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RESEARCH ARTICLE

MiR-27b promotes sheep skeletal muscle satellite cell proliferation by targeting *myostatin* gene

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Abstract. To investigate the role of miR-27b in sheep skeletal muscle development, here we first cloned the sequence of sheep pre-miR-27b, then further investigated its expression pattern in sheep skeletal muscle *in vivo*, the relationship of miR-27b expression and sheep skeletal muscle satellite cell proliferation and differentiation *in vitro*, and then finally confirmed its target gene during this development process. MiR-27b sequence, especially its mature sequence, was conservative among different species. MiR-27b highly expressed in sheep skeletal muscle than other tissues. In skeletal muscle of Suffolk and Bashbay sheep, miR-27b was upregulated during foetal period and downregulated during postnatal period significantly ($P<0.01$), but it still kept a relatively higher expression level in skeletal muscle of postnatal Suffolk sheep than Bashbay. There is a potential target site of miR-27b on 3′-UTR of sheep myostatin (*MSTN*) mRNA, and the double luciferase reporter assay proved that miR-27b could successfully bind on this site. When sheep satellite cells were in the proliferation status, miR-27b was upregulated and *MSTN* was downregulated significantly ($P<0.01$). When miR-27b mimics was transfected into sheep satellite cells, the cell proliferation was promoted and the protein level of *MSTN* was significantly downregulated ($P<0.01$). Moreover, miR-27b regulated its target gene *MSTN* by translation repression at an early step, and followed by inducing mRNA degradation in sheep satellite cells. Based on these results, we confirm that miR-27b could promote sheep skeletal muscle satellite cell proliferation by targeting *MSTN* and suppressing its expression.

Keywords. miR-27b; *myostatin* gene; sheep; skeletal muscle; satellite cell.

Introduction

Skeletal muscle is a highly complex and heterogeneous tissue, and its mass is mainly determined by the number and size of muscle fibres (McCoard *et al.* 2000). During foetal period, the total number of muscle fibres is already fixed, and although the diameter and length of muscle fibres increase after postnatal period, their number remains relatively stabilized (Russell and Oteruelo 1981; Maier *et al.* 1992), except for events like skeletal muscle injury (Dayanidhi and Lieber 2014). Thus, the embryonic period is a primary and important stage that affects animal skeletal muscle mass. Embryonic myogenesis starts when cells, called myoblasts, in embryonic somites acquire myogenic potential and migrate to muscle-forming regions of the trunk and limbs (Miller 1992). Myoblasts are myogenic precursor cells that originate in the embryonic mesoderm (Picard *et al.* 2002). After a period of cell division, the myoblasts withdraw from the cell cycle and fuse with each other to create primary and secondary muscle fibres, and also tertiary muscle fibres in some large agricultural animals (Miller 1992; Wilson *et al.* 1992). During postnatal development, the muscle fibres increase their length and diameter, and some myoblasts enter quiescence and further reside within skeletal muscle as satellite cells (Chargé and Rudnicki 2004). If mature skeletal muscle is damaged, the quiescent-satellite cells would be activated again and differentiate into new muscle fibres to repair the tissue and reestablish muscle homeostasis (Rudnicki *et al.* 2008;

Sacco *et al.* 2008), and this process is similar to embryonic myogenesis (Tajbakhsh 2009).

MicroRNAs (miRNAs) are a class of endogenous, short noncoding RNAs about 22 nucleotides (nt) in length. From the year 1993, when the first miRNA, lin-4, was found (Lee *et al.* 1993) until now, more than 20,000 miRNAs have been identified (http://www.mirbase.org/). These miRNAs play important roles in various biological processes, such as cell proliferation, differentiation, apoptosis, metabolism, development and tumour metastasis (Alvarez-Garcia and Miska 2005; Hwang *et al.* 2006). In humans, more than 35% genes are regulated by miRNAs (Lewis *et al.* 2005). In animals, most miRNAs bind to the 3′-UTR, CDS or 5′-UTR of their target gene mRNAs by —two to eight nt of 5′-end, called seed sequences (Bartel *et al.* 2009; Helwak *et al.* 2013). They can negatively regulate the expression of their target genes by means of inhibition of translation initiation (Mathonnet *et al.* 2007), or other forms of translation repression (Liu *et al.* 2005; Chu and Rana 2006) as well as by mRNA degradation (Yekta *et al.* 2004).

Increasing evidence has confirmed the involvement of miRNAs in the regulatory networks modulating muscle gene expression, myoblast proliferation and differentiation. MiR-133 targets serum response factor (*SRF*), a critical factor for muscle cell proliferation and differentiation *in vitro* and *in vivo*, and enhances myoblast proliferation (Chen *et al.* 2006). MiR-133 also targets the *Prdm16* gene and controls the differentiation of skeletal muscle satellite cells into either the myogenic lineage or brown adipocytes (Yin *et al.* 2013). MiR-1 and miR-206 may promote muscle-cell differentiation by inhibiting histone deacetylase 4 (*HDAC4*), *Hand2* and connexin 43 (*Cx43*) (Zhao *et al.* 2005; Anderson *et al.* 2006; Chen *et al.* 2006). In Texel sheep, the 3′-UTR of the myostatin (*MSTN*) gene mutated and creates a potential target site of miR-1 and miR-206, thus its expression is inhibited and the suppression of skeletal muscle growth is accordingly removed, therefore this sheep breed shows double-muscled phenotype (Clop *et al.* 2006). MiR-29 can inhibit myoblast proliferation and facilitate differentiation by suppressing *Akt3* (Wei *et al.* 2013).

