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Myostatin Gene Knockdown by Myostatin-specific Short Interfering Hairpin RNAs Increases MyoD Expression in C2C12 Myoblasts*

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Abstract Myostatin is a TGF-β superfamily member that negatively regulates the growth of the skeletal muscle mass. Remarkable muscle increase was observed in myostatin-knockout mice. Injection and electroporation of myostatin-targeting shRNA into rat tibialis anterior resulted in an increase in its weight, fiber size, and MHC Ⅱ expression. Two siRNAs targeting mouse myostatin were identified to block mouse myostatin expression upon co-transfection with a myostatin-expressing plasmid into HEK293 cell culture. These siRNAs were cloned into shRNA expression vectors and transferred into C2C12 myoblasts. ShRNA-positive cells were screened by neomycin selection and flow cytometry. By using real-time PCR, it was determined that the endogenous myostatin mRNA expression decreased by 10.2% and 35.5% in Mst-shRNA1-treated and Mst-shRNA2-treated C2C12 myoblasts, respectively. Western blot analysis indicated that the myostatin protein expression level decreased by 29.3% and 64.7%, respectively, in the two groups. it was also demonstrated that downstream MyoD pathway was affected by myostatin blockade, as evidenced by the 24.4% and 40.4% upregulation of MyoD expression in shRNA-treated cells. The results indicate that myostatin-targeting siRNA produced endogenously could efficiently downregulate myostatin expression. This RNAi-based method of increasing muscle mass could provide an alternative strategy to gene knockout methods for genetic breeding and may be useful in improving the economic properties of livestock.

Key words myostatin, RNA interference, siRNA, shRNA, MyoD
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Myostatin, also called GDF-8, is a TGF-β superfamily member that was discovered in 1997. It functions by negatively regulating skeletal muscle growth[1]. In mice, the deletion of myostatin by gene targeting leads to a widespread and remarkable increase in skeletal muscle mass. The increase is manifested both in the muscle number (hyperplasia) and the size of existing muscle fibers (hypertrophy) with individual muscles weighing 2～3 times as much as those of wild-type mice[1–2].

In fact, the excessive muscle growth in some "double-muscled" cattle breeds, such as Belgian Blue, Piedmontese and others, is due to mutations in the myostatin gene[3–7]. Recently, there were reports on a child and dog breed that exhibited a similar "muscling" phenotype; both carried an inactivating mutation in myostatin[8–9]. Cell proliferation and protein synthesis are reportedly restrained by myostatin in C2C12 muscle cells[6–7]. In contrast, a decrease in muscle mass and fiber size was observed in myostatin-overexpressing transgenic mice[10]. All these evidences support the notion that myostatin specifically functions as a negative growth factor that regulates skeletal muscle mass, suggesting that muscle mass may be increased by suppressing myostatin expression.

Myostatin propeptide, a natural inhibitor of myostatin, administered through weekly intraperitoneal injections for one month or by gene delivery with adeno-associated virus serotype 8 vectors enhanced the muscle mass in mice[11–12]. Blocking myostatin by

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injecting anti-myostatin antibodies into the peritoneum resulted in increased muscle mass and muscle size in mouse muscle[12-13]. RNA oligonucleotides that targeted the myostatin mRNA also increased muscle growth in both normal and cancer cachexia mice by upregulating MyoD expression[14].

However, further studies are required to understand the molecular mechanisms by which myostatin regulates muscle mass. There is evidence to suggest that the skeletal muscle mass is mainly regulated by myostatin through the Akt/mammalian target of the rapamycin (mTOR) pathway[15-16]. Based on the structural and biochemical characteristics of myostatin, it appears that the active myostatin ligand can bind to receptors and activate a signal transduction cascade in the target cell[17]. Myostatin signaling begins with the binding of the active myostatin ligand to an activin type Ⅱ receptor (ActR-ⅡA or ActR-ⅡB). This ligand/type Ⅱ receptor complex then binds to a type Ⅰ receptor (ALK-5 or ALK-4) and phosphorylates its kinase. The activated type Ⅰ receptor kinase then phosphorylates Smad2 and Smad3, followed by oligomerization with Smad4. Next, the activated Smad complex mediates intracellular signaling by translocating to the nucleus to regulate the transcription of downstream genes such as MyoD and other myogenesis-related genes[17-18].

Muscle regulatory factors (MRFs) are considered to be critical transcriptional regulatory proteins in developmental myogenesis regulation due to their ability to convert nonmyogenic fibroblast cells into myogenic cells[19] and the presence of their functional *cis* elements within many skeletal muscle-specific promoters and enhancers[20]. MyoD, a member of the MRF family, is one of the earliest markers of myogenic commitment and plays a key role in regulating muscle differentiation. Its main function in development is to transform mesoderm cells to skeletal muscle cells and subsequently regulate the myogenic process.

