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The critical role of myostatin in differentiation of sheep myoblasts

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ABSTRACT

Myostatin [MSTN, also known as growth differentiation factor 8 (GDF8)], is an inhibitor of skeletal muscle growth. Blockade of MSTN function has been reported to result in increased muscle mass in mice. However, its role in myoblast differentiation in farm animals has not been determined. In the present study, we sought to determine the role of MSTN in the differentiation of primary sheep myoblasts. We found that ectopic overexpression of MSTN resulted in lower fusion index in sheep myoblasts, which indicated the repression of myoblast differentiation. This phenotypic change was reversed by shRNA knockdown of the ectopically expressed MSTN in the cells. In contrast, shRNA knockdown of the endogenous MSTN resulted in induction of myogenic differentiation. Additional studies revealed that the induction of differentiation by knocking down the ectopically or endogenously expressed MSTN was accompanied by up-regulation of MyoD and myogenin, and down-regulation of Smad3. Our results demonstrate that MSTN plays critical role in myoblast differentiation in sheep, analogous to that in mice. This study also suggests that shRNA knockdown of MSTN could be a potentially promising approach to improve sheep muscle growth, so as to increase meat productivity.

1. Introduction

Myostatin (MSTN), also known as growth differentiation factor 8 (GDF8), is a member of the TGFβ superfamily. Previous studies reported that disruptive mutations in the coding region of bovine MSTN gene were associated with dramatic and widespread increase of skeletal muscle mass, which was characterized as "double muscling" [1,2]. Similar double muscling trait was also observed in human, accompanied by naturally occurred loss-of-function mutations in the MSTN gene [3]. In line with these findings, knockout of MSTN in mice resulted in significant hyperplasia (increase in number of muscle fibers) and hypertrophy (increase in size of muscle fibers) [4]. In contrast, over expression of MSTN in mice showed decreased skeletal muscle mass [5] and the development of cachectic-like muscle wasting [6]. These studies demonstrate that MSTN plays a critical role in muscle growth and development.

Based on accumulating evidence, it is conceivable that inhibition of MSTN function in farm animal myoblasts could effectively increase muscle mass so as to increase meat productivity. This has been an interest to many investigators. Several studies revealed that dominant negative proteins, such as MSTN prepropeptide and follistatin, can suppress MSTN activity and result in increased muscle mass [7,8]. However, these dominant negative proteins may cause undesirable immune response and result in perturbation of their inhibitory functions. Though MSTN knockout mice have been successfully obtained [9], gene knockout in farm animals has still been time consuming and labor intensive. RNA interference (RNAi) is a promising method to specifically and efficiently silence gene expression [10]. It has been widely used in gene function analysis and potential therapeutics of diseases [11]. A recent study suggested that using RNAi to silence the MSTN gene could be an effective approach to improve meat productivity [12].

In this study, we used both ectopic overexpression and shRNA knockdown approaches to investigate the role of MSTN in the differentiation of primary ovine myoblasts. The results of our study document that MSTN inhibits sheep myoblast differentiation. Further, our study also suggests that shRNA knockdown of MSTN could be a potentially promising approach to improve sheep muscle growth, so as to increase meat productivity.

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2. Materials and methods

2.1. Cell culture

TLA-HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Ovine myoblasts were derived from vastus lateralis of 60-day fetus of Xinjiang Merino Sheep as previously described [13]. Briefly, ovine myoblasts were liberated with at least 90% myoblast purity from fresh vastus lateralis by mincing the muscle tissue. Muscle scrap was digested with 0.02% Type 1-A collagenase (Sigma, St Louis, MO) for 45 min at 37 °C, and cultured in DMEM with 1×105 IU/liter penicillin, 100 mg/liter streptomycin (Sigma) and 10% FBS in 37 °C humidified atmosphere containing 5% CO_2. This study has been approved by the Animal Research Safety and Ethics Committee of Xinjiang Academy of Animal Science.