Mir-27b is a key regulator in animal muscle development and growth. By applying array technology, we have detected 22 miRNAs in sheep skeletal muscle, including miR-27b, their expression accounted for 89% of the total detected miRNAs (Zhang *et al.* 2015). In mouse myogenesis, miR-27b plays important roles in embryonic myoblast differentiation and satellite cell activation in mature skeletal muscle by targeting *Pax3* (Crist *et al.* 2009). In Piedmontese cattle, miR-27b could bind the 3′-UTR of *MSTN* mRNA, downregulates its expression significantly, and finally induces skeletal muscle hypertrophy in this bovine breed (Silvia *et al.* 2013). But in sheep, its sequence and functions in myogenesis are still unknown. In our previous study, by constructing cDNA library and high-throughput sequencing, we found one target site of miR-27b in the 3′-UTR of *MSTN* mRNA (Wei Zhang, Shang-Quan Gan, Shi-Yin Wang, Expression patterns and target genes of miRNAs in sheep skeletal muscle during foetal and postnatal periods *unpublished data*). Here, we further validated that miR-27b could target the 3′-UTR of *MSTN* mRNA in sheep skeletal muscle satellite cells to promote cell proliferation, and may be associated with skeletal muscle mass of different sheep breeds.

Materials and methods

Animals and sample collection

To investigate the expression patterns of miR-27b in sheep breeds with different meat production performances, we choose two breeds, Suffolk and Bashbay sheep. Suffolk is the famous sheep breed world wide, and has great meat production performance. Bashbay sheep is a Chinese local breed which grows gradually and has relatively poor meat production performance.

The limb skeletal muscles of these two-sheep breeds were collected from foetal stage of the 40th, 60th, 80th, 100th day and postnatal stage of newborn, 3, 6, 9 and 12 months old. Meanwhile, the tissues of the heart, liver, spleen, lung, kidney, skin, stomach, intestine and brain of the 80th day foetus were also collected. All samples were snap-frozen in liquid nitrogen, and then stored at −80°C.

All research involving animals were performed according to the regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004). The Institute Ethics Committee of the Xinjiang Agricultural Professional Technological College approved the relevant ethic issues in this study.

RNA extraction

Total RNA was extracted from the sample and cultured cells using TRIzol (Invitrogen, USA) in accordance with the manufacturer's protocol. The DNase I was used to digest the traces amount of DNA in total RNA. The purity of total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent, USA), according to the OD_{260}/OD_{280} ratio. RNA degradation was examined by agarose gel electrophoresis and detected the 28S and 18S rRNA bands.

Cloning sheep miR-27b and sequence alignment

Sequence of sheep miR-27b is currently not known, thus here we referred to the sequence of *Bos taurus* miR-27b (MI0004760) designing primers (table 1) to clone sheep miR-27b. Total RNA was reverse-transcribed with a specific primer CL-miR-27b by using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's

Table 1. Primers used in this study.

Name	Sequence (5′ → 3′)	Note
CU-miR-27b	ACCTCTCTGACGAGGTGCAGA	Cloning pre-miR-27b
CL-miR-27b	CACCTTCTCTTCAGGTGCAGA	
MiR-27bRT	CTCGACTGAGTTGCCGTGAGTCGGCAACTCAGTCGAGGCAGAACT	Reverse transcription qRT-PCR
MiR-27bqU	TGAGTTGCCGTGAGTCGGCAACTCA	
MiR-27bqL	ACACTCCAGCTGGGTTCACAGTGGCTA	
5sRT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAGCCTAC	Reverse transcription qRT-PCR
5sqU	CTGGTGTCGTGGAGTCGGCAATTCAG	
5sqL	ATACTCCAGCTGGGGAATACCGGGTGCT	
P-3′-UTR-wU	CC***CTCGAG***GGATAAGGCCAATTACTGCTCTG	Cloning MSTN 3′-UTR
P-3′-UTR-wL	CC***AAGCTT***GGAATTCACCAGAAGACAAGGAG	
P-3′-UTR-mU	TTTGAACGGCTGAAATTATGTACCACGGGC	Mutating the target site in MSTN 3′-UTR
P-3′-UTR-mL	TTCAGCCGTTCAAAATTGTTGAGGGGAAGAC	

The bases of primer miR-27bRT and 5sRT with underlines were complementary with eight bases of 3′ end sequence of miR-27b and 5s, the ones of primer miR-27bqL and 5sqL were 13 bases of 5′ end sequence of miR-27b and 5s, the ones of primer P-3′-UTR-wU and P-3′-UTR-wL were restriction enzyme cutting sites (italics and bold) and protective bases of *Xho*I and *Hind*III, the ones of primer P-3′-UTR-mU and P-3′-UTR-mL were mutated bases of the miR-27b target site in MSTN 3′-UTR.

instructions. Then the PrimeSTAR HS DNA Polymerase (TAKARA, Japan) and cloning primers, CU-miR-27b and CL-miR-27b, were used to amplify the sequence of pre-miR-27b. The PCR products were purified using gel, dA was added to the 3′ end of sequence using a Mighty TA-cloning Reagent Set (TAKARA) and then connected with a pGEMT Easy Vector (Promega, USA) by the TA cloning method. Finally, the vectors were transformed into competent cells and white colonies were chosen to sequence.

Further, the stem-loop structure of pre-miR-27b was analysed using RNA structure software, and its sequence was compared with bta-miR-27b (MI0004760), hsa-mir-27b (MI0000440), mmu-mir-27b (MI0000142), rno-mir-27b (MI0000859), gga-mir-27b (MI0001274) and ssc-mir-27b (MI0013109) to analyse the sequence conservation.