There is considerable evidence that myostatin negatively regulates the expression of MyoD and other MRFs. It was reported that myostatin overexpression in C2C12 cells inhibited the myogenic process by downregulating the mRNA levels of the muscle regulatory factors myoD and myogenin as well as the activity of their downstream target creatine kinase, thereby regulating myogenic differentiation[21]. MyoD expression was found to be significantly upregulated by the suppression of myostatin with a resulting increase in muscle growth in normal and cachectic mice[14].

RNA interference, another efficient and accurate method of gene silencing, was discovered to be an evolutionarily conserved mechanism in 1998[13]. Subsequent studies demonstrated that chemically synthesized small interfering RNAs (siRNAs) could specifically inhibit targeted gene expression[22], while a long-term inhibitory effect could be achieved by hairpin siRNAs (shRNAs) expressed under pol Ⅲ or pol Ⅱ promoters in plasmid or viral vectors[23].

Myostatin gene knockdown, concomitant with enhanced muscle growth, has been achieved by using both siRNA and shRNA. Myostatin-specific siRNA delivered into normal or diseased mice both locally and systemically through nanoparticle formation of chemically unmodified siRNAs with atelocollagen resulted in a remarkable increase in muscle mass within a few weeks[24]. A myostatin-targeting short hairpin RNA plasmid vector silenced myostatin expression in cell culture and rat skeletal muscle *in vivo* and consequently increased muscle mass after local intramuscular electroporation[25]. Silencing of the myostatin gene by RNAi was also reported to result in a giant phenotype in zebrafish[26].

The current study aimed to identify efficient targets of myostatin gene silencing and examine, at the muscle cell level, whether a plasmid vector that was integrated into mouse C2C12 myoblasts expressing myostatin-targeting shRNA could continuously suppress endogenous myostatin expression through the MyoD pathway. If this occurred, it could increase the muscle mass in a long-term and stable manner. Two shRNA constructs, each carrying an siRNA region that could target myostatin for degradation, were found to silence myostatin expression after integration into C2C12. This was accompanied by the upregulation of muscle regulatory factor MyoD. This data helps in elucidating the mechanisms of muscle mass regulation by myostatin. It also suggests that shRNA-based myostatin gene silencing could potentially increase the muscle mass, thereby providing an alternative approach for increasing the meat yield in animals.

1 Materials and methods

1.1 Identification of a myostatin-targeting siRNA

The mouse myostatin gene sequence (GenBank accession No. NM 010834) was analyzed by GenChem

Ltd. (Shanghai, China) to identify potential siRNA targets, and three regions were found to be the most likely targets for inhibition by siRNAs (nucleotide position [target #]: 246 [#1], 756 [#2] and 1020 [#3]). Double-stranded siRNAs and a negative control siRNA that did not target any sequence were synthesized by GenChem. A mouse myostatin-expressing plasmid pN1-mMSTNcDNA, which was constructed by inserting mouse myostatin cDNA into the pN1 vector, was cotransfected with the individual siRNA into HEK293 cell cultures using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The HEK293 cell cultures were grown in DMEM (GIBCO, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA). The inhibitory activity of each siRNA was tested at concentrations of 20 and 40 nmol/L.

Nucleic acid (in the form of 1 μg pN1-mMSTNcDNA and an appropriate concentration of the siRNA) was applied to 6-well plates at a nucleic acid to liposome ratio of 1∶1, and the cells were incubated for 48 h. The cell lysates were collected in a protein extraction reagent (Western and IP Cell Lysis Reagent, Beyotime, Haimen, Jiangsu, China), and Western blots were performed using a monoclonal antibody specific to green fluorescent protein (GFP; Chemicon, Billerica, MA, USA).

1.2 Construction of the shRNA plasmid vector

Two inhibitory siRNA sequences, i.e., Mst-siRNA1 (5′ CACGAGGTACTCCAGAATA 3′) and Mst-siRNA2 (5′ ACAGCCTGAATCCAACTTA 3′), were synthesized as short hairpin DNA sequences and cloned into the siRNA-expressing plasmid vector pGCsi-U6/neo/DsRed (Mst-shRNA1, 5′ GGATCCC-CACGAGGTACTCCAGAATATTCAAGAGATATT-CTGGAGTACCTCGTGTTTTTTGGAAAGCTT 3′; Mst-shRNA2, 5′ GGATCCCACAGCCTGAATCCA-ACTTATTCAAGAGATAAGTTGGATTCAGGCTG-TTTTTTTGGAAAGCTT 3′). Both these sequences contain a *Bam*HⅠ DNA restriction site, sense strand, 9 nucleotide loop, antisense strand, RNA polymeraseⅢ terminator, and *Hin*dⅢ DNA restriction site from 5′ to 3′. Moreover, a randomer shRNA that does not block any mammalian mRNA sequence (pGCsi.U6/neo/RFP-R) was also produced and used as a control. All of the constructs were sequenced to ensure that the inserts had the exact shRNA sequences required.

