MicroRNA-27a promotes porcine myoblast proliferation by downregulating *myostatin* expression


Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Institute of Animal Nutrition, Sichuan Agricultural University, Chengdu, Sichuan 611130, P. R. China

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**MicroRNAs** are endogenous ~22nt RNAs that negatively regulate gene expression at the posttranscriptional level via binding to the 3′-untranslated region (3′UTR) of target mRNAs. The microRNA miR-27a was reported to depress the expression of myostatin, a critical inhibitor of skeletal myogenesis, by binding to its 3′UTR in mouse. In this study, we cloned the full-length 3′UTR of porcine myostatin by rapid amplification of 3′cDNA ends (3′-RACE) and demonstrated that the 3′UTR of porcine myostatin is targeted by miR-27a. The phenomenon that the level of myostatin inversely correlated with miR-27a was observed in fat and heart of pigs and also in proliferating porcine myoblasts. Besides, overexpression of miR-27a in porcine myoblasts promoted cell proliferation by reducing the expression of myostatin. Our data suggest that miR-27a positively regulates porcine myoblast proliferation via targeting myostatin.

**Keywords:** miR-27a, porcine *myostatin*, 3′UTR, cloning, porcine myoblast proliferation

**Implications**

Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, is a critical autocrine/paracrine inhibitor of skeletal muscle growth. Myostatin plays an important role in myoblast proliferation and differentiation. In this study, a miR-27a binding site in the 3′-untranslated region of porcine *myostatin* was identified. There was an inverse correlation between miR-27a and *myostatin* expression in fat and heart of pigs as well as in proliferating porcine myoblasts. We identified that miR-27a could positively regulate porcine myoblast proliferation via targeting *myostatin*. This work may lead to new strategies for blocking porcine myostatin activity and improving lean meat percentage of pigs.

**Introduction**

Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, is a critical autocrine/paracrine inhibitor of skeletal muscle growth, and is expressed in developing muscle of embryos and muscle of adult animal (McPherron *et al.*, 1997; Lee, 2004). Myostatin plays a crucial role in myoblast proliferation and differentiation (Huang *et al.*, 2011). However, the regulatory mechanisms of myostatin during myoblast proliferation and differentiation remain largely unknown.

MicroRNAs (miRNAs) are endogenous ~22nt RNAs that negatively regulate gene expression at the posttranscriptional level via binding to the 3′-untranslated region (3′UTR) of target mRNAs (Gladka *et al.*, 2012). MicroRNAs are able to suppress translation and/or induce mRNA degradation (Tanzer and Stadler, 2004). Translational control via the 3′UTR can affect formation of the closed loop translation initiation complex, ribosome binding or a post-initiation step (de Moor *et al.*, 2005). The sequence of mature miRNAs are well conserved, whereas the sequence and length of 3′UTR of miRNAs are different among mammalian species (Bartel, 2004). Therefore, it seems especially essential to explore miRNAs regulating target gene expression starting from the animal itself.

MiR-27a, a member of miR-27 family, which has negative impacts on kinds of cells through different pathways, has been demonstrated to promote murine myoblast proliferation and differentiation through targeting *myostatin* in our previous studies (Huang *et al.*, 2012; Chen *et al.*, 2014). Nevertheless, we have no idea whether miR-27a could also promote porcine myoblast proliferation via downregulation of *myostatin* expression. In this study, we cloned the full-length...
3'UTR of porcine myostatin by rapid amplification of 3'-cDNA ends (3'-RACE), demonstrated that the 3'UTR of porcine myostatin is targeted by miR-27a through luciferase reporter assay, and further confirmed that miR-27a suppresses myostatin expression, thereby accelerating porcine myoblast proliferation.

Material and methods

Cloning of porcine myostatin 3'UTR
To obtain the full-length of porcine myostatin 3'UTR, 3'-RACE experiment was performed using a 3'-Full RACE Core Set Ver.2.0 Kit (TaKaRa, Dalian, China) according to the manufacturer’s directions. The gene-specific primer used in 3'-RACE was 5'-GATTTCACTATAGG-3' (forward) and the 3' RACE inner primer (5'-CGCGGATCCTCCACTAGT GATTTCATAGG-3') (reverse). The purified PCR product was cloned into pMD19-T vector (TaKaRa) and sequenced, resulting in pMD19-T- pMyostatin3'UTR.