2.2. Construction of lentiviral shRNA and MSTN expression constructs

shRNAs targeting ovine MSTN mRNA (NM001009428) were designed by using the Dharmacon siDESIGN Center database (http://www.dharmacon.com/sidesign/siRNA). Six shRNAs targeting the MSTN coding region at nucleotide positions 198, 218, 539, 540, 548, and 622, respectively, were selected based on ranking criteria of Reynolds [14]. A shRNA targeting the firefly luciferase gene was designed as the non-targeting control. All the shRNAs were cloned into pLL3.7 lentiviral vector (Kindly provided by Dr. Wenlai Zhou, Howard Hughes Medical Institute, Department of Medicine, University of California, San Diego, USA) as shown in Fig. 1. The MSTN specific shRNAs were designated as MSTN-shRNAs, while the non-targeting control was designated as NTC-shRNA (Table 1).

For MSTN overexpression, total RNA were extracted from primary ovine myoblasts using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNAs were prepared from 1 μg total RNA using a RT-PCR kit (Takara Bio Inc., Otsu, Shiga, Japan). Ovine MSTN transcripts were amplified by using forward (5′-GTCCGGATCCAAAACCATGCAAAAACTGCAAATCT-3′) and reverse (5′-GTTATCTCGAGTCAAGCGTAGTCTGGGACGGTATGGGTATGAGCACCCACAGCGATCTAC-3′) primers. The underlined sequence represents a HA tag. The amplicons were digested with *XhoI* and *BamHI* and subcloned into a lentiviral expression vector (pLex-MCS, Open Biosystems, Huntsvile, AL) and the resulting construct was named pLex-MSTN. Disruptive mutations in the siRNA targeting regions of the ovine MSTN gene were introduced to test the specificity of MSTN-shR218 and MSTN-shR548 according to the RNAi rescue method [15]. MSTN constructs with site-directed disruptive mutations were designated as pLex-mt-MSTN.

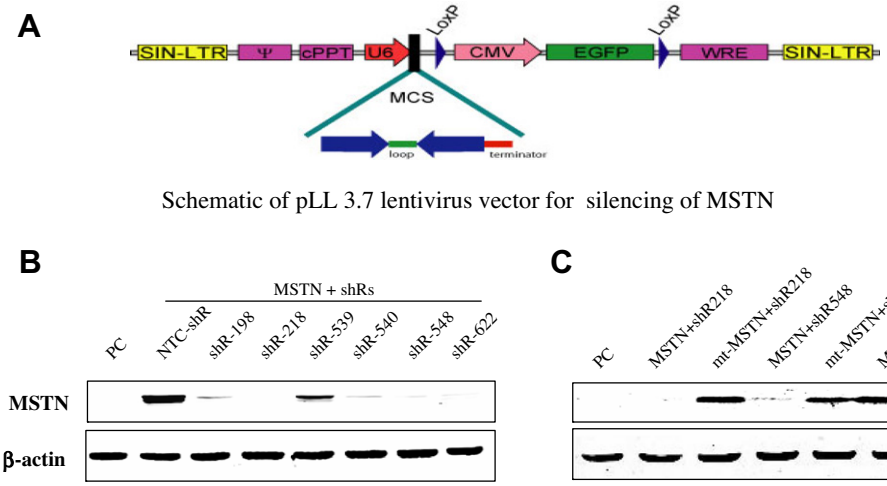
2.3. Identification of the most effective and specific shRNA for MSTN knockdown

293T cells were used to screen the MSTN-shRNAs. Cells were seeded in six-well plates in complete medium (DMEM containing 10% FBS) one day prior to transfection. For transfection, 1 μg of each shRNA construct was co-transfected with 1 μg of pLex-MSTN into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells were then harvested and whole cell lysates were extracted for Western blotting using an anti-HA antibody (Abcam, Cambridge, MA). The co-transfections were performed in duplicate for each MSTN-shRNA construct. To test the specificity of the shRNA constructs, MSTN-shR218 and MSTN-shR548 constructs were co-transfected with pLex-mt-MSTN as described above.