One microgram of pGCsi-U6/neo/DsRed-Mst shRNA1 and shRNA2 or pGCsi.U6/neo/RFP-R was cotransfected with 1 μg pN1-mMSTNcDNA into 6-well plates containing cultured HEK293 cells using 10 μl Lipofectamine 2000 for 48 h. The cells were photographed 24 h posttransfection. Western blotting was performed to test the silencing. The protein extract (40 μg) was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels using a 1∶1 000 mouse monoclonal anti-myostatin primary antibody (Chemicon, Billerica, MA, USA), and the washed membranes were then incubated with a 1∶10 000 dilution of anti-mouse horseradish peroxidase-linked secondary antibody (ZSBIO, Beijing, China).

1.3 Silencing of myostatin in C2C12 myoblasts

To test the activity of the two myostatin shRNA constructs in inhibiting endogenous myostatin gene expression, 4 μg of plasmid was introduced into C2C12 cell cultures that had reached 90% confluence in 6-well plates. G418 (600 mg/L) was added to the medium at 48 h posttransfection to screen for neo-positive cells, and the medium was refreshed every 3 d. After 14 d of G418 selection, the cells were sorted by flow cytometry, and cells exhibiting red fluorescence were purified. Each cluster was left to grow until it reached 100% confluence in T25 culture flasks. It was then assayed by reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting analysis.

Myostatin mRNA expression was determined by the real-time RT-PCR of isolated C2C12 myoblasts. Total RNA was isolated from the cells using the Trizol procedure (Invitrogen). 0.2 μg total RNA from myostatin shRNA-treated and randomer shRNA-treated C2C12 myoblasts was reverse transcribed using Superscript Ⅲ RNase H⁻ reverse transcriptase (Invitrogen) and 0.25 μg random hexamers. One microliter of the RT reaction product was added to a reaction mixture consisting of 1 × qPCR Mastermix Plus(Eurogentec, San Diego, CA, USA) and 300 μmol/L each of the forward and reverse primers (forward: 5′ GGAAACAATCATTACCATGC 3′ at nucleotide positions 451～470; reverse: 5′ ATCCACAGTTGG-GCTTTTAC 3′ at nucleotide positions 560～579)[25] in a 25 μl reaction mixture. Real-time RT-PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, Warrington, UK). The control reference gene was β-actin(GenBank accession No.NM_007393). The β-actin primers were as follows: the forward primer was 5′ TGTGATGGTGGGAA-T GGGTCAG 3′ at nucleotide positions 205～226,

and the reverse was 5′ TTTGATGTCACGCACGAT-TTCC 3′ at nucleotide positions 697～718. Each RT was amplified in triplicate, and the results were expressed as the $\bar{x} \pm s$. The relative ratios were calculated based on the $2^{-\Delta\Delta CT}$ method[25].

The myostatin, myoD, and β-actin proteins were detected by Western blotting. For endogenous myostatin expressed in C2C12 cell cultures, 50 μg protein extracts was electrophoresed on 12% SDS-PAGE gels using a 1∶1 000 mouse monoclonal anti-myostatin primary antibody (Chemicon, Billerica, MA). For myoD, a 1∶2000 dilution of a mouse monoclonal anti-myoD antibody (Abcam, Cambridge, MA, USA) was used. The secondary anti-mouse antibody (ZSBIO, Beijing, China) linked to horseradish peroxidase was diluted 1∶10 000. For β-actin, a 1∶2 500 dilution of an anti-actin polyclonal antibody (ZSBIO, Beijing, China) was used, and the washed membranes were incubated with a 1∶10 000 dilution of an anti-rabbit horseradish peroxidase-linked secondary antibody (ZSBIO, Beijing, China). The immunoreactive bands were visualized using the Pierce® ECL Western Blotting Substrate detection system (Pierce, Rockford, IL, USA). Band intensities were estimated using Quantity One (Bio-Rad, Hercules, CA, USA) and corrected on the basis of the respective β-actin band intensities.

