MicroRNA-27a promotes myoblast proliferation by targeting myostatin

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ABSTRACT

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that play critical roles in skeletal muscle development as well as in regulation of muscle cell proliferation and differentiation. However, the role of miRNAs in myoblast proliferation remains poorly understood. Here we found that the expression of miR-27a was increased during proliferation of C2C12 myoblasts. Moreover, overexpression of miR-27a in C2C12 cells promoted myoblast proliferation by reducing the expression of myostatin, a critical inhibitor of skeletal myogenesis. In addition, the miR-27a was confirmed to target myostatin 3′UTR by a luciferase reporter analysis. Together, these results suggest that miR-27a promotes myoblast proliferation through targeting myostatin.

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1. Introduction

Skeletal muscle development (myogenesis) is a multistep process that commences with the commitment of multipotent precursor cells to myoblasts, followed by proliferation, withdrawal from the cell cycle, differentiation and fusion into multinuclear myotubes and then myofibers [1,2]. During the highly orchestrated process, myoblast proliferation is an early cellular event critical for myogenesis, which is controlled by a multitude of signaling cascades initiated by various autocrine/paracrine growth factors and cytokines [3]. Among them, myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, has been well established to be a critical inhibitor of skeletal myogenesis and play a crucial role in myoblast proliferation [4–9]. However, the regulatory mechanisms of myostatin during myoblast proliferation remain unclear.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs of ∼22 nucleotides, which negatively regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions (3′UTRs) of target mRNAs [10,11]. miRNAs have been shown to play critical roles in skeletal muscle development as well as in regulation of muscle cell proliferation and differentiation [reviewed in Ref. [12]]. Studies using C2C12 myoblasts showed that miR-133 promoted their proliferation [13], whereas miR-1 and miR-206 enhanced their differentiation [13,14]. Moreover, miR-24, miR-26a, miR-322/424, miR-503 and miR-486 were shown to positively regulate myoblast differentiation [15–18], whereas miR-221/miR-222 and miR-125b were found to have the opposite effects [19,20]. It would be reasonable to speculate that more miRNAs that govern myoblast proliferation and differentiation are yet to be discovered.

The miR-27 family consists of two members (miR-27a and miR-27b) located at different chromosomes (mouse chromosome No. 8 and human chromosome No. 19 for miR-27a; mouse chromosome No. 13 and human chromosome No. 9 for miR-27b). It has been shown that both miR-27a and miR-27b could inhibit various aspects of adipogenesis [21–23] and promote myeloblast differentiation [24]. Moreover, it has been demonstrated that miR-27a could facilitate the growth of cancer cells [25,26]. However, a function for miR-27a in myoblast proliferation has not been reported. In the present study, we investigated the role of miR-27a in myoblast proliferation. We demonstrated that miR-27a can target the 3′UTR of *myostatin* transcript and downregulate *myostatin* expression, thereby promoting myoblast proliferation.

2. Materials and methods

2.1. Cell culture and transfection

Mouse C2C12 myoblasts (CRL-1772) and human embryonic kidney (HEK) 293T cell line (CRL-11268) were from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), and antibiotics (100 U/mL penicillin and 100 μg/L streptomycin) (Invitrogen) at 37 °C in the 5% CO_2, humidified atmosphere. C2C12 and HEK 293T cells were transfected with plasmid DNA, miR-27a mimics or miR-27a inhibitor (RiboBio, Guangzhou, China) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

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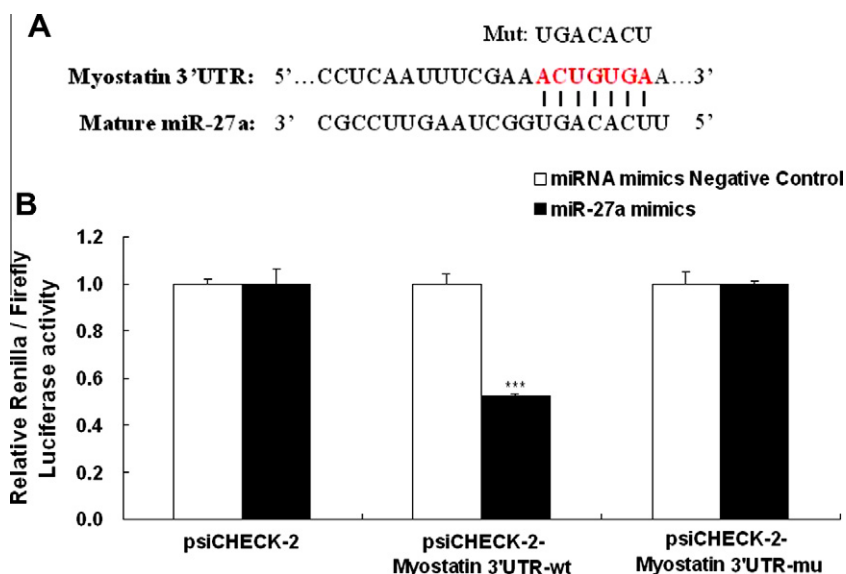E-mail address: dwchen@sicau.edu.cn (D. Chen).