2.4. Generation of MSTN overexpression and shRNA knockdown stable clones in primary ovine myoblasts

To produce pLex-MSTN lentiviral particles, 293T cells were seeded in a 100 mm dish at a density of 60,000 cells/cm^2 and co-transfected with plex-MSTN (12 μg) along with packaging plasmids (3.5 μg pMD2.G and 9 μg psPAX2) using Lipofectamine 2000 (Invitrogen). To produce shRNA lentiviral particles, MSTN-shR218 and NTC-shRNA, were co-transfected with packaging plasmids (REV, pMDL, and VSVG, 6 μg each), respectively, as mentioned above. Forty-eight hours post-transfection, the supernatant containing lentivirus particles was collected and filtered through a 0.45-μm syringe filter.

To generate MSTN overexpressing or shRNA knockdown stable clones, primary ovine myoblasts were seeded in 100 mm plates at a density of 15,000 cells/cm^2 one day prior to lentiviral infection. The lentiviral particles and polybrene (10 μg/ml final concentration, Sigma) were added to the culture of primary ovine myoblasts



Schematic of pLL 3.7 lentivirus vector for silencing of MSTN

Fig. 1. Identification of the optimal MSTN-shRNA in 293T cells. (A) Schematic representation of the pLL 3.7 lentiviral vector for silencing MSTN. (B) Each of MSTN-shRNA or the NTC-shRNA construct was co-transfected with pLex-MSTN vector into 293T cells. (C) The MSTN-shR218, shR548, or NTC-shRNA construct was co-transfected with plex-MSTN or pLex-mt-MSTN vector into 293T cells. Protein levels of MSTN were measured by Western blotting using an anti-HA antibody. β-actin levels were examined as the loading control. MSTN, cells transfected with MSTN plasmid; PC, parental 293T cell control; NTC, luciferase shRNA non-targeting control; shR198 to shR548, shRNA constructs designed to target MSTN at five positions based on MSTN mRNA sequence.

Table 1
Sequences of MSTN-shRNAs.

Name	Sequences of MSTN-shRNA (pLL 3.7)
MSTN-shRNA-198	5′-TGGAAACAGCTCCTAACATCTTCAAGAGAGATGTTAGGAGCTGTTTCCTTTTTTC-3′
	5′-TCGAGAAAAAAGGAAACAGCTCCTAACATCTCTCTTGAAGATGTTAGGAGCTGTTTCCA-3′
MSTN-shRNA-218	5′-TGCAAAGATGCTATAAGACATTCAAGAGATGTCTTATAGCATCTTTGCTTTTTTC-3′
	5′-TCGAGAAAAAAGCAAAGATGCTATAAGACA TCTCTTGAATGTCTTATAGCATCTTTGCA-3′
MSTN-shRNA-539	5′-TTGAAAGACGGTACAAGGTATTCAAGAGA TACCTTGTACCGTCTTTCATTTTTTC-3′
	5′-TCGAGAAAAAATGAAAGACGGTACAAGGTATCTCTTGAATACCTTGTACCGTCTTTCAA-3′
MSTN-shRNA-540	5′-TGAAAGACGGTACAAGGTATTTCAAGAGAATACCTTGTACCGTCTTTCTTTTTTC-3′
	5′-TCGAGAAAAAAGAAAGACGGTACAAGGTATTCTCTTGAAATACCTTGTACCGTCTTTCA-3′
MSTN-shRNA-548	5′-TGTACAAGGTATACTGGAATTTCAAGAGAATTCCAGTATACCTTGTACTTTTTTC-3′
	5′-TCGAGAAAAAAGTACAAGGTATACTGGAATTCTCTTGAAATTCCAGTATACCTTGTACA-3′
MSTN-shRNA-622	5′-TGTGAAGACAGTGTTGCAAATTCAAGAGATTTGCAACACTGTCTTCACTTTTTTC-3′
	5′-TCGAGAAAAAAGTGAAGACAGTGTTGCAAATCTCTTGAATTTGCAACACTGTCTTCACA-3′
MSTN-shRNA-NTC	5′-TCGGTATGATTGATGAGAATTTCAAGAGAATTCTCATCAATCATACCGTTTTTTC-3′
	5′-TCGAGAAAAAA CGGTATGATTGATGAGAATTCTCTTGAAATTCTCATCAATCATACCGA-3′

and incubated for 24 h. The infected cells were then washed twice with fresh complete medium and cultured in the presence of puromycin (for pLex constructs) or neomycin (for pLL3.7 constructs) to select the cells. The resulting puromycin or neomycin resistant cells were used to examine the effects of MSTN on ovine myoblast differentiation.

