Science in China Series C: Life Sciences

Functional analysis of the *Myostatin* gene promoter in sheep

DU Rong[1,2], AN XiaoRong[1†], CHEN YongFu[1] & QIN Jian[3]

[1] State Key Laboratory for Agrobiotechnology, College of Biological Science, China Agricultural University, Beijing 100094, China;
[2] College of Animal Science and Technology, Shanxi Agricultural University, Taigu 030801, China;
[3] College of Modern Education Technology, Shanxi Agricultural University, Taigu 030801, China

Compared with the understanding for the functional mechanism of the *myostatin* gene, little is known about the regulatory mechanism of the *myostatin* gene transcription and expression. To better understand the function of the *myostatin* gene promoter (*MSTN*pro) in the transcriptional regulation of the *myostatin* gene and to further investigate the transcriptional regulation mechanism of the *myostatin* gene, the promoter region of the *myostatin* gene in sheep has been cloned in our recent study (AY918121). In this study, the wild (*W*) type *MSTN*Pro^{W}-*EGFP* vectors and E-box (E) (CANNTG) mutant (*M*) type *MSTN*Pro$^{E(3+5+7)M}$-*EGFP* vectors were constructed and the transcriptional regulation activities were compared by detecting the fluorescent strength of EGFP (enhanced green fluorescent protein) in C2C12 myoblasts (or myotubes) and sheep fibroblasts transfected with the vectors. Results showed that the 0.3—1.2 kb sheep *myostatin* promoter could activate the transcription and expression of *EGFP* gene in C2C12 myoblasts to different extent and the 1.2 kb promoter was the strongest. However, fluorescence was not observed in the sheep fibroblasts transfected with the 1.2 kb sheep *myostatin* promoter. These results suggested that the specific nature of the *myostatin* gene expression in skeletal muscle was attributed to the specific nature of the *myostatin* promoter activity. The increasing growth density of C2C12 myoblasts inhibited the transcriptional regulation activity of the wild type sheep *myostatin* promoter by a mechanism of feedback. The transcriptional regulation activity of the 1.2 kb wild type sheep *myostatin* promoter increased significantly after C2C12 myoblasts were differentiated, while the activity of 1.2 kb E(3+5+7)-mutant type *myostatin* promoter had no obvious change. This result suggested that MyoD may be responsible for the difference of the *myostatin* gene transcription and expression between growing and differentiating conditions by binding to E-box of the *myostatin* promoter.

sheep, *myostatin* promoter, transcriptional regulation activity, C2C12

Myostatin (MSTN), also named growth/differentiation factor 8 (GDF8), a member of the transforming growth factor β (TGF-β) superfamily, is expressed almost exclusively in skeletal muscle and acts as an autocrine negative regulator of skeletal muscle growth and development in mammals[1,2]. Overexpression of the *myostatin* gene can induce muscular atrophy and mutations of the *myostatin* gene are responsible for the double-muscled phenotype of animals including human[3−8]. Like other TGF-β family members, myostatin may exist in a latent complex with the propeptide and on activation myostatin may function by binding to activin type II receptors (ActRII)[9]. The combination of myostatin and ActRII induced Smad signal pathway[10−12], affecting the expressions of some muscle regulatory factors (MRFs) such as myogenic differentiation 1 (MyoD), myogenic

Received October 31, 2006; accepted July 4, 2007
doi: 10.1007/s11427-007-0085-2
†Corresponding author (email: xra@cau.edu.cn)
Supported by the National Hi-Tech R&D Program ("863" Program) of China (Grant No. 2002AA206311)

factor 5 (Myf5) and myogenin. Then the myoblast proliferation and differentiation were inhibited[10,13]. Although myostatin showed rapid sequence evolution in animals such as ruminants before domestication[14], the identical or highly similar sequences of myostatin protein's C-terminal active region in animals (humans, rats, mice, pigs, chickens, turkeys, cattle and sheep) suggested that the function of myostatin was extremely conserved throughout the evolution[3,15]. In fish, myostatin exhibited more differences in structure, distribution and role[16−20].