Fig. 1. Targeting site of miR-27a in the myostatin 3′UTR. (A) Predicted miR-27a target site in the 3′UTR of mouse myostatin. The wild type mouse myostatin mRNA sequence is shown with potential binding sites indicated in red. The highly conserved mature miR-27a sequence in mammals and potential binding between the miR-27a seed region to the mouse myostatin 3′UTR sequence are shown and mutated bases are indicated above. (B) Mouse myostatin 3′UTR reporter, its miR-27a site mutant, or vector was cotransfected with either miR-27a mimics or miRNA mimics negative control into HEK 293T cells, followed by cell lysis and luciferase assays 48 h later. Data were presented as mean ± SE (n = 3). ***P < 0.001 as compared with negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Plasmid construction

For myostatin 3′UTR reporter assay, the entire 3′UTR of mouse myostatin was PCR-amplified from mouse genomic DNA and cloned into psiCHECK-2 dual-luciferase reporter plasmid (Promega, Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene to generate psiCHECK-2-Myostatin3′UTR-wt. The mutant mouse myostatin 3′UTR reporter, designated as psiCHECK-2-Myostatin3′UTR-mu, was created by mutating the seed region of the predicted mmu-miR-27a site (ACUGUGA to UGACACU) by nested PCR.

2.3. Luciferase reporter assay

For luciferase reporter assay, HEK 293T cells (2.0×10^4 cells per well) were plated in a 24-well plate (Corning) 24 h before transfection. Cells were cotransfected with 0.5 μg of either the psiCHECK-2-Myostatin3′UTR-wt, psiCHECK-2-Myostatin3′UTR-mu or empty vector psiCHECK-2, and 50 nM of either miRNA mimics Negative Control or miR-27a mimics (RiboBio). After a transfection of 48 h, cells were lysed in Passive Lysis Buffer (Promega) and activities of *Firefly* and *Renilla* luciferase were measured with a GloMax® 20/20 Luminometer (Promega) using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocols.

2.4. RNA isolation and reverse transcription

Total RNA was extracted from the adherent cultured C2C12 cells using RNAiso Plus reagent (TaKaRa, Dalian, China) according to the manufacturer's directions. The concentrations of total RNA were determined spectrophotometrically using a Beckman Coulter DU800 (Beckman Coulter, Fullerton, CA, USA). A first-strand complementary DNA was prepared from 1 μg of total RNA from each sample using a PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa) according to the protocols of the manufacturer.

2.5. Quantitation of mRNA expression

Real-time quantitative PCR was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the
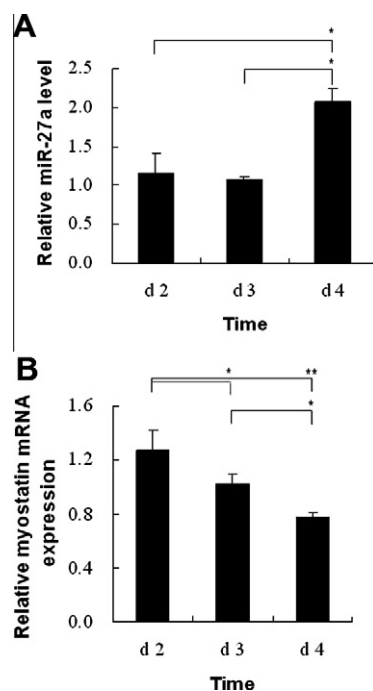

Fig. 2. Relative expressions of endogenous miR-27a and myostatin during myoblast proliferation. C2C12 cells were seeded in 35 mm dishes at 4.0×10^4 cells per dish. The cells were harvested 2–4 days after initial plating and RNA was extracted. miR-27a level (A) and *myostatin* mRNA (B) were determined using real-time quantitative PCR. The amount of miR-27a and *myostatin* mRNA were normalized to the amount of U6 snRNA and *GAPDH* mRNA, respectively. Data were the mean and standard errors from three independent experiments performed in duplicate. *P < 0.05 and **P < 0.01 as compared with negative control.