2 Results

To identify an siRNA that could specifically inhibit myostatin expression, HEK293 cells were cotransfected with the myostatin-expressing plasmid pN1-mouse myostatin and 20 or 40 nmol/L of each siRNA that had been designed according to the mouse myostatin gene sequence. Following transfection and Western blot analysis using an anti-GFP antibody that recognizes the myostatin-GFP fusion protein, two myostatin-targeting siRNAs, i.e., Mst-siRNA1 and Mst-siRNA2, were identified. At a concentration of 40 nmol/L, these could effectively target myostatin for degradation (Figure 1a). No inhibition of the housekeeping gene β-actin was detected in the cells that had been incubated with the Mst-siRNAs. We next tested vectors containing short hairpin DNAs that represented the corresponding regions of Mst-siRNA1 and Mst-siRNA2. Western blot analysis revealed that following cotransfection of pN1-mouse myostatin and each pGCsi-U6/neo/DsRed plasmid (1:1 ratio; 2 μg each), Mst-shRNA1 and Mst-shRNA2 blocked 50.6% and 76.2% of the expression of myostatin, respectively (Figure 1b). These results were similar to the silencing results obtained using the corresponding double-stranded siRNAs.

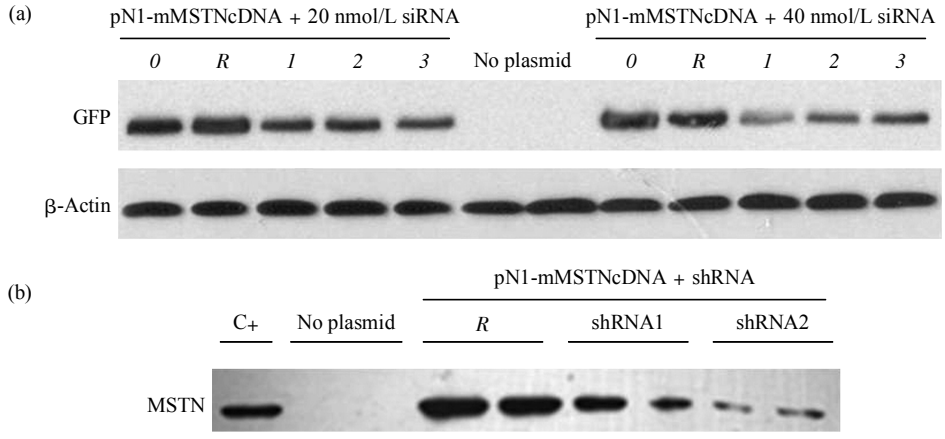

Fig. 1 Effect of mouse myostatin siRNA and shRNA on myostatin protein expression in HEK293 cell cultures

(a) The myostatin-GFP protein level was examined by Western blot analysis after cotransfecting a myostatin-expressing plasmid pN1-MSTNcDNA and three double-stranded myostatin-targeting siRNAs at concentrations of 20 and 40 nmol/L. The expression level of the housekeeping protein β-actin was also determined from the protein profile. No plasmid: Untransfected HEK293 cell culture; *0*: Transfected with myostatin-expressing plasmid; *R*: Randomer siRNA that does not target any sequence; *1*～*3*: siRNA*1*～*3*. (b) The myostatin expression level was examined by Western blot analysis after cotransfection of a myostatin-expressing plasmid and plasmids expressing myostatin-targeting shRNAs. Two independent transfections are shown for each assay. C+: Mouse control skeletal muscle protein lysate; No plasmid: Untransfected cell cultures; *R*: Randomer shRNA cotransfected with the myostatin-expressing plasmid.

The silencing efficiency of Mst-shRNAs with respect to endogenous myostatin was determined by RT-PCR and Western blot analysis after the transfection of Mst-shRNA1 or Mst-shRNA2 into

C2C12 myoblasts. After correcting for the mRNA level of the housekeeping gene β-actin, myostatin mRNA expression in Mst-shRNA1-treated and Mst-shRNA2-treated myoblasts was decreased by 10.2% and 35.5%, respectively (Figure 2), while the myostatin protein level, as demonstrated by Western blot, decreased by 29.3% and 64.7%, respectively, in comparison to the randomer-shRNA-treated myoblasts (Figure 3a).

Evidence from other myostatin-related studies has shown that the downregulation of myostatin can increase muscle-specific gene expression. Western blotting analysis revealed that the myoD protein level increased by 24.4% and 40.4% in Mst-shRNA1-treated and Mst-shRNA2-treated myoblasts, respectively, in comparison to the randomer-shRNA-treated control cells (Figure 3c).

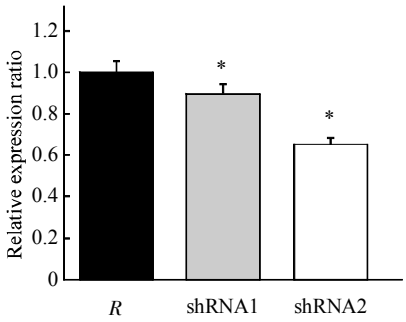

Fig. 2 Real-time RT-PCR of myostatin mRNA expression in randomer-shRNA- and Mst-shRNA-treated C2C12 myoblasts

The myostatin expression was normalized to β-actin expression and denoted as the myostatin/β-actin ratio. *$P < 0.05$.