2.5. Quantification of ovine myoblast differentiation

The MSTN overexpression or shRNA knockdown stable clones derived from primary ovine myoblasts were seeded at a density of 40,000 cells/cm^2 in 6-well plates in complete growth medium and cultured for 24 h. The growth medium was then replaced with differentiation medium (DMEM containing 2% horse serum) for 72 h and then used for measuring fusion index, which was well established as indicator for myoblast differentiation. Fusion index was calculated by the ratio of nuclear numbers within multinucleated myotubes to total numbers of nuclei. Data were presented as mean ± SD from three independent experiments

2.6. Real time RT-PCR quantification of gene transcripts

Total RNAs were extracted from ovine myoblasts with TRIzol reagent (Invitrogen). cDNAs were synthesized from 1 μg of total RNA using a RT-PCR kit (Takara Bio Inc.) and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Ovine *MSTN*, *18s rRNA*, *MyoD*, *Myogenin* and *Smad3* transcripts were quantitated using a LightCycler 1.5 Real-time PCR machine (Roche, Indianaopolis, IN), as described previously [16]. PCR reactions contained 2 μl purified cDNA or standard plasmid, 4 mM $MgCl_2$, 0.5 μM each of primers, and 2 μl FastStart DNA Master mix (Roche). Primers are shown in Table 2. Real-time PCR results were presented as means from 3 independent experiments using the same cDNA preparation and normalized to 18s rRNA.

Table 2
Sequences of realtime RT-PCR primers and sizes of amplicons.

Gene	Primer sequence	Size (bp)
Myostatin	Sense : 5′-ATCCATCTCTGAAACTTGACATG-3′	129
	Antisense: 5′-CAGTCCATCTTCTTCTGGTTCTG-3	
Smad3	Sense : 5′-AGACATTCCACGCCTCCCAG -3′	121
	Antisense: 5′-GTCAGCTCCACAGCCGCGTT-3′	
Myod	Sense: 5′-AGGGTCCCTCGCGCCCAAAAG -3′	122
	Antisense: 5′-TGCGGGAGGCGGAAACACAACAGT-3′	
Myogenin	Sense : 5′-AGGTGAATGAAGCCTTCGAG-3′	139
	Antisense: 5′-TCCTGGTTGAGGGAGCTGAG -3′	
18s rRNA	Sense: 5′-GCTTGAACCCCATTCGTG-3′	121
	Antisense: 5′-AGCGACGGGCGGTGTG-3′	

2.7. Western blot analysis

Proteins were extracted from 293T cells or primary sheep myoblasts and subjected to SDS–polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc, Rockford, IL) and immunoblotted with anti-HA or anti-β-actin antibodies (Abcam, Cambridge, MA), as described previously [17]. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE), as described by the manufacturer.

2.8. Immunofluorescent microscopy

Primary ovine myoblasts were cultured on glass-bottomed dishes in DMEM containing 10% FBS. The attached cells were fixed with 4% paraformaldehyde, washed by TBS and blocked with 5% BSA. After washing, cells were incubated with mouse anti-Myosin heavy chain (Sigma) monoclonal antibody at 1:400 dilution overnight at 4 °C. The FITC labeled goat anti-mouse IgG (FITC-IgG) secondary antibody was added to the cells in the next day. DAPI was incubated with the cells at 100 ng/ml for 1 h at room temperature to specifically stain nuclei. Cells were visualized under UV or bright field at 10 × 20 magnitude under a fluorescent microscopy.