SsoFast EvaGreen Supermix (Bio-Rad) in a final volume of 20 μL. The thermal cycling conditions started with 98 °C for 2 min followed by 45 cycles of 98 °C for 2 s and 58 °C for 5 s. The following primers were used: mouse *myostatin* (forward) 5′-GATGGGAC TGGATTA TCGC-3′ and (reverse) 5′-GCACAAGATGAGTATGCGG-3′; mouse *GAPDH* (forward) 5′-AGGGCATCTTGGGCTA CAC-3′ and (re-verse) 5′-TGGTCCAGGGTTTCTTACTCC-3′. A single sharp peak and a single band of the expected size were observed in the melting curve and in the agarose gel, respectively. Identities of the products were confirmed by DNA sequencing. Relative mRNA expression was quantified using the efficiency-corrected comparative Ct method [27] with GAPDH as an endogenous control.

2.6. Detection of miRNA expression

Total RNA was extracted from cultured cells using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). The concentration of each sample was determined using a Beckman Coulter DU 800 Spectrophotometer (Beckman Coulter). Ten nanograms of total RNA was reverse transcribed using a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster, CA, USA). Expression of mature miR-27a was assayed using TaqMan® MicroRNA Assay kit (Applied Biosystems) specifically designed for miR-27a in a CFX96 Real-Time PCR Detection System (Bio-Rad) following the

manufacturer's recommendations. Relative miRNA expression level was analyzed using the comparative Ct method [28], with U6 snRNA (Applied Biosystems) as the internal control for normalization.

2.7. EdU proliferation assay

Proliferating C2C12 cells were determined by using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) according to the manufacturer's protocol. Briefly, cells were incubated with 10 μM EdU for 3 h before fixation, permeabilization, and EdU staining. Cell nuclei were stained with Hoechst 33342 (Invitrogen) at a concen-tration of 5 μg/mL for 30 min.

EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analog of thy-midine that is incorporated into DNA during active DNA synthesis only by proliferating cells. After incorporation, a fluorescent mole-cule was added that reacted specifically with EdU, making possible fluorescent visualization of proliferating cells.