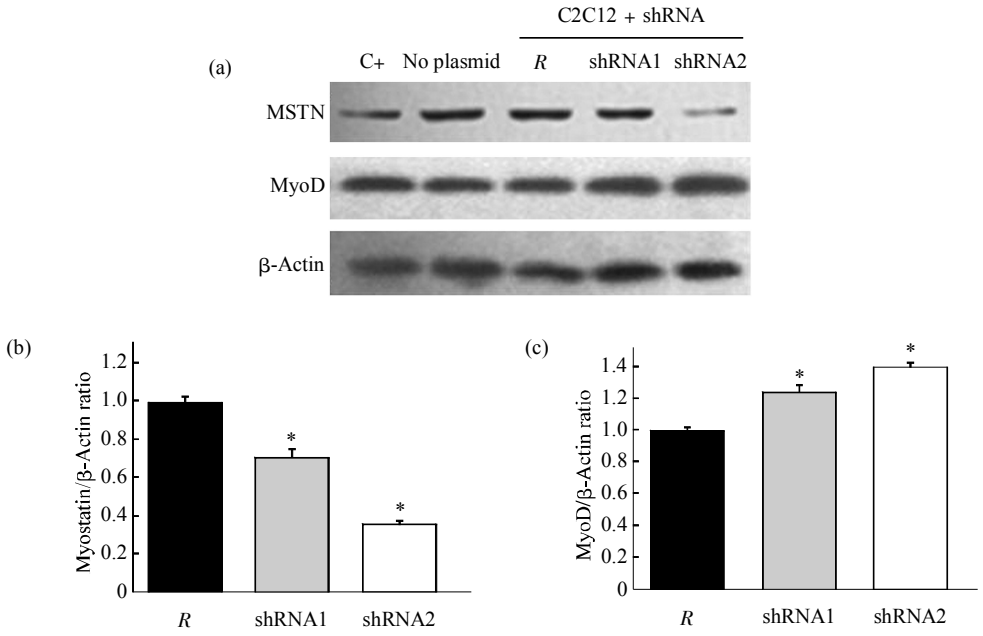

Fig. 3 Expression of myostatin and MyoD in C2C12 myoblasts following shRNA gene transfer

(a) Western blotting analysis of myostatin, MyoD, and β-actin in myostatin-targeting shRNA-treated C2C12 myoblasts. (b) Densitometric analysis of the myostatin/β-actin ratio expression determined by Western blot analysis. *$P < 0.05$. (c) Densitometric analysis of the MyoD/β-actin ratio expression determined by Western blot analysis. *$P < 0.05$.

3 Discussion

Myostatin negatively regulates muscle growth during fetal skeletal muscle development and adult skeletal muscle growth. It has been proven that the muscle mass can be increased by counteracting myostatin expression and/or activity by gene knockout or by blocking the myostatin protein using myostatin antagonists, anti-myostatin antibodies, or a myostatin propeptide. Additionally, RNA oligonucleotides can be used to target myostatin mRNA[11–12, 14, 27–28].

More recently, the suppression of myostatin expression by RNA interference (RNAi) using myostatin-targeting siRNA or shRNA was reported, thereby providing a new method for myostatin administration[24–25]. RNAi is a conserved biological

mechanism that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids and regulates gene expression by silencing the target gene with great specificity and efficiency. It has become a robust tool in gene function analysis, gene therapy, and virus protection[13, 29]. In one study, myostatin-targeting shRNA was injected and electroporated into rat tibialis anterior. After 2 weeks, the myostatin-targeting shRNA reduced the myostatin mRNA and protein expression levels by 27% and 48%, respectively, while the tibialis anterior weight, fiber size, and MHC Ⅱ levels increased by 10%, 34% and 38%, respectively. This demonstrated that myostatin shRNA gene transfer is a potential strategy for increasing the muscle mass[25]. Another study reported the effectiveness of *in vivo* siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen(ATCOL). ATCOL-mediated local application of myostatin-targeting siRNA in mouse skeletal muscles or intravenous administration resulted in a marked increase in the muscle mass within a few weeks of application[24].

