. Our studies therefore indicated that miR-148a promoted myogenic differentiation by targeting the ROCK1 gene.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 myoblast cells were purchased from the Cell Resource Center in IBMS in CAMS/PUMC. C2C12 myoblasts were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin and 1% penicillin/streptomycin. Myogenic differentiation was induced by replacing the medium of the subconfluent cells with DMEM supplemented with 2% horse serum (Hyclone) and 1% penicillin/streptomycin. The primary muscle cells were enzymatically isolated from skeletal muscles of the hind- and fore-limbs form 2-day-old mice as described (53). Mouse primary muscle cells were cultured in Ham’s F-10 supplemented with 20% FBS and 2.5 ng/ml basic FGF and 1% penicillin/streptomycin in a collagen type 1-coated plate and differentiated using DMEM with 2% horse serum.

RNA Isolation—The total RNA was extracted using the TRIzol reagent (Invitrogen), as recommended by the manufacturer. Only RNA preparation with an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio greater than 2.0 were used for subsequent analysis.

TagMan® miRNA Expression Assays—Single-stranded cDNA was synthesized from total RNA samples using specific miRNA stem-loop primers and the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Mature miRNA expression was measured with TagMan MicroRNA Assays (Applied Biosystems) according to the manufacturer’s instructions with the Applied Biosystems 7500 Real-Time PCR system. U6 was used to normalize miRNA expression.

Immunoblotting and Immunofluorescence—Immunoblotting was performed using standard procedures and antibodies to MHC (MF20, DSHB), myogenin (F5D, Santa Cruz Biotechnology), myoD (Santa Cruz Biotechnology), ROCK1 (Cell Signaling Technology), α-tubulin (Santa Cruz Biotechnology), and GAPDH (Santa Cruz Biotechnology). For immunostaining, C2C12 cells treated in 6-well plates were fixed in 4% formaldehyde for 10 min and then washed three times for 10 min each in PBS. The cells were then permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 5% skim milk in PBS, the cells were incubated with the primary antibody MF20 (1:40 dilution) for 1 h at 37 °C. The Cy-3-conjugated anti-mouse IgG (1: 400 dilution) was incubated for 1 h at 37 °C. The nuclei of the cells were visualized using DAPI staining.

RNA Oligonucleotides and Transfection—The miRNA mimics (double-stranded RNA oligonucleotides) and negative control duplexes were synthesized by GenePharma. 2′-O-methyl antisense oligonucleotides against the target miRNAs and a negative control were synthesized by Invitrogen. The miR-148a inhibitor sequence was: 5′-ACAAAAGUCUGAUGUCAGUGAC-3′. Transfection was performed with the Lipofectamine 2000 reagent (Invitrogen) combined with 150 nm of miRNA mimics and 200 nm 2′-O-methyl antisense oligonucleotide.

Cell Proliferation Assay—C2C12 cells transfected with the miR-148a mimics or the negative control duplexes were seeded at 3 × 10³ cells/well in a 48-well plate and cultured in growth medium for 4 days. The cell proliferation assay was performed by adding 20 μl of Cell-Counting Kit-8 (CCK-8) reagents (Dojindo) to the cells for 1 h. Absorbance at 450 nm was measured using the SpectraMax M5 microplate spectrophotometer.

Flow Cytometry Analysis of the Cell Cycle—C2C12 cells were collected 36 h after transfection with the miR-148a mimics or the negative control duplexes. The collected cells were then washed in PBS and fixed in 75% ethanol at −20 °C. For cell cycle analysis, 2–5 × 10⁵ cells from each sample were stained with 50 μg/ml propidium iodide (Invitrogen) containing 10 μg/ml RNaseA (Takara) and then analyzed in a FACSCalibur flow cytometer (BD Biosciences).