2.8. Statistical analysis

Data expressed as mean ± SE were compared by one-way ANOVA followed by Tukey's tests (SPSS Inc., Chicago, IL, USA). Differences were regarded as significant at a level of $P < 0.05$.
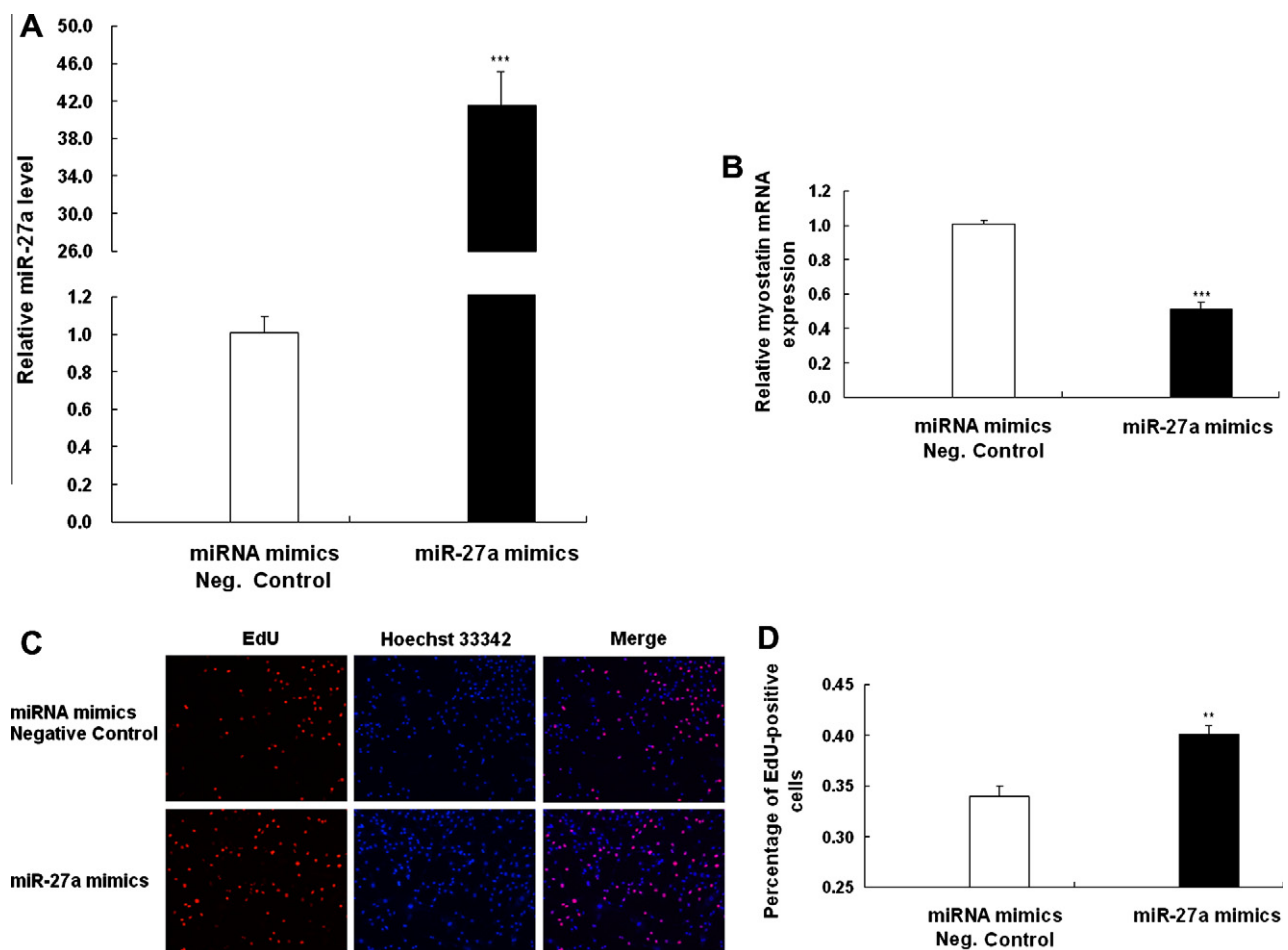

Fig. 3. Overexpression of miR-27a promotes myoblast proliferation. C2C12 cells were seeded in a 24-well plate at a density of 1.0×10^4 cells per well. After 48 h, the cells were transfected with 50 nM of either miRNA mimics negative control or miR-27a mimics. Transfection mix was removed 6 h later and cells were grown for another 18 h in DMEM/0.5%FBS medium. (A) The amount of miR-27a against U6 snRNA was measured by real-time quantitative PCR. Data were the mean and standard errors from three independent experiments performed in duplicate. (B) The amount of *myostatin* mRNA against *GAPDH* mRNA was measured by real-time quantitative PCR. Results were the mean and standard errors from three independent experiments performed in duplicate. (C) Proliferating C2C12 cells were labeled with EdU. The Click-it reaction revealed EdU staining (red). Cell nuclei were stained with Hoechst 33342 (blue). The images are representative of the results obtained. (D) The percentage of EdU-positive C2C12 cells were quantified. Data were presented as mean ± SE ($n = 6$). **$P < 0.01$ and ***$P < 0.001$ as compared with negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Targeting site of miR-27a in the myostatin 3′UTR

To identify miRNAs which could modulate myoblast proliferation, we have screened miRNAs which potentially suppress myostatin expression because myostatin is a critical inhibitor for skeletal myogenesis. miRNA target site prediction for mouse myostatin was performed with the use of the following algorithms: TargetScan (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi), MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), miRanda (http://www.microrna.org/microrna/getGeneForm.do) and miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi). We found a binding site for miR-27a in the 3′UTR of mouse *myostatin* (Fig. 1A). To confirm mouse myostatin as a real miR-27a target, the entire wild-type 3′UTR of mouse *myostatin* or the mutant 3′UTR with a 7 bp mutation in the seed region was inserted downstream of the luciferase gene and assayed in HEK 293T cells. As shown in Fig. 1B, cotransfection of miR-27a mimics with the mouse *myostatin* 3′UTR reporter resulted in a highly significant decrease (48%) in luciferase activity. Consistent with the data, no decrease in luciferase activity was observed when miR-27a mimics was transfected into HEK 293T cells together with the mutant reporter (Fig. 1B), indicating that the predicted site is a direct target of miR-27a and it is solely responsible for miR-27a targeting of the mouse *myostatin* 3′UTR.