Myostatin is specifically expressed in developing and adult skeletal muscle, while very little myostatin expression has been detected in lipocytes and other tissues[30-31]. Although the increase in the muscle mass in myostatin-knockout mice was attributed to both hypertrophy and hyperplasia, some studies have indicated that myostatin mainly functions by controlling the number of myofibers during fetal skeletal muscle development and satellite cell proliferation during adult skeletal muscle growth [2,17,32-34]. On the other hand, myostatin blockade during embryonic development leads to some side effects. Both myostatin-mutant breeds and knockout mice exhibit reduced female fertility[27, 35-37] and abnormal overheating during exercise, which were related to excessive muscle mass [38]. Considering this, it is important to control/limit the muscle increase within a moderate degree, which will ease the excess-muscle-caused side effects.

Myostatin regulates muscle mass in a dose-dependent manner. The muscles of myostatin-deficient mice weighed approximately 2 ～3 times more than those of wild-type mice, while to a lesser degree, the weight of individual muscles isolated from the heterozygous mutant only exhibited a 25% increase in comparison with those isolated from wild-type littermates [17]. It is widely accepted that RNAi rarely results in total elimination of gene expression, and si/shRNAs that target different locations of one gene will have a varying silencing effect. This serves the purpose of partly inhibiting myostatin so that a state of homeostasis is achieved between the increasing muscle mass and its side effects. In other words, we can theoretically increase muscle growth to any extent required by using si/shRNAs with different inhibitory efficiencies.

Considering that the expression of myostatin occurs almost exclusively in the muscle, direct injection of myostatin antagonists, si/shRNA, antibodies, myostatin propeptide, or RNA oligonucleotides into muscle has been considered for the purpose of myostatin inhibition. This method has a wider applicability since it would not affect tissues other than the skeletal muscle where the endogenous myostatin would still be significantly involved in other functions. However, it is difficult to sustain long-term inhibition by injecting antagonists into muscle. Although this method avoids side effects to tissues other than muscles, the difficulty is that separate multiple injections into several muscles would be impractical. In such a case, the injection into each muscle may not be uniform, and the uptake may be inefficient. Moreover, repeated injections may be required to increase the overall uptake. In this case, it might be applicable in postnatal gene therapy blockage of myostatin expression. However, to improve the economic properties of farm animals using this strategy, in other words, for genetic breeding, a more stable and long-lasting method is required.

Recent advances involving the use of vector-based RNA interference technologies promise alternative approaches for the stable silencing of genes in different animal species, including mammals. ShRNA constructs, expressed in plasmid or viral vectors, have been shown to effectively cause gene silencing[39-44]. The expression constructs can be stably incorporated into the genome due to the vector characteristics and can therefore be used in cell cultures and the production of transgenic animals. Some reports have verified the use of this technique in mouse ES cells or early stage embryos to create transgenic mice that exhibit a phenotype analogous to that of the knockout animal. Furthermore, it has been shown that this RNAi-based suppression could be passed through the germ line to the offspring as a

dominant trait, which is important for trait preservation in transgenic animals[45-48].

In comparison with gene knockout techniques, RNAi-based gene silencing *in vivo* is independent of homologous recombination or ES cells. This technique would save both labor and time in the production of transgenic animals in livestock species. A strategy to create a cloned transgenic goat fetus with dramatically reduced PrP expression by using RNAi-based techniques has been described, and evidence has been presented that these techniques can also be adapted to cattle, indicating that RNA interference can be applied to the creation of genetically engineered animals that resist viral diseases or have improved agricultural traits[49].

In this study, we designed two siRNAs that target myostatin, i.e., Mst-siRNA1 and Mst-siRNA2. These effectively block myostatin expression in HEK293 cells transfected with a plasmid construct expressing mouse myostatin. We cloned short hairpin versions of these two siRNAs, referred to as Mst-shRNA1 and Mst-shRNA2, and transfected each of these into C2C12 myoblasts to examine their ability to silence endogenous myostatin expression. Myostatin mRNA and protein expression levels were decreased by 10.2% and 29.3%, respectively, by Mst-shRNA1 in C2C12 cells. These levels were decreased by 35.5% and 64.7%, respectively, in C2C12 cells integrated with Mst-shRNA2. MyoD expression was also found to be increased in these two groups. This data supports the conclusion that myostatin regulates muscle growth through the Akt/MyoD pathway and that neutralizing myostatin expression through the shRNA approach could enhance muscle growth. Taken together with the vector characteristics of shRNA expression constructs, this raises the possibility that RNAi technology in combination with somatic cell nuclear transfer technology can be introduced to the genetic breeding field with the aim of increasing the muscle mass/meat yields and muscle-fat ratio and improving the economic properties of livestock. Moreover, considering the high evolutionary conservation of the myostatin gene and protein product across mice, pigs, cows, and chickens, si/shRNA that target myostatin should facilitate myostatin gene silencing studies in these species as well. However, it is to be noted that RNAi is a relatively new technology. More effort is required to optimize this approach in animal models prior to its potential administration.