Stem-loop quantitative real-time polymerase chain reaction (qRT-PCR)

In this study, the stem-loop qRT-PCR method (Chen *et al.* 2005) was used to detect the expression of miR-27b in skeletal muscle of different development stages of Suffolk and Bashbay sheep, and in different tissues of Suffolk sheep during the 80th foetal period. Total RNA was reverse-transcribed with a specific primer miR-27bRT and 5sRT using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using a LightCycler 480 instrument (Roche, Germany), with SYBR Green SuperMix (Qiagen, Germany) and two primers (part of the stem-loop primer, and an oligonucleotide plus part of the miRNA sequence, table 1). 5S ribosomal RNA was used as an endogenous control, and the expression levels of miR-27b were normalized to 5S.

The expression levels of miR-27b in heart and skeletal muscle of a newborn were considered as controls, respectively, and the relative fold expression differences were calculated using the $2^{-\Delta\Delta ct}$ method, where $\Delta Ct = Ct(miRNA) - Ct(5S)$ and $\Delta\Delta Ct = \Delta Ct$ miRNA(other tissue or step) $- \Delta Ct$ miRNA(heart or newborn). All reactions were run in triplicate. Graphs were generated using MS Excel. Bar graphs show the means±SD.

Construction of wild-type and mutant MSTN 3′-UTR dual-luciferase reporter vectors

Wild-type *MSTN* 3′-UTR fragments (NM_001009428.2) containing putative target site of miR-27b were PCR amplified from *Ovis aries* genomic DNA using PrimeSTAR HS DNA Polymerase (TAKARA) and P-3′-UTR-wU and P-3′-UTR-wL primers. Mutant-type *MSTN* 3′-UTR fragments, in which the putative target site of miR-27b was mutated, were achieved by overlap extension PCR. Briefly, using the wild-type *MSTN* 3′-UTR fragments as a template, the up-stream and down-stream fragments of the

mutant *MSTN* 3′-UTR, including the mutant target site of miR-27b, were amplified by P-3′-UTR-wU and P-3′-UTR-mL or P-3′-UTR-mU and P-3′-UTR-wL, respectively. Further by using both up-stream and down-stream fragments as templates, the mutant *MSTN* 3′-UTR fragments were amplified by primers P-3′-UTR-wU and P-3′-UTR-wL. Finally, the wild-type and mutant-type *MSTN* 3′-UTR fragments were inserted into the PGL4 luciferase reporter vectors (Promega, Madison, USA) between the 5′ *Xho*I and 3′ *Hin*dIII sites. The plasmids were transformed into competent cells and cultured on LB plates containing ampicillin at 37°C for 16–18 h. The enzyme digestion and sequencing were used to confirm if the wild-type and mutant-type fragments were correct or not. The recombinant wild-type and mutant-type plasmids were named pGL4-MSTN-wt and pGL4-MSTN-mut, respectively.

Cell transfection and luciferase reporter assay

HeLa cells were seeded in six-well plates (2×10^5 cells/well), and after 24 h Lipofectamine 2000 (Invitrogen) was applied to cotransfect the recombinant plasmids, miR-27b mimics or negative control mimics into HeLa cells according to the manufacturer's protocol. Further, the cells were harvested after further cultured for 48 h, and the luciferase activity was measured using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Skeletal muscle satellite cell cultures and induced differentiation

Skeletal muscle satellite cells of Suffolk sheep, obtained by the primary culture method from skeletal muscle, were seeded in six-well plates (1×10^5 cells/well), and cultured using proliferation medium (DMEM+10% foetal calf serum+10% equinum serum+1% penicillin–streptomycin) in a 5% CO_2 incubator at 37°C. When the cells covered about 70–80% of bottom of well, six wells were harvested, and then total RNA and protein were extracted respectively and stored at −80°C. The rest six well cells were further cultured in inducing differentiation medium (DMEM+0.5% foetal calf serum+0.5% equinum serum+1% penicillin–streptomycin). When the satellite cells fused with each other and the myotubes were formed, the cells were harvested, and then total RNA and protein were extracted respectively and stored at −80°C. qRT-PCR and Western blot were applied to detect the expression of miR-27b and *MSTN* in RNA and protein levels during these two different statuses.

Upregulation or downregulation of miR-27b in sheep skeletal muscle

Lipofectamine 2000 (Invitrogen) was used to transfect miR-27b mimics, mimics negative control, miR-27b inhibitor or inhibitor negative control into sheep skeletal muscle satellite cells respectively, and transfect nothing as a blank control according to the manufacturer's instructions. Their sequences are listed in table 2.

The transfected cells were seeded in 96 well plates (about 2000 cells/well), each transfected cell was seeded into six wells and the cells were further cultured in complete medium (DMEM+15% foetal calf serum+1% penicillin–streptomycin). Every 24 h, the cells in one well were digested respectively and counted to investigate the effect of upregulated or downregulation of miR-27b on proliferation of sheep skeletal muscle satellite cells.

The miR-27b mimics or mimics negative control transfected cells were seeded in six wells respectively (about 2×10^5 cells/well), and the cells were further cultured in complete medium. Every 12 h, cells in one well were harvested and the total RNA was extracted to detect the mRNA level of *MSTN* using qRT-PCR, to examine if the upregulation of miR-27b would lead to *MSTN* mRNA degradation.