2.9. Statistical analysis

Statistical analyses were performed by using the SPSS 13.0 for Windows. Data are shown as mean ± SD. One-way analysis of variance was performed to determine differences among groups. $P < 0.05$ (one-tailed) was considered statistically significant.

3. Results

3.1. Identification of the most effective and specific shRNA to knockdown MSTN

Six shRNAs were designed by using the Dharmacon siDESIGN Center database and cloned into the pLL3.7 lentiviral vector (Table 1 and Fig. 1A). To determine the efficacies of these shRNA of knocking down MSTN, the MSTN-shRNA constructs were co-transfected with pLex-MSTN into 293T cells. Western blotting was performed to measure MSTN protein levels in the transfected cells by using an anti-HA antibody. Interestingly, all the shRNAs except for shR539, caused substantial down-regulation of MSTN

expression compared to that of NTC-shRNA. Among the five effective shRNAs, shR218 and shR548 showed the best efficacy with up to approximately 90% reduction of MSTN protein (Fig. 1B, lanes 3–8). Efforts were then undertaken to evaluate the specificity of shR218 and shR548. Co-transfection of MSTN-shR218 or MSTN-shR548 with corresponding pLex-mt-MSTN constructs, which contain site-directed mutations in the shR218 and shR548 targeting regions, showed no change on MSTN protein levels (Fig. 1C, lanes 3 and 5). These studies demonstrated that specific and efficient silence of ovine MSTN can be achieved by shRNA. ShR218 was selected as the optimal shRNA to knockdown ovine MSTN in the rest of our studies.

3.2. Overexpression of MSTN reversibly suppressed myogenic differentiation

To determine the role of MSTN in ovine myoblast differentiation, primary ovine myoblasts were infected with pLex-MSTN lentivirus and selected with puromycin to generate stable clone for overexpression of MSTN. This resulted in approximate 100-fold increase of both protein and mRNA levels of MSTN (Fig. 2A). Overexpression of MSTN also resulted in repression of multinucleated myotube formation and the repression could be reversed by shR218 (Fig. 2B), which indicated that overexpression of MSTN led to the inhibition of ovine myoblast differentiation. Fusion index as an indicator of differentiation showed the 45.3% decrease in MSTN cells compared to parental cells (Figs. 2C, $p \leqslant 0.05$). These phenotypic changes was reversed by knocking down the ectopically overexpressed MSTN with shR218. In contrast, the NTC-shRNA cells showed no change of fusion index (Fig. 2C). These results suggest that MSTN can reversibly suppress myogenic differentiation in ovine myoblasts.

3.3. shRNA knockdown of endogenous MSTN promoted ovine myoblast differentiation

To further investigate the role of MSTN in myogenic differentiation, shRNA stable clones of primary ovine myoblasts were generated by infecting the cells with the MSTN-shR218 lentivirus (designated shR218 cells). Cells stably expressing the NTC-shRNA (designated NTC-shR cells) were also generated as control.

Under the differentiation conditions, the shR218 cells showed persistent reduction of MSTN transcripts compared to the NTC-shR cells (Fig. 3A). Morphology and differentiation indicators in