References

[1] McPherron A C, Lawler A M, Lee S J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature, 1997, **387**(6628): 83-90

[2] Grobet L, Pirottin D, Farnir F, *et al*. Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. Genesis, 2003, **35**(4): 227-238

[3] Grobet L, Martin L J R, Poncelet D, *et al*. A deletion in the bovine myostatin gene causes the double muscled phenotype in cattle. Nat Genet, 1997, **17**(1): 71-74

[4] Grobet L, Poncelet D, Royo L J, *et al*. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double muscling in cattle. Mamm Genome, 1998, **9** (3): 210- 213

[5] Kambadur R, Sharma M, Smith T P L, *et al*. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res, 1997, **7**(9): 910-915

[6] McPherron A C, Lee S J. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA, 1997, **94** (23): 12457- 12461

[7] Gill J L, Bishop S C, McCorquodale C, *et al*. Associations between the 11-bp deletion in the myostatin gene and carcass quality in Angus-sired cattle. Anim Genet, 2009, **40**(1): 97-100

[8] Schuelke M, Wagner K R, Stolz L E, *et al*. Myostatin mutation associated with gross muscle hypertrophy in a child. N Engl J Med, 2004, **350**(26): 2682-2688

[9] Shadan S. Run, whippet, run. Nature, 2007, **447**(7142): 275

[10] Reisz-Porszasz S, Bhasin S, Artaza J N, *et al*. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. Am J Physiol Endocrinol Metab, 2003, **285**(4): E876-E888

[11] Wolfman N M, McPherron A C, Pappano W N, *et al*. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. Proc Natl Acad Sci USA, 2003, **100**(26): 15842-15846

[12] Qiao C, Li J, Jiang J, *et al*. Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice. Hum Gene Ther, 2008, **19**(3): 241-254

[13] Fire A, Xu S, Montgomery M K, *et al*. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature, 1998, **391**(6669): 806-811

[14] Liu C M, Yang Z, Liu C W, *et al*. Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice. Gene Ther, 2008, **15**(3): 155-160

[15] Amirouche A, Durieux A C, Freyssenet D, *et al*. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. Endocrinology, 2009, **150**(1): 286-294

[16] Trendelenburg A U, Meyer A, Rohner D, *et al*. Myostatin reduces AKT/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. Am J Physiol Cell Physiol, 2009, **296** (6):

C1258-1270

[17] Lee S J. Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol, 2004, **20**: 61-86

[18] Kollias H D, McDermott J C. Transforming growth factor-beta and myostatin signaling in skeletal muscle. J Appl Physiol, 2008, **104**(3): 579-587

[19] Pownall M E, Gustafsson M K, Jr Emerson C P. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol, 2002, **18**: 747-783

[20] Black B L, Olson E N. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol, 1998, **14**: 167-196

[21] Rios R, Carneiro I, Arce V M, *et al*. Myostatin is an inhibitor of myogenic differentiation. Am J Physiol Cell Physiol, 2002, **282**(5): C993-C999

[22] Elbashir S M, Harborth J, Lendeckel W, *et al*. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 2001, **411**(6836): 494-498

[23] Brummelkamp T R, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002, **296**(5567): 550-553

[24] Kinouchi N, Ohsawa Y, Ishimaru N, *et al*. Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass. Gene Ther, 2008, **15**(15): 1126-1130

[25] Magee T R, Artaza J N, Ferrini M G, *et al*. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med, 2006, **8**(9): 1171-1181

[26] Acosta J, Carpio Y, Borroto I, *et al*. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol, 2005, **119**(4): 324-331

[27] Whittemore L A, Song K, Li X, *et al*. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun, 2003, **300**(4): 965-971

[28] Bogdanovich S, Krag T O, Barton E R, *et al*. Functional improvement of dystrophic muscle by myostatin blockade. Nature 2002, **420**(6914): 418-421

[29] Hannon G J. RNA interference. Nature, 2002, **418**(6894): 244-251

[30] Ji S, Losinski R L, Cornelius S G, *et al*. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am J Physiol Regul Integr Comp Physiol, 1998, **275** (4pt2): R1265-R1273

[31] Sharma M, Kambadur R, Matthews K G, *et al*. Myostatin, a transforming growth factor-β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol, 1999, **180**(1): 1-9

[32] Thomas M, Langley B, Berry C, *et al*. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem, 2000, **275**(51): 40235-40243

[33] Gonzalez-Cadavid N F, Bhasin S. Role of myostatin in metabolism. Curr Opin Clin Nutr Metab Care, 2004, **7**(4): 451-457