The transfected cells were seeded in six wells (about 2×10^5 cells/well), each transfected cell was seeded into three wells, and then the cells were further cultured in complete medium. After 72 h, the cells were lysed in RIPA lysis buffer containing proteinase inhibitor (Thermo, USA), and incubated for 30 min on ice, then centrifuged at $12{,}000\times g$ for 10 min at 4°C. The protein concentration in the supernatants from cell lysates was determined using the BCA protein assay. A total of 100 μg total proteins were resolved by 12% SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Further, the membrane was blocked in milk in PBST and probed overnight at 4°C with primary antibodies: mouse monoclonal anti-GDF8/11 (sc-398333, 1:500; Santa Cruz Biotechnology, Santa Cruz, USA), or mouse monoclonal α-tubulin (sc-47778, 1:500; Santa Cruz Biotechnology) as an endogenous control. The membrane was washed four times in TBS-T, and incubated with secondary antibody (goat anti-mouse, Santa Cruz) for 1 h at room temperature in TBS-T. Then the membrane was washed four times in TBS-T and examined.

Statistical analysis

All experiments were repeated thrice and the results are presented as means±SD. SPSS 13.0 software (SPSS, Chicago, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to evaluate the significance of differences between groups. Differences with a $P<0.05$ were determined as statistically significant.

Results and analysis

The sequence of sheep pre-miR-27b

By applying TA cloning (figure 1a) and sequencing, the sequence of sheep pre-miR-27b, composed of 97 nt,

Table 2. Sequence of miR-27b mimics, miR-27b inhibitor and negative control.

Name	Sequence (5′ → 3′)
MiR-27b mimics	UUCACAGUGGCUAAGUUCUGC
Mimics negative control	CAGUACUUUUGUGUAGUACAA
MiR-27b inhibitor	GCAGAACUUAGCCACUGUGAA
Inhibitor negative control	UUCUCCGAACGUGUCACGUTT

was obtained. Its mature sequence was 21 nt in length (figure 1b). The pre-miR-27b could be folded into a typical stem-loop structure, and the mature miR-27b was on its 3′ arm (figure 1c). Then, the pre-miR-27b sequences of seven species were compared using DNAMAN software (figure 1c), and the result showed that pre-miR-27b was highly conservative between *O. aries* and *B. taurus*. Although there were some different nucleotides in pre-miR-27b among *O. aries* and the rest five species, their mature sequences were entirely identical (figure 1d).

The expression of sheep miR-27b in vivo and in vitro

The result of qRT-PCR showed that, compared with the expression in the heart, miR-27b expressed highly in sheep skeletal muscle, a relatively high level in the stomach and intestine, and a very low level in the liver, spleen, lung, kidney, brain and skin (figure 2a). Then the Suffolk sheep, a typical sheep breed with great meat production trait, and the Bashbay sheep, a local sheep breed in China with poor meat production performance, were chosen to further investigate the difference in miR-27b expression in skeletal muscle of these two-sheep breeds using qRT-PCR. Choosing newborn stage as the control, the expression of miR-27b in skeletal muscle of foetal period was significantly higher than that in postnatal period of these two-sheep breeds ($P<0.01$). There was no significant difference in the expression of miR-27b between two breeds during foetal period, but at postnatal period, their expression was different, miR-27b was significantly downregulated than the newborn stage in Bashbay sheep ($P<0.05$), but still kept a relatively high-expression level in skeletal muscle of Suffolk sheep (figure 2b). Based on these data, we could speculate that the target genes of miR-27b may play an inhibition role in sheep myogenesis.

To further understand the function of miR-27b in sheep myogenesis, its expression in sheep skeletal muscle satellite cells was studied *in vitro*. The satellite cells were cultured in growth or differentiation medium, and achieved the cells in proliferation or differentiation status, respectively (figure 2c). Then, the levels of miR-27b were detected using qRT-PCR in two different statuses. The expression level of miR-27b was significantly higher in the proliferation status than in the differentiation status ($P<0.01$) (figure 2d), thus miR-27b may promote sheep myogenesis by stimulating proliferation of skeletal muscle cells.

In our previous study, we found a potential target site in 3′-UTR of sheep *MSTN* gene mRNA (Wei Zhang, Shang-Quan Gan, Shi-Yin Wang, Expression patterns and target genes of miRNAs in sheep skeletal muscle during foetal and postnatal periods *unpublished data*), and we predicted that miR-27b may regulate the expression of *MSTN* gene to affect myogenesis in sheep. The result of Western blot showed that when sheep skeletal muscle satellite cells were in the proliferation status, the protein level of *MSTN* gene was significantly lower than that in the differentiation status ($P<0.01$) (figure 2, e&f). Thus, we speculated that the *MSTN* gene was a candidate target gene of miR-27b, and miR-27b may promote sheep satellite cell proliferation by suppression *MSTN* expression.

MiR-27b can bind on the target site in 3′-UTR of sheep MSTN mRNA

To further validate if miR-27b could bind to the potential target site in 3′-UTR of sheep *MSTN* mRNA and affect its expression, double luciferase reporter plasmids which contained either wild-type or mutant 3′-UTR fragments of *MSTN* were constructed (figure 3a). Thus, the wild-type or mutant reporter plasmids were cotransfected into HeLa cells along with miR-27b mimics or mimics negative control. In HeLa cells, which were cotransfected with pGL4-MSTN-mut, no relative luciferase activity difference was found between the cells cotransfected with miR-27b mimics and cells with mimics negative control. But in cells cotransfected with pGL4-MSTN-wt, the relative luciferase activity of cells cotransfected with miR-27b mimics was significantly lower compared with cells cotransfected with mimics negative control ($P<0.01$) (figure 3b). Thus, we confirmed that miR-27b could bind on the 3′-UTR of sheep *MSTN* mRNA and affect its expression.

Effect of miR-27b expression on cell proliferation and MSTN expression in sheep satellite cells

There was a potential target site of miR-27b in 3′-UTR of sheep *MSTN* mRNA (NM_001009428.2) (figure 4a).

