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

(Received 14 March 2014; Accepted 27 May 2014; First published online 9 July 2014)

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 positive 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 superfamily, is a negative regulator 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 mRNAs 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′-GTAAGATCGCTGTTGGTTACATGA-3′, which was designed according to the porcine *myostatin* coding sequence (GenBank accession no. NM_214435). The full-length 3′UTR of porcine *myostatin* was PCR amplified from the total RNA isolated from the longissimus lumborum muscle of a 28-day-old, 7.6 kg female Duroc × Landrace × Yorkshire (DLY) pig using the gene-specific primer (forward) and the 3′RACE inner primer (5′-GGCGGATCTCTCAGATGATTTCATATAGG-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′-GGCGGATCTAGATGATTTCATATAGG-3′ and reverse primer 5′-GGCGGATCTCTCAGATGATTTCATATAGG-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 cotransfected 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/l 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 directions. 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.
**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 GAATGAGAACGACGCAA-3' and (reverse) 5'-TCGTTTCCG TCAGAGCGTA-3'; porcine β-actin (forward) 5'-CATCCTG ACCGCAAAT-3' and (reverse) 5'-TGTCACCTCAAACGTCC-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 Ct 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.
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 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 (Linet et al., 2009; Wang et al., 2013). MiR-27a shares the same seed region with miR-27b, and strongly inhibits adipogenesis (Linet et al., 2009; Wang et al., 2013). In a recent study, miR-27b was reported to target to bovine myostatin (Miretti et al., 2012). The reciprocal modulation of miR-27a and MSTN and porcine myoblast proliferation

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31272459), the National Basic Research Program of China (No. 2012CB124701), the Sichuan Youth Science and Technology Foundation (No. 2012JQ0049) and the Specific Research Supporting Program for Academic Sustentation Research Team in Sichuan Agricultural University.

Conflicts of Interest

None.

Supplementary material

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

References