[34] Tobin J F, Celeste A J. Myostatin, a negative regulator of muscle mass: implications for muscle degenerative diseases. Curr Opin Pharmacol, 2005, **5**(3): 328-332

[35] Patel K, Amthor H. The function of myostatin and strategies of myostatin blockade-new hope for therapies aimed at promoting growth of skeletal muscle. Neuromuscul Disord, 2005, **15**(2): 117-126

[36] Wiener P, Smith J A, Lewis A M, *et al*. Muscle-related traits in cattle: The role of the myostatin gene in the South Devon breed. Genet Sel Evol, 2002, **34**(2): 221-232

[37] Hanset R. The major gene of muscular hypertrophy in the Belgian Blue cattle breed//Owen J B, Axford R F E. Breeding for Disease Resistance in Farm Animals. Bangor, UK:CAB International, 1991: 467-478

[38] Chupin D. Analysis of reproduction problems in double muscle females//King J W B, Menissier F. Muscle Hypertrophy of Genetic Origin and Its Use to Improve Beef Production. Dordrecht:Kluwer, 1982

[39] Stegmeier F, Hu G, Rickles R J, *et al*. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. Proc Natl Acad Sci USA, 2005, **102**(37): 13212-13217

[40] Seibler J, Kuter-Luks B, Kern H, *et al*. Single copy shRNA configuration for ubiquitous gene knockdown in mice. Nucleic Acids Res, 2005, **33**(7): e67

[41] Paddison P J, Caudy A A, Bernstein E, *et al*. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev, 2002, **16**(8): 948-958

[42] Paddison P J, Caudy A A, Hannon G J. RNAi functions in cultured mammalian neurons. Proc Natl Acad Sci USA, 2002, **99**(3): 1443-1448

[43] Paddison P J, Hannon G J. RNA interference: the new somatic cell genetics?. Cancer Cell, 2002, **2**(1): 17-23

[44] Siolas D, Lerner C, Burchard J, *et al*. Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol, 2004, **23**(2): 227-231

[45] Carmell M A, Zhang L, Conklin D S, *et al*. Germline transmission of RNAi in mice. Nat Struct Biol, 2003, **10**(2): 91-92

[46] Rubinson D A, Dillon C P, Kwiatkowski A V, *et al*. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet, 2003, **33**(3): 401-406

[47] Tiscornia G, Singer O, Ikawa M, *et al*. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci USA, 2003, **100**(4): 1844-1848

[48] Tiscornia G, Tergaonkar V, Galimi F, *et al*. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Proc Natl Acad Sci USA, 2004, **101**(19): 7347-7351

[49] Michael C G, Charles R L, Michelle A C, *et al*. Suppression of prion protein in livestock by RNA interference. Proc Natl Acad Sci USA, 2006, **103**(14): 5285-5290

小鼠肌肉生长抑制素基因短发夹 RNA 促进 MyoD 在 C2C12 细胞中的表达 *

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摘要　肌肉生长抑制素 (myostatin，MSTN) 属于转化生长因子 -β (transforming growth factor-β，TGF-β) 超家族，主要功能为负向调节骨骼肌的生长．肌肉生长抑制素基因敲除小鼠肌肉出现显著增加，而将干涉该基因的短发夹 RNA 注射并电击转化入大鼠胫前肌则引起肌肉重量、肌纤维以及 MHC Ⅱ表达的增加．通过与小鼠肌肉生长抑制素基因表达载体共转染 HEK293 细胞，筛选到两条能够高效抑制小鼠肌肉生长抑制素基因表达的小干涉 RNA．构建了这两条小 RNA 的表达载体 Mst-shRNA1 和 Mst-shRNA2，用其分别转染小鼠 C2C12 成肌细胞，并通过 G418 药物筛选和流式细胞仪富集整合了短发夹 RNA 表达载体的阳性细胞．通过采用 Real-time PCR 和 Western blot 分析，检测到在分别整合了 Mst-shRNA1 和 Mst-shRNA2 的 C2C12 细胞中，内源性肌肉生长抑制素基因的 mRNA 水平分别下降了 10.2%和 35.5%，蛋白质表达则分别下降了 29.3%和 64.7%．同时，在这两组中 MyoD 的表达上升了 24.4%和 40.4%，证明通过 RNA 干涉实现的肌肉生长抑制素基因的抑制导致了下游 MyoD 基因表达的上调．这些结果表明内源性产生的肌肉生长抑制素基因小干涉 RNA 能够有效抑制该基因的表达．这种以 RNA 干涉技术为基础促进肌肉生长的方法为遗传育种提供了一种新技术，能够在对家畜经济性状的改良中发挥重要作用．

关键词　肌肉生长抑制素，RNA 干涉，小干涉 RNA，短发夹 RNA，MyoD

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