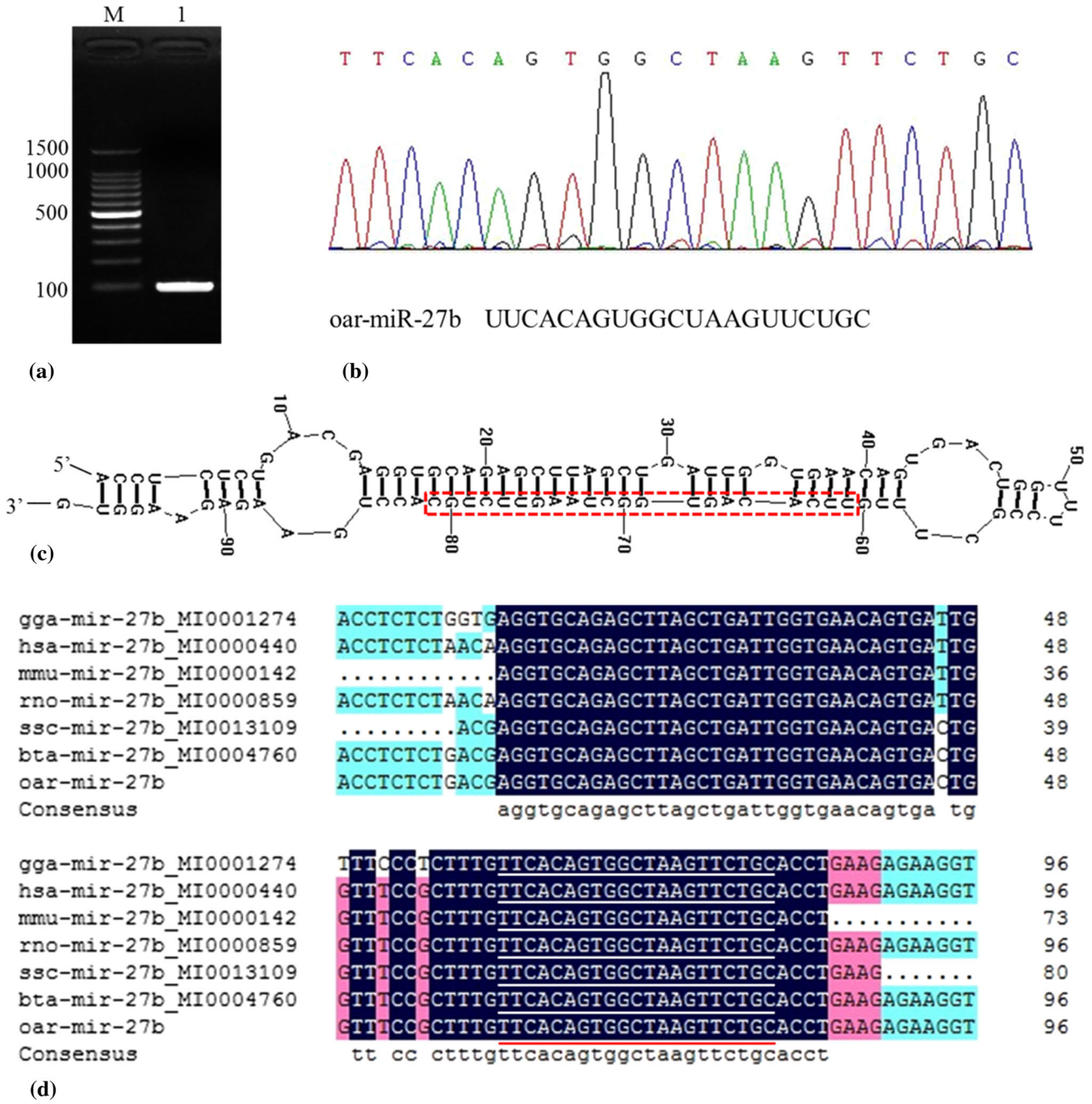

Figure 1. The sequence and stem-loop structure of sheep pre-miR-27b. (a) Using agarose gel electrophoresis, the pre-miR-27b in the T-vector was detected; M lane, 100-bp marker; 1, PCR products of pre-miR-27b including some sequences of the T-vector. (b) Sequence of miR-27b. (c) Stem-loop structure of pre-miR-27b. (d) Sequence alignment of seven species pre-miR-27b sequences; the boxed nucleotides are mature sequence of miR-27b.

Applying double luciferase reporter assay, we had validated that miR-27b could bind this target site and affect the luciferase activity. In sheep skeletal muscle satellite cells, whether miR-27b could regulate the expression of *MSTN* gene or not and its influence on cells were other important questions which need to be further studied.

When miR-27b was upregulated by transfecting miR-27b mimics into sheep skeletal muscle satellite cells, the proliferation of cells were promoted significantly compared with the blank control, miR-27b mimics negative control, miR-27b inhibitor and miR-27b inhibitor negative control groups in which there were no significant effects on cell proliferation (figure 4b). Thus, we can conclude that the upregulation of miR-27b could promote the proliferation of sheep skeletal muscle satellite cells *in vitro*.