Plasmid construction
The wild type 3'UTR of porcine myostatin was PCR-amplified using the specific primers (forward primer 5'-CCGCTCGAG GATTATATTTGCTTACATC-3' and reverse primer 5' -CCTCGAGTCTTACTGAGG-3') and the recombinant plasmid pMD19-T-pMyostatin3'UTR as a template. The forward and reverse primers introduced Xho I and Not I sites (underlined), respectively. After digestion with Xho I and Not I, the PCR product was cloned into psiCHECK-2 dual-luciferase reporter vector (Promega, Madison, WI, USA) to generate psiCHECK-2-pMyostatin3'UTR-wt. The mutant porcine myostatin 3'UTR reporter, designated as psiCHECK-2-pMyostatin3'UTR-mu, was created by mutating the seed region of the predicted ssc-miR-27a site (ACUGUGA to UGACACU) by nested PCR.

Luciferase reporter assay
The human embryonic kidney (HEK) 293T cell line (CRL-11268) was from the American Type Culture Collection (Rockville, MD, USA). Luciferase reporter assay was performed as previously described (Huang et al., 2012). Briefly, the HEK 293T cells were plated into a 24-well plate and maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) at 37°C, 5% CO2 in a humidified atmosphere. After 24 h, cells were transfected with 0.5 µg of either the psiCHECK-2-pMyostatin3'UTR-wt, psiCHECK-2-pMyostatin3'UTR-mu or empty plasmid psiCHECK-2, and 50 nM of either miRNA mimics Negative Control or miR-27a mimics (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. Forty-eight hours after the transfection, cells were lysed in Passive Lysis Buffer (Promega) and Firefly and Renilla luciferase activities were measured with a GloMax 20/20 Luminometer (Promega) using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Animals and tissue sample collection
Three 10-week-old female DLY pigs (BW of 31 ~ 31.6 kg) were slaughtered in a humane manner according to protocols approved by the Animal Care Advisory Committee of Sichuan Agricultural University. The heart, fat, psoas major (PM) muscle, longissimus lumborum (LL) muscle, extensor digitorum longus (EDL) muscle and Soleus (SOL) muscle were removed and immediately snap frozen in liquid nitrogen before being stored at −80°C for RNA isolation.

Cell isolation and culture
Porcine myoblasts were isolated from longissimus lumborum muscle of 3-day-old male DLY pigs. The pig obtained from the farm of Sichuan Agriculture University was slaughtered according to protocols approved by the Animal Care Advisory Committee of the University. Briefly, muscle was removed and washed twice in cold PBS (pH 7.4) under sterile conditions, and then cut into small pieces with scissors. The dissected muscle was digested with 0.2% collagenase type I (Sigma, St. Louis, MO, USA) for 2 h at 37°C in a water bath. A single cell suspension was obtained after filtering the samples through 37-µM mesh, and then collected cells were purified by differential adhesion method. The cell suspension were plated on rat-tail collagen coated-flask and cultured in DMEM/Ham’s F-12 (1 : 1) medium (Invitrogen) supplemented with 20% FBS (Invitrogen), 5 nM FGF-basic (Invitrogen) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Invitrogen). Cells were maintained at 37°C in a saturated humidly atmosphere containing 95% air and 5% CO2, with the medium being renewed every 2 days. The cells were identified by immunofluorescence with anti-myosin heavy chain antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA isolation and reverse transcription
Total RNA was extracted from the collected tissue samples and adherent cultured porcine myoblasts using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The concentrations of total RNA were determined spectrophotometrically using a Beckman Coulter DU800 (Beckman Coulter, Fullerton, CA, USA). Reverse transcription was done with 500 ng total RNA from each sample using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer’s instructions.

miR-27a overexpression
Porcine myoblasts were cultured in 24-well plates (Corning) and transfected with 50 nM of miR-27a mimics or 50 nM of miRNA mimics Negative Control (Ribobio) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions.
miR-27a, MSTN and porcine myoblast proliferation

Real-time quantitative PCR
Real-time quantitative PCR was performed in a 7900HT real-time PCR system (384-cell standard block) (Applied Biosystems, Foster, CA, USA) using the iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) in a final volume of 10 μl. The primers for quantification of mRNA expression as follows: porcine myostatin (forward) 5′-TCT GAATGAGAACGCGAGCAA-3′ and (reverse) 5′-TCGTAGCCGTA-3′; porcine β-actin (forward) 5′-CATCGTCCA ACCGCAAT-3′ and (reverse) 5′-TGTCACCTTACCGTCC-3′. The Bulge-Loop™ miRNA qPCR Primer Sets specific for miR-27a and U6 were purchased from Ribobio. The PCR conditions were as follows: predenaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. A single sharp peak was detected in the melting curve. Relative expression of the transcript/miRNA was calculated using comparative C_{T} method (Livak and Schmittgen, 2001) and was normalized by β-actin or U6 expression, respectively.