Compared with the understanding for the functional mechanism of the *myostatin* gene, little is known about the regulatory mechanism of the *myostatin* gene transcription and expression. Earlier studies for *myostatin* mRNA indicated that the *myostatin* gene expression appears to be regulated at the transcriptional level[1,4,21]. Some limited researches on the *myostatin* promoter in mouse, human and bovine were carried out and some transcriptional factors (such as MyoD, MEF-2, FoxO and Smad) were perceived to regulate the transcription and expression of the *myostatin* gene[12,22−26]. However, many unclear fields still need to be investigated about the function of the *myostatin* promoter (*MSTN*pro) in the transcriptional regulation of the *myostatin* gene. Moreover, there were greater differences for the *myostatin* promoter region sequence among different animals compared with the *myostatin* gene coding region sequence according to our recent study and other studies[15,27,28], which suggested that the possible differences of the *myostatin* transcriptional regulation exist among different animals. To better understand the function of the *myostatin* promoter, we cloned the 1.211 kb *myostatin* promoter region from Small Tail Han Sheep (a local Chinese breed of *Ovis aries*) (AY918121)[27], selected the more intuitionistic (easy to observe) reporter gene *EGFP* (enhanced green fluorescent protein) which has not been used for the study of the *myostatin* promoter in mammals, and constructed various *MSTN*Pro-*EGFP* vectors. The transcriptional regulation activities in various conditions were compared by detecting the fluorescent strength of EGFP in C2C12 myoblasts (myotubes) or sheep fibroblasts transfected with the vectors.

1 Materials and methods

1.1 Construction of *MSTN*Pro-*EGFP* vector

Six *myostatin* promoter fragments with different lengths (1.2, 1.0, 0.9, 0.7, 0.4 and 0.3 kb) were generated by polymerase chain reaction (PCR) from the plasmid (T-*MSTN*pro) with the 1.517 kb *myostatin* promoter region of sheep (Small Tail Han *Ovis aries*, a local Chinese breed)[27]. The PCR reaction was performed in a 25-μL reaction containing 20 ng plasmid (T-*MSTN*pro), 1×Taq reaction buffer, 5 nmol dNTPs, 20 pmol of each primer, and 0.25 U Taq DNA polymerase (Tiangen). The PCR program was carried out for an initial 5 min 94℃ denaturing step, 30 cycles (each cycle included 30 s at 94℃, 30 s at 56℃, and 1 min at 72℃), and a final 10 min extension at 72℃ in a Biometra® T-gradient thermocycler. The primers are shown in Table 1.

Table 1 The primers used to amplify the sheep *myostatin* promoter fragment with different lengths

Fragment size	Sense (S) or anti-sense (A) primer
1211 bp (1.2 kb)	S: 5′-ATCAAGCTTAGACCTTACCCCAAATCC-3′
1037 bp (1.0 kb)	S: 5′-CTCAAGCTTCTGTGTTCACAATGTTTG-3′
855 bp (0.9 kb)	S: 5′-ATTAAGCTTCACATCCTCTAAGCCACA-3′
702 bp (0.7 kb)	S: 5′-CGCAAGCTTGCTAAGAATTTATTCAGG-3′
429 bp (0.4 kb)	S: 5′-CGCAAGCTTGTTTCACATATAAGGATG-3′
272 bp (0.3 kb)	S: 5′-ATCAAGCTTCTGTTTGGTGACTTGTGA-3′
0.3 kb−1.2 kb	A: 5′-CGCGGATCCGGTTTTAAAATCAATACA-3′

The bases underlined indicate *Hin*d III or *Bam*H I sites introduced by PCR.