Microarray—The Whole Mouse Genome Micorarray 4 × 44K array (G412F, Agilent Technologies) was used in this study. C2C12 cells were transfected with miR-148a mimics, miR-143 mimics and a negative control duplex. Total RNAs form each sample were isolated 48 h after transfection. The cDNA was synthesized from the RNA samples and then used to synthesize aminoacyl-UTP (aaUTP, Ambion)-labeled cRNA. The cRNA samples were purified using the Qiagen RNeasy® Mini Kit and then labeled using the a Low Input Linear Amplifi-
miR-148a Is Up-regulated during C2C12 Myoblast Differentiation—We used the C2C12 myoblast (MB) cell line as a model system to identify the functional characteristics of miR-148a in skeletal myogenesis. We assessed miR-148a expression during myogenesis using an miRNA TaqMan RT-qPCR assay (Applied Biosystems). The results indicated that miR-148a was significantly up-regulated during C2C12 myoblast differentiation (Fig. 1).

miR-148a Positively Regulates Myoblast Differentiation—To investigate the function of miR-148a in myoblast differentiation, we introduced a synthetic RNA duplex (mimics) of miR-148a into C2C12 myoblasts. Overexpression of miR-148a markedly increased the number of MHC-positive cells (Fig. 2A), and the protein levels of MHC and myogenin (Fig. 2B). Moreover, these changes were accompanied by significant increases in the mRNA expression levels of the myogenic marker genes MHC, myogenin, and skeletal α-actin (Fig. 2C). These results indicated that miR-148a accelerated myoblast differentiation. To determine whether miR-148a was essential for myogenesis, we treated C2C12 cells with 2′-O-methyl antisense oligonucleotides against miRNA-148a. The inhibition of miR-148a suppressed myoblast differentiation, as indicated by a decrease in the mRNA and protein expression of myogenic markers (Fig. 2, D and E). However, overexpression or inhibition of miR-148a had no effect on MyoD expression (supplemental Fig. S1).

In addition, we introduced miR-148a mimics into the primary muscle cells and found that overexpression of miR-148a enhanced the expression level of MHC and myogenin (Fig. 2F). Taken together, we concluded that miR-148a was an essential positive regulator of myogenesis.

The Introduction of miR-148a Enhances Cell Cycle Arrest—We further examined the role of miR-148a on myoblast proliferation by overexpressing miR-148a. We transfected C2C12 cells cultured in GM with miR-148a mimics or the negative control duplexes. We then monitored the transfected cell lines for alterations in proliferation using WST-8 assay with the Cell Counting Kit-8. The results demonstrated that miR-148a had no significant effect on cell proliferation (p > 0.05), indicating that it was not involved in modulating myoblast proliferation (Fig. 3A).

Cell cycle arrest is a critical step during myoblasts differentiation, therefore, we also tested the effect of miR-148a on cell cycle progression. The cell cycle was analyzed using fluorescence-activated cell sorter (FACS) after propidium iodide staining. C2C12 MBs cultured in GM were transfected with miR-148a mimics or control duplexes and then harvested at
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36 h after transfection. Propidium iodide staining revealed that transfection of miR-148a approximately increased the G1 population of cells by 10% \((p < 0.05)\), suggesting that miR-148a enhances the G0/G1 arrest (Fig. 3B).

**Gene Expression Changes under miRNA-148a Overexpression**—Microarrays analysis revealed a large number of mRNA changes in response to miR-148a overexpression (Fig. 4A). A total of 89 genes were up-regulated and 43 genes were down-regulated. To explore the biological meaning of the differentially expressed genes, the DAVID Functional Annotation Tool was employed. The Gene Ontology data base indicated that these genes were distributed in cell proliferation, cell differentiation, the circulatory system, muscle development, immune system processes, stimulus responses, and cellular complex assembly (Fig. 4B). Interestingly, some of the muscle-specific genes were significantly up-regulated, including myosin light polypeptide 4 (Myl4), myosin heavy polypeptide 7 (Myh7), myosin heavy polypeptide 3 (Myh3), troponin T3 (TnnT3), myoglobin (Mb), myosin light chain phosphorylatable fast skeletal muscle (Mylpf), myomesin family member 3 (Myom3), and...
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Myogenin (supplemental Table S2). However, in our microarray analysis, MyoD also showed no significant change upon overexpression of miR-148a. We verified six of the up-regulated genes (Tnnt3, Mb, Mylpy, Myom3, myogenin, and IGF2) by using RT-qPCR (Fig. 4C). The up-regulation of these muscle-specific genes seemed to be related to the positive effect of miR-148a on myogenic differentiation.