EdU proliferation assay
EdU (5-ethyl-2′-deoxyuridine) is a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis only by proliferating cells. Proliferating porcine myoblasts were determined by using the Click-IT EdU Alexa Fluor 594 Imaging Kit (Invitrogen). EdU proliferation assay was performed as previously described (Huang et al., 2012), with minor modification. Briefly, porcine myoblasts were incubated with 10 μM EdU for 6 h before fixation, permeabilization, and EdU staining. Cell nuclei were stained with Hoechst 33342 (Invitrogen) at a concentration of 5 μg/ml for 30 min.

Statistical analysis
Data expressed as mean ± s.e. were compared by one-way ANOVA followed by Tukey’s tests (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to be statistical significant.

Results

Cloning and sequence analysis of porcine myostatin 3′UTR
DNA sequencing showed that the length of amplified fragment is 638 bp. The nucleotide sequence of porcine myostatin 3′UTR has been deposited in GenBank database under accession number KC299915. Sequence analysis showed that porcine myostatin 3′UTR cloned in this study shares 99.36% homology with the known porcine myostatin 3′UTR (GenBank accession no. AF033855). A polyA tail was observed at 14 nucleotides downstream from typical polyadenylation site (AATAAA). A potential binding site for miR-27a was found at its N-terminus (ACTGTGA) (63–70 bp), indicating that porcine myostatin might be a miR-27a target.

The 3′UTR of porcine myostatin is targeted by miR-27a
To examine whether porcine myostatin 3′UTR can be directly targeted by miR-27a, the entire wild-type 3′UTR of porcine myostatin or the mutant 3′UTR with 7 bp mutation in seed region was cloned downstream of the luciferase gene and assayed in HEK 293T cells. As shown in Figure 1, a highly significant decrease (56%) was observed in luciferase activity when miR-27a mimics and the wild-type porcine myostatin 3′UTR reporter were cotransfected into HEK 293T cells. By contrast, cotransfection of miR-27a mimics with the mutant porcine myostatin 3′UTR reporter didn’t result in decrease in luciferase activity (Figure 1), indicating that the predicted site is a direct target of miR-27a.

miR-27a level inverse correlates with porcine myostatin mRNA expression in vivo and in vitro
The expression profiles of porcine myostatin mRNA and miR-27a were determined by real-time quantitative PCR in various tissues. As shown in Figure 2, porcine myostatin mRNA was abundant in the EDL muscle, LL muscle, SOL muscle and PM muscle, followed by the heart and fat. MiR-27a level was most abundant in the fat and was least in the heart in examined tissues (Figure 2). There was an inverse correlation between myostatin and miR-27a expression in fat and heart of pigs (Figure 2).

To determine the correlation with expressions of myostatin mRNA and miR-27a in vitro, porcine myoblasts were seeded
in a 24-well plate at $2 \times 10^4$ cells per well. After 8 days, the cells reached ~100% confluence (Supplementary Figure S1). Then, we detected the expressions of myostatin mRNA and miR-27a at day 2, 4, and 6. As show in Figure 3, the expression of mature miR-27a was up-regulated and myostatin was downregulated during porcine myoblasts proliferation.