Like other muscle-specific genes, the *myostatin* gene in sheep has multiple E-boxes (CANNTG) in its promoter region[27] to cooperatively regulate gene transcription by binding to the basic helix-loop-helix myogenic regulatory factors (MRFs, including the MyoD, Myf5, myogenin, and MRF4 transcription factors). Site-directed mutations in the E-box (E) motifs (E3+E5+E7) were introduced by PCR. The same template and condition but different primers (Table 2) with the above were used for generating the E-mutant fragments. Briefly, the target element sequence was replaced with a suitable restriction site and the fragments flanking the target element were amplified respectively (Figure 1).

The p*EGFP*-N1 (4.1 kb) vector without promoter was gained by removing the cytomegalovirus (CMV) promoter from p*EGFP*-N1 (4.7 kb) vector (Clontech). Then the wild type *MSTN*Pro^{W}-*EGFP* vectors and the E(3+5+7)-mutant type *MSTN*Pro$^{E(3+5+7)M}$-*EGFP* vector were constructed by inserting the sheep *myostatin* promoter region with different lengths or inserting successively the E-mutant *myostatin* promoter fragments amplified above into the corresponding restriction sites of the p*EGFP*-N1 (4.1 kb) vector.

Table 2 The primers used to amplify the sheep *myostatin* gene promoter fragments with mutant-E(3+5+7)

Fragment	Primer	Fragment	Primer
E3*M*R(s)	5′-AAT*GGTACC*AATCAGCTCACCCTTGAC-3′	E5E7*M* (s)	5′-CGC*GAATTC*GAATAAAGATATTATTTA-3′
E3*M*R(a-s)	5′-CGCGGATCCGGTTTTAAAATCAATACA-3′	E5E7*M* (a-s)	5′-GCA*CTGCAG*CACTTAGATCTTATTCAT-3′
E3E5*M* (s)	5′-CGC*CTGCAG*TTATTGTTACTAAAGTTT-3′	E7*M*L(s)	5′-ACTAAGCTTAGACCTTACCCCAAATCC-3′
E3E5*M* (a-s)	5′-CGC*GGTACC*ACTACTTCTTAAAAGGAA-3′	E7*M*L(a-s)	5′-CGG*GAATTC*AAAATTGCATTTCAGTTT-3′

The bases underlined indicate the restriction sites introduced by PCR. The italics indicate the E-box sequence mutated into restriction sites. E*M*L (E*M*R) indicates the left (right) *myostatin* promoter fragment flanking the mutant-E; E3E5*M* (E5E7*M*) indicates the *myostatin* promoter fragment between two mutant E-boxes. "s" or "a-s" indicates the sense (anti-sense) primers.

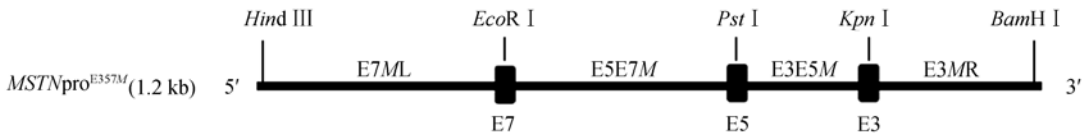

Figure 1 Site-directed mutations in the E-box (E3+E5+E7) motifs were introduced by replacing the target element sequence with a suitable restriction site in PCR. E3*M*R, E3E5*M*, E5E7*M* and E7*M*L indicate the right (left) *myostatin* promoter fragments flanking the mutant-E.

1.2 Cell culture and transfection

The fibroblasts were isolated from sheep tissues and the C2C12 myoblasts (MB) were obtained from ATCC. Cells were routinely cultured in DMEM-F12 (Gibco) supplemented with 10% FBS (TBD), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Alternatively, after the above passage, C2C12 cells were cultured in DMEM-F12 containing 2% horse serum (Gibco) for 4—5 d, and then the C2C12 myoblasts were differentiated and the myotubes (MT) were harvested.

