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The critical role of myostatin in differentiation of sheep myoblasts

Chenxi Liu [a,b], Wenrong Li [b,c,d], Xuemei Zhang [b,c,d], Ning Zhang [b,c,d], Sangang He [b,c,d], Juncheng Huang [b,c,d], Yubin Ge [e], Mingjun Liu [b,c,d,*]

[a] College of Life Science and Technology, Xinjiang University, Urumqi, China
[b] Xinjiang Laboratory of Animal Biotechnology, Urumqi, China
[c] Laboratory of Grass-fed Animal Genetics, Breeding & Reproduction of Ministry of Agriculture, Urumqi, China
[d] Animal Biotechnological Research Center, Xinjiang Academy of Animal Science, Urumqi, China
[e] The State Engineering Laboratory of AIDS Vaccine, College of Life Science, Jilin University, Changchun, China

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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.

* Corresponding author at: Biotechnology Center, Xinjiang Academy of Animal Sciences, Urumqi, Xinjiang, China. Fax: +86 991 4841417.
E-mail address: xjlmj2004@yahoo.com.cn (M. Liu).

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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.

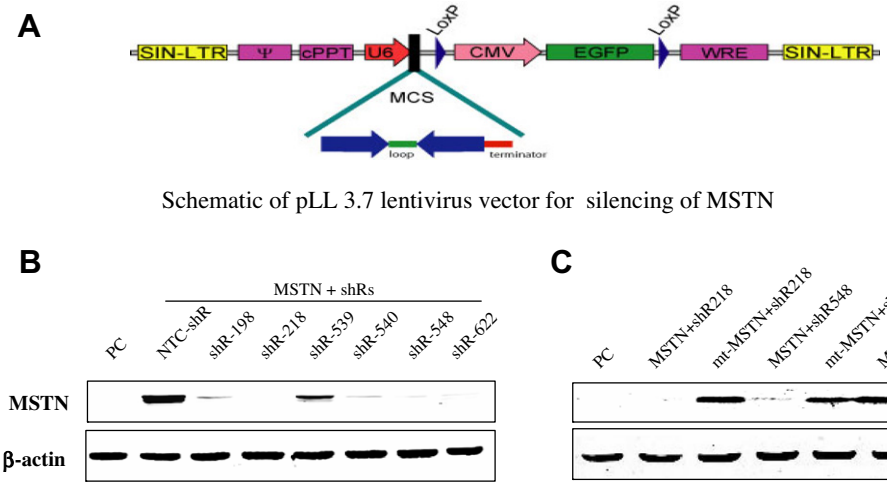
2.3. Identification of the most effective and specific shRNA for MSTN knockdown

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.