**Overexpression of miR-27a promotes proliferation of porcine myoblasts**

A miR-27a binding site in the porcine myostatin 3'UTR and negative correlation between mature miR-27a and porcine myostatin transcription gave us the motion to examine whether miR-27a could modulate proliferation of porcine myoblasts. In order to verify this point, porcine myoblasts were transfected with miR-27a mimics or miRNA mimics Negative Control and EdU incorporation experiments were performed to assess its proliferation. As shown in Figure 4a, compared to the cells transfected with miRNA mimics Negative Control, miR-27a mimics transfected cells had a significance increased in miR-27a level. By contrary, level of porcine myostatin transcript reduced 35% (Figure 4b). In EdU proliferation assay, miR-27a mimics transfected cells had higher proliferation (Figure 4c). These data suggest that the miR-27a targeted 3'UTR of porcine myostatin and suppressed porcine myostatin expression, then promoted proliferation of porcine myoblasts.
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Discussion

Myostatin belongs to the TGF-β superfamily and is known to play an essential role in the regulation of skeletal muscle mass (Jouila-Ekaza and Cabello, 2006). The complete CDS of myostatin has been cloned from swine (Ji et al., 1998), cattle (Grobet et al., 1997), horse (Hosoyama et al., 2002) and sheep (Sharma et al., 1999). miRNAs negatively regulate gene expression at the posttranscriptional level via binding to the 3'UTR of target mRNAs (Bartel, 2004; Tanzer and Stadler, 2004; de Moor et al., 2005; Gladka et al., 2012). The myostatin 3'UTRs of some species have been known, such as 1552 bp (1262–2814 bp) in goats, 1506 bp (1261–2767 bp) in cattle, 498 bp (1234–1732 bp) in rat (data from GenBank database) and so on. In this study, we cloned the full-length porcine myostatin 3'UTR (638 bp) by 3'-RACE. These results suggested that the complete CDS of myostatin is well conserved among mammalian species, but the length of myostatin 3'UTR is different among different species.

Myostatin has been reported to be regulated by several miRNAs, such as miR-208a (Callis et al., 2009), miR-27b (Allen and Loh, 2011) and miR-499 (Zhang et al., 2012). In Texel sheep, the myostatin allele characterized by a G to A mutation in myostatin 3'UTR creates a target site for miR-1 and miR-206 (Clop et al., 2006). High expression of miR-1/206 in muscle inhibits transcript level of myostatin, which contributes to muscular hypertrophy (Clop et al., 2006). In our previous work, we demonstrated that miR-27a suppresses transcript level of mouse myostatin (Huang et al., 2012). It should be pointed out that mature miR-27a sequence (5'-UUCAUGGCUAGUUGCGC-3') is well conserved among mammalian species. In the known myostatin 3'UTR of rat, dog, horse and cattle, the potential miR-27a binding site (5'-ACUGUGA-3') is found, suggesting that miR-27a might regulate myostatin expression of these species at the posttranscriptional level. In this study, we found a potential miR-27a target site in 3'UTR of porcine myostatin, and we demonstrated that the porcine myostatin is a bona fide target of miR-27a, by luciferase reporter assay. Besides, we also observed an inverse correlation between expressions of myostatin mRNA and miR-27a in fat and heart of pigs and in proliferating porcine myoblasts. The reciprocal expression of miR-27a and myostatin has also been reported in other studies (Allen and Loh, 2011; Huang et al., 2012; Chen et al., 2014).

The mir-27 family consists of two members (miR-27a and miR-27b) located at different chromosomes. It has been reported that miR-27 enhances differentiation of osteoblasts and strongly inhibits adipogenesis (Lin et al., 2009; Wang and Xu, 2010). MiR-27b is a regulatory hub in lipid metabolism through repressing critical regulators of lipid homeostasis, such as PPARα, HMGCR (Kida et al., 2011, Vickers et al., 2013). In a recent study, miR-27b was reported to specifically target to bovine myostatin (Miretti et al., 2013). MiR-27a shares the same seed region with miR-27b, but with a C to U mutation at C-terminal. MiR-27a modulates the growth of breast and liver cancer cells by targeting FOXO1 and FZD7/β-catenin, respectively (Guttilla and White, 2009; Chen et al., 2013). Besides, miR-27a also suppresses adipocyte differentiation through targeting PPARγ (Kim et al., 2010). We previously demonstrated that miR-27a inhibits myostatin expression then promotes proliferation of C2C12 myoblasts (Huang et al., 2012). In this study, we showed that miR-27a enhances proliferation of porcine myoblasts through downregulation of myostatin expression.

In summary, we cloned the full-length of porcine myostatin 3'UTR and confirmed that miR-27a positively regulates porcine myoblast proliferation via targeting myostatin. In the future, it will be interesting to determine whether miR-27a could modulate the differentiation of porcine myoblasts.

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Conflicts of Interest

None.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1751731114001694

References