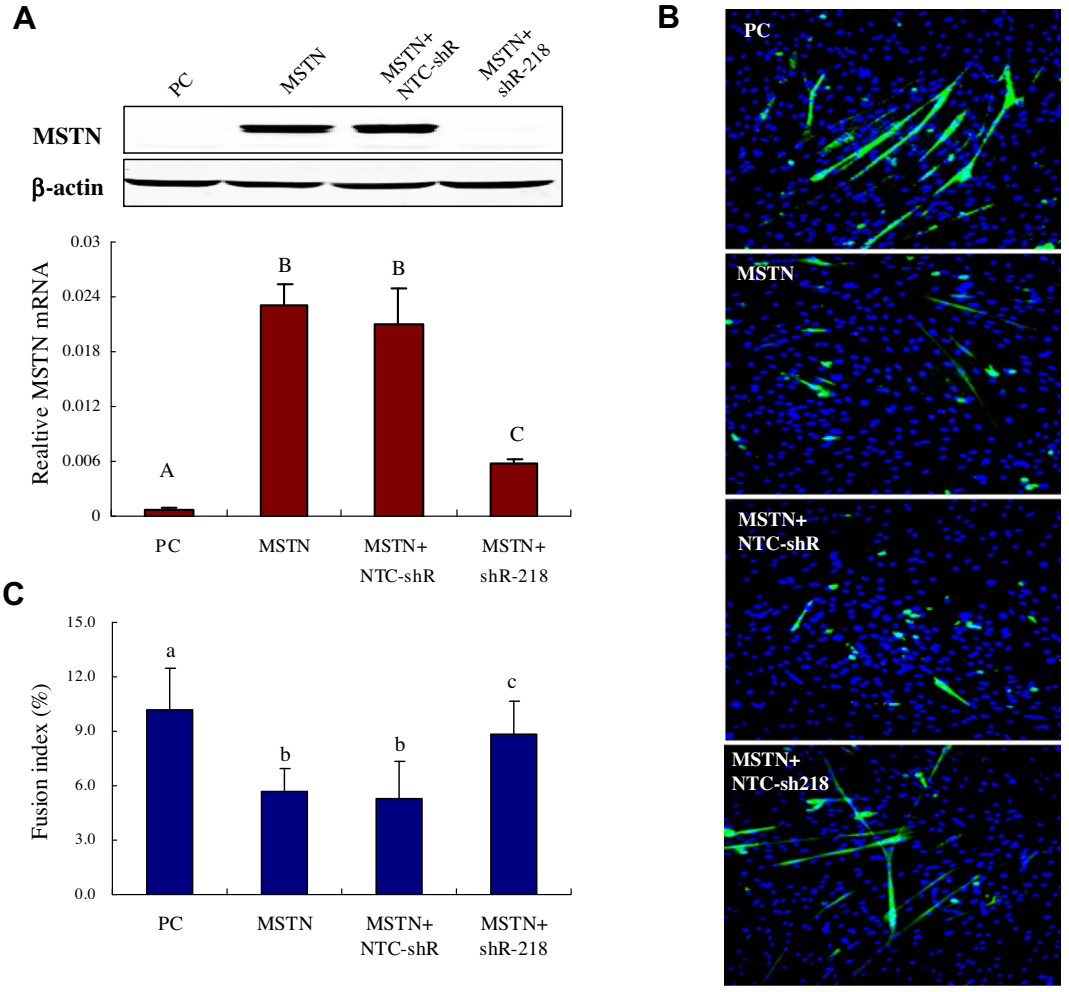


Fig. 2. Ectopic overexpression of MSTN in primary ovine myoblasts significantly suppressed myoblast differentiation. (A) Primary ovine myoblasts were transfected with pLex-MSTN to generate stable clone overexpressing MSTN. The cells were transfected with MSTN-shR218 or NTC-shR construct to generate double-stable clones. Protein and mRNA levels of MSTN were determined by Western blotting (upper panel) and Realtime RT-PCR (lower panel). (B) The stable clones generated in (A) were cultured in differentiation medium for 72 h. The cells were then fixed and incubated with mouse anti-Myosin heavy chain (MyHC) monoclonal antibody or DAPI, as described in the Materials and Method. Images were taken under a fluorescence microscopy under UV field at 10 × 20 magnitude and merged. (C) Fusion index values were determined as described in the Materials and Methods. All data are presented as mean ± SD from three independent experiments. PC, parental sheep myoblast control; MSTN, transfected only with MSTN; NTC-shR, co-transfected with MSTN and non-targeting control; shR-218, co-transfected with MSTN and shR218.