The *MSTN*pro-*EGFP* vectors were transfected into C2C12 myoblasts or sheep fibroblasts cultured in 12-well plates using lipofectamine (Invitrogen). Briefly, the cells were washed and 300 μL fresh DMEM-F12 (serum-free, penicillin-free and streptomycin-free) was added before the DNA-lipofectamine mixture was added. Plasmids and lipofectamine were first diluted respectively with 100 μL DMEM-F12 (serum-free, penicillin-free and streptomycin-free) and incubated for 5 min at room temperature. Then the diluted plasmids and lipofectamine were mixed and incubated for 20 min at room temperature before the mixture was added to the cells. After being cultured for 4 h at 37℃ (5% CO_2), the transfected cells were added with 500 μL DMEM-F12 containing 20% FBS and cultured further for 36 h till fluorescence analysis was done. To achieve equal molar concentration of each promoter construct used for transfection, the amounts of plasmids with different lengths were adjusted by the following formula[22], where 4127 is the size of the basic vector p*EGFP*-N1 (4.1 kb) and 1211 is the size of the longest *MSTN*pro fragment:

Plasmid(μg)=0.5 μg×(*MSTN*pro size+4127)/(1211+4127).

To get the C2C12 myoblasts transfected stably with the 1.2 kb *MSTN*pro, the longest wild type or E(3+5+7)-mutant type *MSTN*pro-*EGFP* vectors were transfected into the C2C12 myoblasts cultured in 60 mm plates according to the above procedures. The C2C12 myoblasts transfected stably were selected for their resistance to geneticin (G418) (600 μg/mL).

1.3 Fluorescence assay and statistical analysis

The fluorescent strengths were analyzed by Flow Cytometry (FACSCalibur) and Fluorescence Spectrophotometer (Hitachi F-4500). For C2C12 cells transfected transiently, the collected and diluted cell suspensions were used directly for analysis. But for C2C12 cells transfected stably, the proteins were obtained after the cells were lyzed by three freeze-thraw cycles. The total protein was estimated by the BioPhotometer (Germany) and the fluorescence strength was analyzed. Each experiment was performed for six times. The data, which were values of test groups (transfected with vectors) minus values of blank groups (untransfected with any vector), were analyzed by the procedure of SAS statistical software, $P<0.01$ or $P<0.05$ being the level of statistical significance.