Further, the effect of miR-27b upregulation or downregulation on expression of *MSTN* gene was investigated using qRT-PCR and Western blot. Compared with the

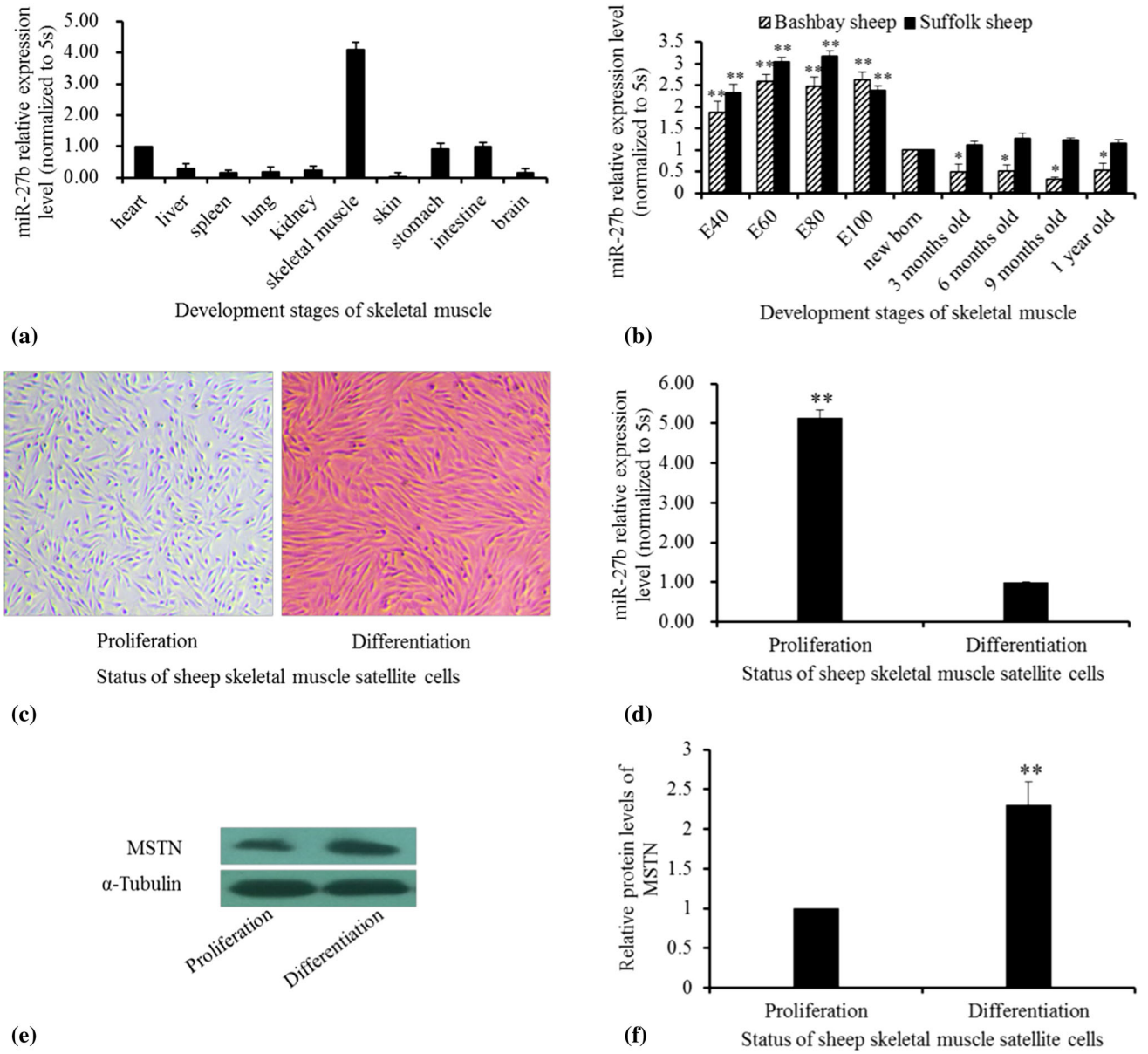

Figure 2. The relative expression of sheep miR-27b *in vivo* and *in vitro*. (a) Relative expression of miR-27b in main tissues of sheep foetal during the 80th day, the expression in the heart was chosen as the control. (b) Relative expression of miR-27b in skeletal muscle of different development stages of Suffolk and Bashbay sheep, the expression during newborn period was chosen as the control. (c) Sheep satellite cells in the proliferation (left) and differentiation (right) status (200×). (d) Relative expression of miR-27b in sheep satellite cells in the proliferation and differentiation status, the expression in differentiation cells was chosen as the control. (e) And (f) protein levels of MSTN in sheep satellite cells in the proliferation and differentiation status. Data are expressed as means±SD of three independent experiments ($^{*}P<0.05$, $^{**}P<0.01$).

mimics negative control group, when miR-27b mimics were transfected into satellite cells, the mRNA level of *MSTN* gene did not decline significantly during the beginning of 24 h. But, the level rapidly downregulated to a relatively low level after 24 h (figure 4c). When the transfected cells were further cultured for 72 h, the protein levels of the *MSTN* gene were detected by applying Western blot. Compared with the blank control group, the protein levels of *MSTN* gene were significantly downregulated in the cells which transfected miR-27b mimics ($P<0.01$), but the other groups did not change significantly ($P>0.05$) (figure 4, d&e).