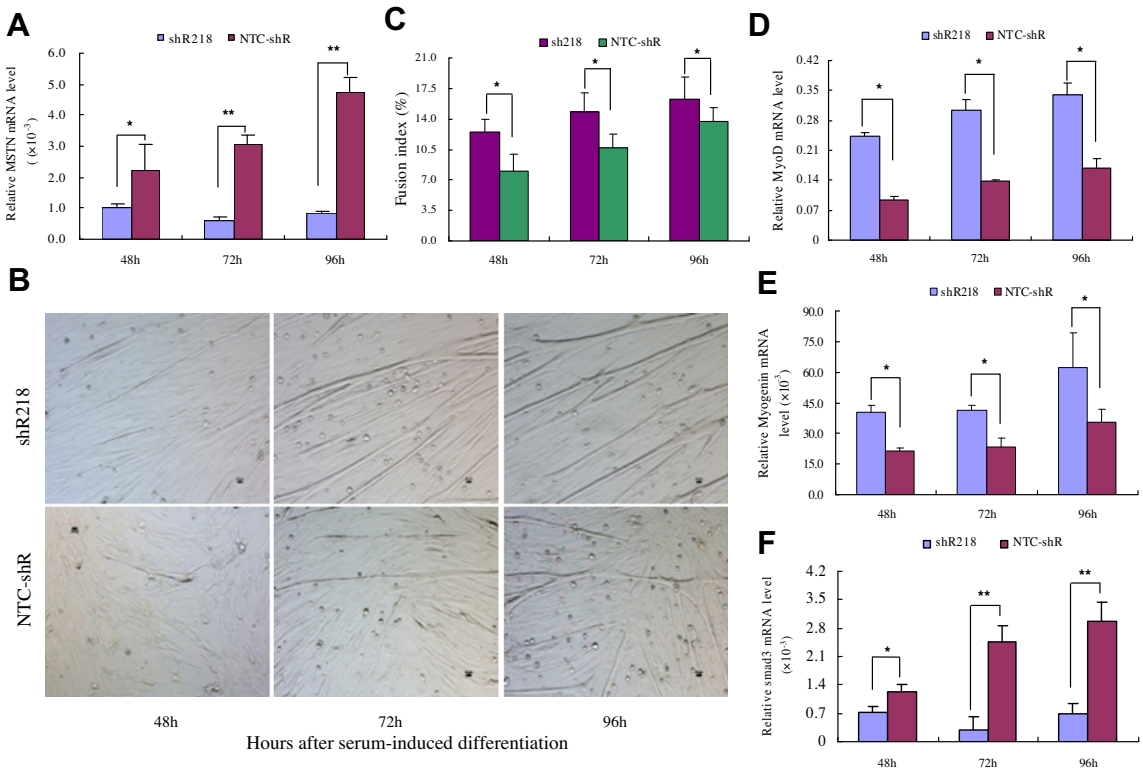


Fig. 3. Effects of shRNA knockdown of endogenous MSTN on myogenic differentiation. (A) Inhibition of endogenous MSTN expression by shR218 in ovine myoblast. (B) Morphology observation of ovine myoblast under the inhibition of endogenous MSTN expression. Multinucleated myotube as a typical feature for myoblast differentiation was observed from 48 to 96 h in the shR218 and NTC-shR cells. (C) Fusion index of shR218 and NTC-shR measured at 48, 72, and 96 h. (D–F) Transcription levels of MyoD, Myogenin and Smad3 in shR218 or NTC-shR were quantitated by real-time RT-PCR and normalized to 18S rRNA. shR218, primary ovine myoblast transfected with shR218; NTC-shR, primary ovine myoblast transfected with non-targeted shRNA control. Data are presented as mean ± SD from three independent experiments. *indicates $p < 0.05$; **indicates $p < 0.01$.

the shR-218 and NTC-shR cells were then examined. Interestingly, multinucleated myotubes could be observed as early as at 48 h in the shR218 cells, and substantially increased from 48 h to 96 h (Fig. 3B, upper panels). In contrast, multinucleated myotubes did not emerge until 72 h in the NTC-shR cells (Fig. 3B, lower panels). To quantitate the extent of differentiation, fusion index were measured in these cells. After cultured in the differentiation media for 48 h, the shR-218 cells showed 40.7 ± 7.8% increase of fusion index, compared to that of the NTC-shR cells (Fig. 3C). Identical results were also obtained from 72 h to 96 h under the same conditions. These results demonstrate that shRNA knockdown of endogenous MSTN can promote ovine myoblast differentiation.