2 Results

2.1 Transcriptional regulation activity of the sheep *myostatin* promoter in myoblasts or fibroblasts

The C2C12 myoblasts transfected with the p*EGF*P-N1

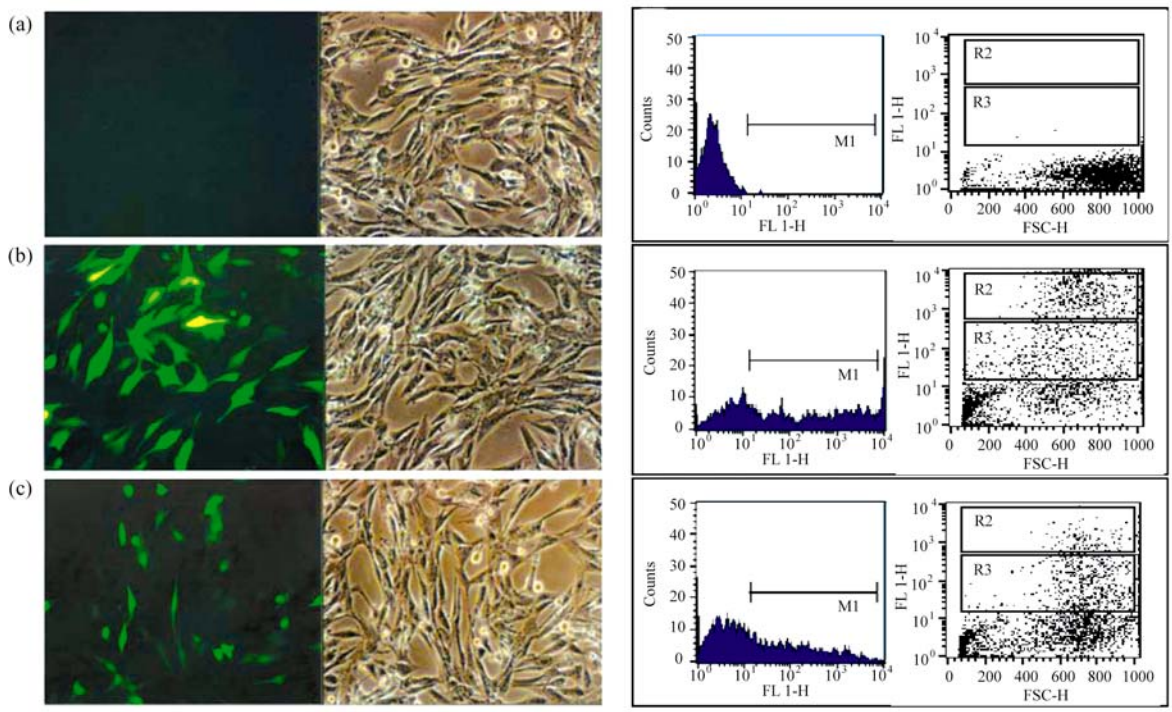

Figure 2 Transcriptional regulation activity of the sheep *myostatin* promoter (1.2 kb) in C2C12 myoblasts. Fluorescence was observed by a fluorescent microscope (10×10) (left) and detected by flow cytometry (right). (a) The negative control group transfected with p*EGFP*-N1(4.1 kb) vector without promoter; (b) the positive group transfected with p*EGFP*-N1(4.7 kb) with CMV promoter; (c) the test group transfected with *MSTN*pro-*EGFP* with the 1.2 kb wild type *MSTN*pro.

(4.1 kb) (negative group), p*EGFP*-N1 (4.7 kb) (positive group) and *MSTN*pro-*EGFP* (1.2 kb *MSTN*pro) were cultured for 40 h. The results showed that fluorescence was hardly observed and detected in the negative group, but the 1.2 kb sheep *myostatin* promoter could activate the transcription and expression of *EGFP* in C2C12 myoblasts (Figure 2). However, the 1.2 kb sheep *myostatin* promoter could not activate the transcription and expression of *EGFP* in the sheep fibroblasts after we transfected *MSTN*pro-*EGFP* (1.2 kb *MSTN*pro) into the sheep fibroblasts (Figure 3).

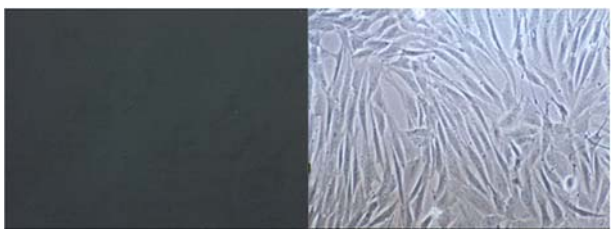

Figure 3 Transcriptional regulation activity of the sheep *myostatin* promoter (1.2 kb) in sheep fibroblasts (10×10)

2.2 Transcriptional regulation activity of the sheep *myostatin* promoter with different lengths in C2C12 myoblasts

The 0.3－1.2 kb sheep *myostatin* promoter could activate the transcription and expression of *EGFP* in C2C12 myoblasts to different extent (1.2 kb>0.7 kb>1.0 kb>0.3 kb>0.9 kb>0.4 kb) and 1.2 kb was the strongest (P<0.01) (Figure 4). Of particular interest were the higher activity of 0.7 kb than 0.9 kb or 1.0 kb and the higher activity of 0.3 kb than 0.4 kb (P<0.01). It suggested that there were negative regulatory motifs along the sheep *myostatin* promoter from 0.7 kb to 0.9 kb or from 0.3 kb to 0.4 kb.