Based on these results, we can finally confirm that miR-27b could target the *MSTN* gene, suppress its expression in sheep skeletal muscle satellite cells and then promote cell proliferation. Owing to the fact that the mRNA of *MSTN* gene did not decline during the beginning of 24 h and declined after 24 h when miR-27b was

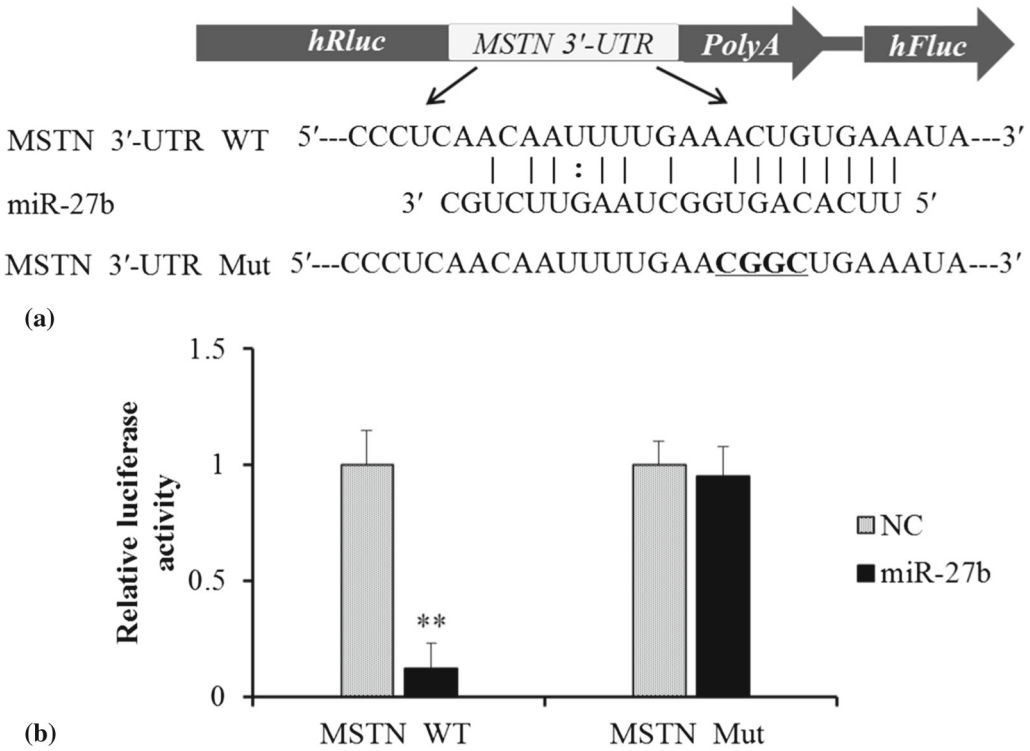

Figure 3. Double luciferase reporter assay. (a) The sequence of miR-27b and its target site on wild-type 3′-UTR of *MSTN*, and the mutant 3′-UTR fragments of *MSTN*, the bases with underline are mutated bases achieved by overlap extension PCR. The wild-type and mutant 3′-UTR fragments of *MSTN* are inserted between Renilla and Firefly luciferases. (b) The relative luciferase activity determined 48 h after transfection. Data are expressed as means ± SD of three independent experiments (**$P<0.01$).

upregulated (figure 4c), we speculated that miR-27b may negatively regulate expression of the *MSTN* gene by translation repression at first, and then inducing its mRNA degradation.

Discussion

Myostatin, which is encoded by *MSTN* gene, is also called ***growth differentiation factor 8*** (*GDF-8*). It is a member of the TGF-β super family of growth and differentiation factors. Myostatin acts as a strong negative regulator of muscle formation, thus the mutations in *MSTN* always cause increase in skeletal muscle mass of animals. In sheep, if the *MSTN* gene was knocked out using a CRISPR/Cas9 system, they show heavier body weight than the wild-type counterparts (Crispo *et al.* 2015). *MSTN* null mice causes hypertrophy and hyperplasia of skeletal muscle mass (McPherron *et al.* 1997), and a deletion in the bovine *MSTN* gene is associated with double-muscled phenotype in Belgian Blue and Piedmontese breeds of cattle (Grobet *et al.* 1997; McPherron and Lee 1997). A mutation in 3′-UTR of Texel sheep*MSTN* mRNA creates a potential target site of miR-1 and miR-206, inhibits the translation of *MSTN* mRNA and finally causes the double-muscled phenotype of Texel sheep (Clop *et al.* 2006). In our present study, we confirmed that miR-27b could target *MSTN* 3′-UTR in sheep skeletal muscle satellite cells. When miR-27b was upregulated, the mRNA and protein levels of *MSTN* were downregulated, and cell proliferation was promoted. Our results are in accordance with the previous studies. In Suffolk sheep, miR-27b still kept a relatively higher level in postnatal skeletal muscle than Bashbay sheep, thus the expression of *MSTN* was suppressed sustainability, and this may be an important factor that causes great meat production performance of Suffolk sheep.

Myoblast proliferation and differentiation are two key stages in myogenesis, and ultimately determine the number of muscle fibres that was important factor affecting skeletal muscle mass (McCoard *et al.* 2000). In this study, when satellite cells were in the proliferation status *in vitro*, we found miR-27b was upregulated and the *MSTN* protein was downregulated (figure 2, e&f), and the proliferation of satellite cells was promoted (figure 4b). Thomas *et al.* (2000) had observed that the proliferation of C2C12 myoblasts decreased with the increasing levels of myostatin, and a further study showed that MSTN specifically upregulated *p21*, a cyclin-dependent kinase inhibitor, and decreased the levels and activity of Cdk2, and then prevented the progression of myoblasts from the G_1- to S-phase of the cell cycle. Thus, they proposed that the double-muscled phenotype of animal caused by *MSTN*