3.2. miR-27a is upregulated and myostatin is downregulated during myoblast proliferation

To characterize the growth rate of C2C12 cells, the cells were seeded in 35 mm dishes at 4.0×10^4 cells per dish and harvested and counted at various times. The result indicated that C2C12 cells entered the logarithmic phase of growth after 2 days and grew extremely slow after 4 days (Fig. S1). Next, we assessed the expressions of miR-27a and myostatin at days 2, 3 and 4. As shown in Fig. 2, mature miR-27a was upregulated and myostatin was downregulated during C2C12 myoblasts proliferation.

3.3. Overexpression of miR-27a promotes myoblast proliferation

The reciprocal expression of miR-27a and myostatin prompted us to examine whether miR-27a could modulate myoblast proliferation. To accomplish this aim, C2C12 cells were transfected with miRNA mimics Negative Control or miR-27a mimics and EdU incorporation experiments were performed to assess its proliferation. As shown in Fig. 3A, miR-27a mimics increased miR-27a level in C2C12 cells. Under these conditions, the level of mouse myostatin mRNA in the miR-27a mimics-transfected cells reduced 49%, compared with cells transfected with the miRNA mimics negative control (Fig. 3B), supporting the idea that miR-27a is capable to target the myostatin 3′UTR to prevent myostatin expression. Importantly, miR-27a mimics accelerated proliferation of C2C12 cells (Fig. 3C). Quantitative analysis demonstrated that this change is statistically significant (Fig. 3D). Together, these results clearly indicate that myostatin 3′UTR targeting miR-27a could suppress myostatin expression, resulting in promotion of myoblast proliferation.

4. Discussion

miRNAs, as endogenous small molecular regulators of gene expression, play important roles in various aspects of skeletal myogenesis [13–20,29]. In the present study, we identified that miR-27a can promote myoblast proliferation by targeting myostatin. Several lines of evidences support this idea. First, expression of miR-27a was increased during myoblast proliferation while myostatin expression was decreased. Second, miR-27a specifically repressed luciferase reporter gene containing the myostatin 3′UTR through its binding site. Finally, overexpression of miR-27a in C2C12 cells potently promoted myoblast proliferation via downregulation of myostatin expression.

As a critical inhibitor of skeletal myogenesis both *in vitro* and *in vivo*, it is not surprising that the expression of myostatin is tightly controlled at multiple levels. At the posttranscriptional level, miR-208a, miR-499 and miR-27b have been previously reported to negatively regulate myostatin expression [30–32]. Moreover, a "G" to "A" transition in the 3′UTR of myostatin creates a target site for miR-1 and miR-206 causing translational inhibition of myostatin expression, resulting in the muscle hypertrophy of Texel sheep [33]. In this study, we confirmed that myostatin is indeed a target of miR-27a and found that the expression of myostatin was negatively regulated by miR-27a. It should be noted that mature miR-27a sequence (5′-UUCACAGUGGCUAAGUUCCGC-3′) is well conserved among mammalian species. The potential miR-27a binding sites (5′-ACUGUGA-3′) are also observed on the known myostatin 3′UTR of rat, dog, horse and cattle, suggesting that miR-27a might regulate myostatin expression of these species at the posttranscriptional level. Taken together, these results indicate that posttranscriptional regulation of myostatin by miRNAs may be a major determinant of myostatin expression.

The functions of mammalian miR-27a and its targets have been reported in several cellular contexts. Kim et al. demonstrated that miR-27a suppressed adipocyte differentiation through targeting PPARγ [23]. Feng et al. showed that miR-27a enhanced differentiation of myeloblasts into granulocytes via posttranscriptional downregulation of Runx1 [24]. Moreover, suppression of miR-27a inhibited the growth of breast and gastric cancer cells by targeting FOXO1 and prohibitin, respectively [25,26]. In this study, we identified for the first time a myogenic role for miR-27a and a new target, myostatin. We showed that miR-27a promoted myoblast proliferation via targeting myostain.

In summary, we identified miR-27a as another miRNA that plays a role in myoblast proliferation. We also provided the first evidence that myostatin is a target of miR-27a. In the future, it will be interesting to determine whether miR-27a could modulate myoblast differentiation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.106.

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