**MiR-148a Regulates ROCK1 Expression at the Protein Level during Myogenesis**—Through computational target prediction programs (TargetScan and miRanda), we identified Rho-associated coiled-coil containing protein kinase 1 (ROCK1) as the potential target of miR-148a. To determine whether miR-148a can directly target ROCK1, we engineered luciferase reporters that included either the wild-type or mutant 3′-UTR of ROCK1 (Fig. 5A). The luciferase reporters were co-transfected with miR-148a mimics or negative control duplexes into C2C12 myoblasts cultured in growth medium at 24 h, 48 h, 72 h, and 96 h. Each value represents the mean of three measurements. β, cells were collected for cell cycle analysis 36 h after transfection. Propidium iodide staining for DNA content and FACS was used to determine the percentage of cells in G1, S, and G2. *, p < 0.05.

The previous studies report that ROCK1 is a negative regulator of skeletal muscle differentiation. In our studies, we also performed ROCK1 knockdown experiment in C2C12 myoblast and primary muscle cells to verify its negative regulatory function on myogenic differentiation. As shown in Fig. 5H, anti-ROCK1 siRNA almost completely diminished its protein expression in C2C12 cells cultured in GM. And the reduction of ROCK1 significantly accelerated C2C12 myoblast differentiation, as indicated by an increase in expression of MHC and myogenin. Similarly, the primary muscle cells transfected with anti-ROCK1 siRNAs showed a reduced expression of ROCK1, unaltered levels of myogenin and a marked increase in MHC expression.

**DISCUSSION**

The development of skeletal muscle is a well-coordinated process of cell proliferation, differentiation and migration that is controlled by evolutionarily conserved networks of myogenic transcription factors (3). Recent observations have revealed the importance of small (20–25 base pair) non-coding RNAs (miRNAs) in the regulation of mammalian skeletal muscle development.

In our work, we identified miR-148a as a novel miRNA that plays a active role in skeletal muscle development. Our study reported for the first time that miR-148a expression was up-regulated during myogenesis and functioned as a positive regulator of myogenic differentiation. The function of miRNAs appears to be in gene regulation. To determine miR-148a effects on global gene expression patterns, we overexpressed miR-148a in C2C12 myoblast cells and employed Agilent microarray analysis to identify differentially expressed genes. Myogenin and IGF2, the critical positive regulators of myogenesis, were significantly up-regulated according to the microarray data, indicating that miR-148a may induce differentiation. The increased expressions of some muscle-fiber genes (Myl4, Myh7, Tnnt3, Mylpy, and Myom3) seemed to be related to the stimulation of myoblast differentiation by miR-148a.

G1 phase cell cycle arrest is a critical step in the differentiation of myoblasts into myotubes. Many miRNAs have been implicated in the cell cycle quiescence and in the differentiation pathways that underlie the transition from myoblast to myotube. For example, miR-206 and miR-214 promote myogenic
differentiation by facilitating the exit from the cell cycle (34, 44). The miR-322/424 and-503 can promote cell cycle quiescence and differentiation via the down-regulation of cell division cycle 25 homolog A (Cdc25A) (54). In our work, we found that miR-148a was involved in the regulation of the cell cycle progression. The FACS profile for DNA content demonstrated that transfecting miR-148a increased the G1 phase population and decreased the S phase population of C2C12 myoblast cells, indicating that it promoted cell cycle quiescence. Thus, the role of miR-148 in cell cycle progression requires further study. However, we were unable to detect any miR-148a-dependent effects on cell proliferation in the cell growth assay. The results suggested that the miR-148a-mediated acceleration of myogenesis was not accompanied by inhibition of cell proliferation.

The identification of targets is critical for deciphering the functional characterization of miRNAs. The functions and targets of miR-148a have been reported in several cancer cells, but our findings revealed for the first time, a myogenic role for miR-148a and a target, ROCK1. The mutation of the seed region of the predicted miR-148a binding site abolished the suppression of the luciferase activity by miR-148a, indicating that miR-148a was a major, if not the only, miRNA regulator of ROCK1. We further examined the effect of miR-148a on the mRNA and protein levels of ROCK1. Our data indicated that
the overexpression of miR-148a decreased ROCK1 expression at the protein level without significantly affecting mRNA levels, suggesting that miR-148a inhibited ROCK1 at a translational level.