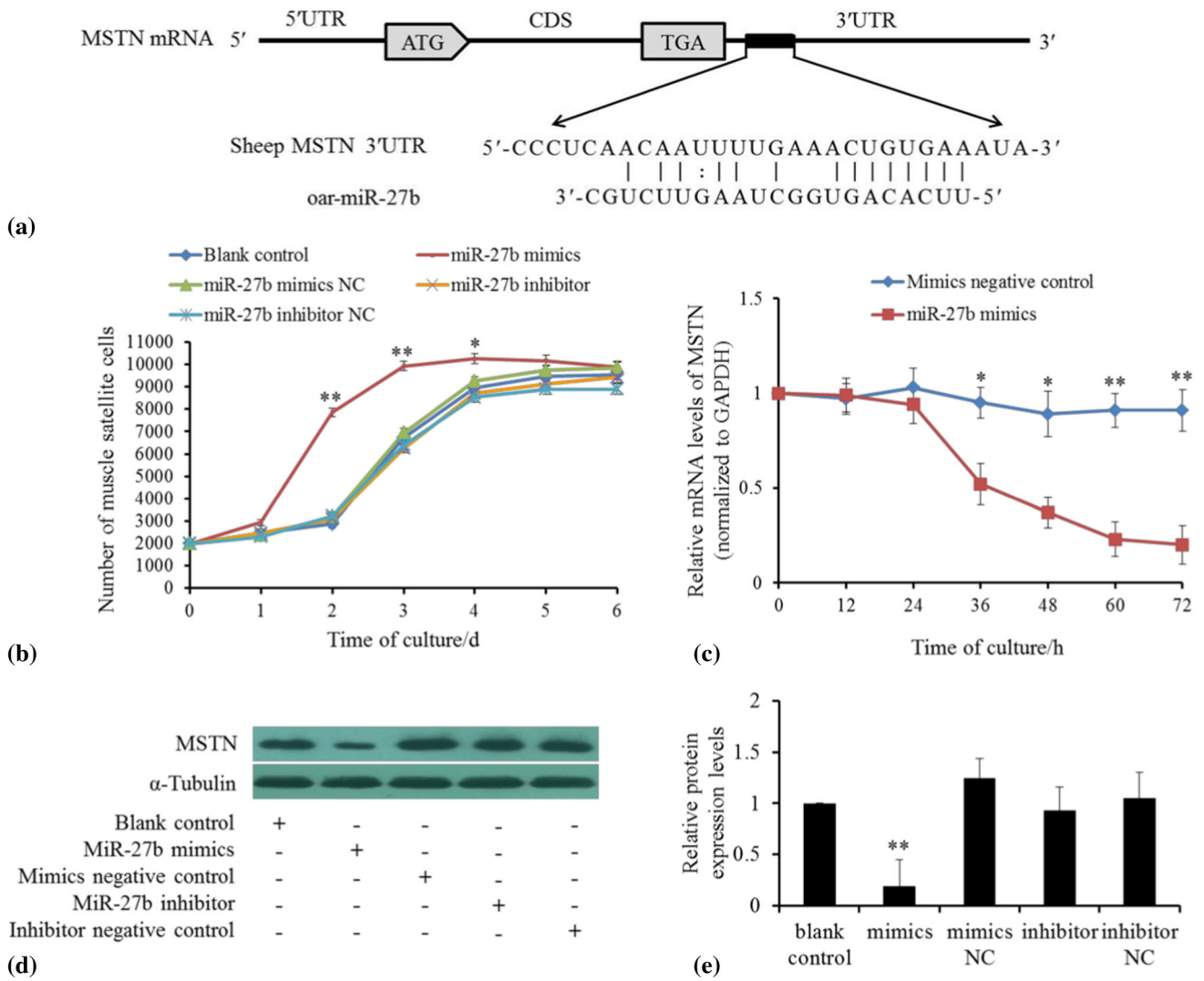

Figure 4. The effect of miR-27b expression on cell proliferation and *MSTN* expression in sheep satellite cells. (a) The target site of miR-27b on 3′-UTR of *MSTN* mRNA. (b) The proliferation speed of sheep satellite cells when miR-27b was upregulated or downregulated. (c) The change of *MSTN* mRNA levels when miR-27b was upregulated in the sheep satellite cells. (d) and (e) The change of *MSTN* protein levels when miR-27b was upregulated or downregulated in sheep satellite cells. Data are expressed as means ± SD of three independent experiments (*$P<0.05$, **$P<0.01$).

gene mutation may be a result of deregulated myoblast proliferation (Thomas *et al.* 2000). Taken together, we speculated that miR-27b suppressed the expression of *MSTN* in proliferation satellite cells, removed its prevention to satellite cell progression from the G_1- to S-phase of the cell cycle and promoted cell proliferation.

The knowledge about the way that miRNA regulate the expression of their target genes is still inconsistent. Some researchers urged that miRNA negatively regulate their target genes by means of translation repression (Chu *et al.* 2006; Mathonnet *et al.* 2007), but others deemed by mRNA degradation (Yekta *et al.* 2004). Djuranovic *et al.* (2012) proved that miRNA regulate gene expression by translation repression at an early step, and followed by mRNA deadenylation and decay (Djuranovic *et al.* 2012). Here we found that the mRNA level of the *MSTN* gene did not decline significantly during the beginning of 24 h, but the level rapidly downregulated to a relatively low level after 24 h (figure 4c) when miR-27b was upregulated in sheep satellite cells. Thus, we speculated that miR-27b regulated its target gene *MSTN* in sheep satellite cells in the same way as Djuranovic and colleagues had described.

In conclusion, miR-27b could promote sheep skeletal muscle satellite cell proliferation by targeting *MSTN* and suppressing its expression. Moreover, miR-27b regulated *MSTN* in sheep satellite cells by translation repression at an early step, and followed by mRNA degradation. Considering the expression of miR-27b in skeletal muscle of Suffolk and Bashbay sheep, we speculated that the

relatively high expression of miR-27b in Suffolk sheep may be the main reason for its great meat production performance.

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