3.4. MSTN inhibits myogenic differentiation through downregulation of MyoD and Myogenin and upregulation of Smad3 in ovine myoblasts

Previous studies demonstrated that myogenic regulatory genes are essential for myoblast differentiation [18]. MyoD and Myogenin play critical roles in myoblast differentiation. Smad3, an important regulator downstream of MSTN/TGF-β cascade, has also been proved to be an important mediator of myoblast differentiation [18]. We hypothesized that MSTN may suppress myogenic differentiation through regulating the expression of these genes in ovine myoblasts. To test this hypothesis, transcription levels of MyoD, Myogenin and Smad3 were measured by Real time RT-PCR in the shR218 and NTC-shR cells. Transcription levels of MyoD and myogenin increased more than 2-fold in the shR218 cells compared to the NTC-shR cells ($p < 0.05$, Fig. 3D and E), while Smad3 transcription level significantly decreased in the shR218 cells compared to the NTC-shR cells (Fig. 3F). These results suggested that MSTN inhibits myogenic differentiation through down-regulation of MyoD and Myogenin, and up-regulation of Smad3 in ovine myoblasts.

4. Discussion

Increase of muscle mass is a consequence of hyperplasia and hypertrophy. This process includes both myoblast proliferation and differentiation. When early muscle progenitors are specified, they can withdraw from the cell cycle to terminally differentiate into functional muscle fibers [19]. Identifying critical regulators is essential to enhance the understanding of the mechanisms of muscle growth and development, and to improve meat productivity of farm animals. MSTN has been considered as a negative regulator for muscle growth through inhibiting myoblast proliferation and differentiation since loss-of-function mutations in the MSTN gene have been associated with dramatically increased skeletal muscle mass [20]. A mutation occurred in the 3′ un-translated region (3′-UTR) of MSTN in Texel sheep was proved to be a putative causal factor for double muscling trait [21]. Based on these studies, stable silencing of MSTN with shRNAs may provide a reliable approach to generate transgenic sheep with increased meat productivity.

The goal of this study was first to investigate the role of MSTN in differentiation of ovine myoblasts by ectopic overexpression and shRNA knockdown approaches, and second to establish a reliable approach to stably silence MSTN expression for generating transgenic sheep to increase meat productivity. Our results showed that overexpression of MSTN in primary ovine myoblasts resulted in significant inhibition of myoblast differentiation, while shRNA knockdown of endogenous MSTN in ovine myoblasts resulted in significantly increased myoblast differentiation. These results shed

important light on the role of MSTN in myoblast differentiation in sheep.

MyoD and Myogenin are important myogenic regulatory factors. Previous studies suggested that MyoD and Myogenin are critical for induction of myoblast differentiation [22]. We hypothesized that MSTN exerts its effects on ovine myoblast differentiation by down-regulating MyoD and Myogenin. Consistent with our hypothesis, shRNA knockdown of MSTN resulted in significantly increased expression of MyoD and myogenin in differentiating ovine myoblasts, which is concomitant with the induction of differentiation. These results are consistent with *in vivo* results in rats [23]. Smad3 is a downstream target of the MSTN receptor complex and is essential for the inhibition of myoblast differentiation by MSTN [24]. Interestingly, decreased expression of Smad3 was observed in the MSTN knockdown cells. Our results suggest that MSTN inhibits differentiation probably by up-regulating Smad3 expression in ovine myoblasts. This is consistent with the results obtained in mice [25].

In summary, our study demonstrates that MSTN suppresses ovine myoblast differentiation, which can be abrogated by shRNA knockdown of the gene. Further, we established a shRNA approach to stably and effectively silence MSTN expression, which provides a promise for the generation of transgenic sheep to improve meat productivity.

Acknowledgments

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