ROCK1 is a well-known downstream effector of RhoA and can be activated when bound to the GTP-bound form of RhoA. In myogenic cells, the RhoA/ROCK pathway has been shown to play a critical role in myogenic differentiation. The RhoA-dependent activation of SRF controls the expression of muscle-specific genes. Thus, RhoA has been proposed to be a positive regulator of myogenesis. In contrast, some studies have reported that the expression of constitutively active RhoA inhibits the muscle differentiation process and that ROCK1 can prevent myoblast exit from the cell cycle and commitment to differentiation (19, 55). ROCK can directly phosphorylate FKHR, an essential regulator of myocyte fusion, resulting in the retention

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FIGURE 5. ROCK1 is a target for miR-148a. A, predicted miR-148a target site in the 3′-UTR of mouse ROCK1. The seed region (in bold) was deleted in the mutant 3′-UTR reporter. B, C2C12 myoblasts were transfected with the ROCK1–3′-UTR mutant or wild-type luciferase reporters and co-transfected with miR-148a mimics or negative control duplexes. The relative luciferase activity was measured 48 h later. The data represent the mean ± S.D. from three independent experiments performed in duplicate (**, p < 0.01). C and D, C2C12 myoblasts were transfected with miR-148a mimics or negative control duplexes. Total RNA and whole cell extracts were harvested at 48 h after transfection and analyzed for ROCK1 mRNA expression using RT-qPCR (C) or protein expression using immunoblots (D). E, C2C12 cells were transfected with miR-148a mimics or negative control duplexes and placed under differentiation conditions. The extracts were analyzed at the indicated times using an anti-ROCK1 antibody or an anti-GAPDH antibody as the control. F, C2C12 myoblasts were transfected with a 2′-O-methyl antisense oligonucleotide inhibitor of miR-148a or the negative control. Total whole cell extracts were harvested 48 h after transfection and analyzed for ROCK1 protein expression using immunoblots. G, primary muscle cells were transfected with miR-148a mimics or negative control duplexes and placed under growth conditions or differentiation conditions. The extracts were analyzed at 48 h using ROCK1 antibody. H, C2C12 myoblasts were transfected with anti-ROCK1 siRNA or negative control duplexes and placed under growth conditions or differentiation conditions for 48 h before immunoblotting for ROCK1, MHC, or myogenin. Primary muscle cells were transfected with anti-ROCK1 or negative control duplexes and placed under differentiation conditions for 48 h before immunoblotting for ROCK1, MHC or myogenin.
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![Diagram](image)

FIGURE 6. A model for the regulatory network involving miR-148a and ROCK1 in myogenesis. The model proposes that miR-148a down-regulates the expression of ROCK1, relieving its suppression of myogenesis.

of FKHR in the cytoplasm (56). When ROCK activity is decreased, FKHR can translocate into the nucleus and form a complex with MRTF-A-Smad, which suppresses the transcription of the Id3 gene. Thus, the inactivation of ROCK is a prerequisite for the FKHR nucleus localization and myogenic differentiation (20, 57). In addition, Lim et al. have reported that the Rhoa/ROCK pathway blocks muscle differentiation by phosphorylating IRS proteins at serine residues, resulting in the decreased IRS-1/2 tyrosine phosphorylation and PI 3-kinase activity (58). Our study demonstrated that the up-regulation of miR-148a significantly accelerated myogenesis and repressed the expression of ROCK1 during myogenic differentiation. And we also confirmed the results that the reduction of ROCK1 by siRNA significantly promoted myogenic differentiation. Thus, the miR-148a-mediated repression of ROCK1 may be an important regulatory mechanism that positively modulates myogenic differentiation (Fig. 6).

In conclusion, we suggested that miR-148a up-regulation is an important step in the G1 quiescence and myogenic differentiation. Differentiation was accelerated by the overexpression of miR-148a but delayed by miR-148a inhibition. We also found that miR-148a directly targeted the ROCK1 gene at the translational level during differentiation. And ROCK1 inhibition with siRNA significantly promoted myogenic differentiation. These results revealed a novel microRNA-mediated regulation mechanism in which miR-148a positively regulated myogenic differentiation via ROCK1 down-regulation.

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