2.3 Effects of cell growth density on the transcriptional regulation activity of the sheep *myostatin* promoter in C2C12 myoblasts

The equal C2C12 myoblasts transfected stably with *MSTN*pro-*EGFP* (1.2 kb *MSTN*pro) were grown in three plates with different areas (9.6, 3.8 and 1.9 cm^2). The fluorescent strength was analyzed after 40 h. The results showed that the increasing growth density of C2C12 myoblasts inhibited the activity of the sheep *myostatin* promoter (P<0.01) (Figure 5).

2.4 Effects of E-box mutation and cell differentiation on the transcriptional regulation activity of the sheep *myostatin* promoter in C2C12 cells

The C2C12 myoblasts transfected stably with the 1.2 kb wild or E(3+5+7)-mutant type sheep *myostatin* promoter

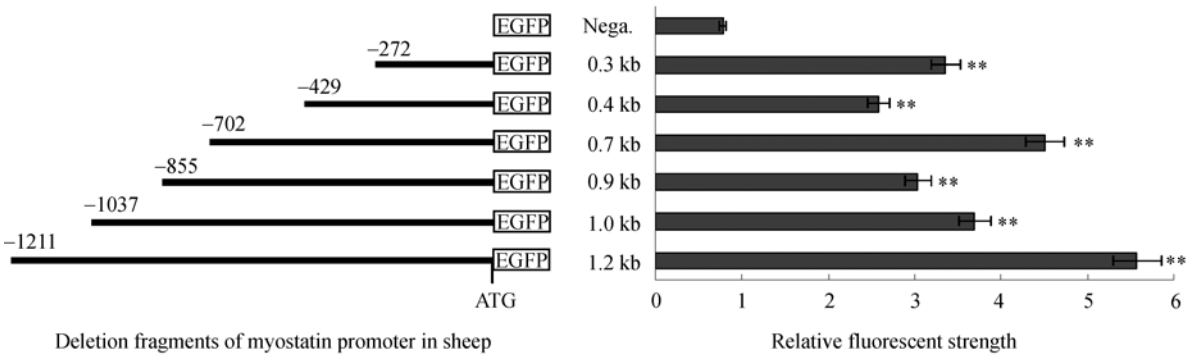

Figure 4 Deletion analysis of the 1.2 kb *myostatin* promoter in sheep. The left and right graphs indicate respectively the vector consruction and corresponding relative fluorescent strength. −272 to −1211 indicate the upstream positions from ATG of the *EGFP*. Nega. indicates the negative vector p*EGFP*-N1 (4.1 kb). Bars indicate means ± standard deviations for six replicates. ** shows that there was significant difference compared with the negative group (P<0.01).

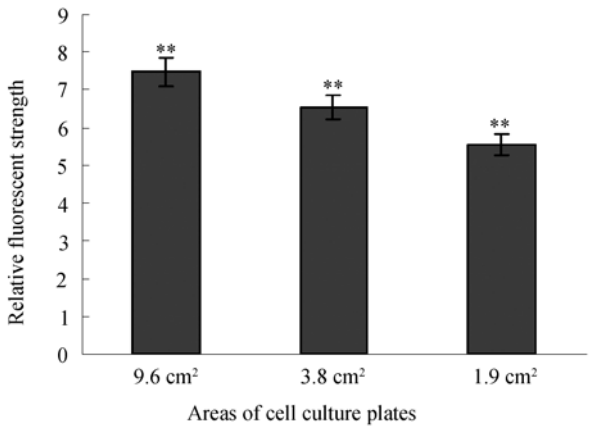

Figure 5 Effects of growth density on the transcriptional regulation activity of the sheep *myostatin* promoter in C2C12 myoblasts. Bars indicate means ± standard deviations for six replicates. ** shows that there were significant differences among the three groups (P<0.01).

were induced to differentiate using 2% horse serum and the fluorescence of the undifferentiated quiescent reserve cells was too faint to observe (Figure 6 (a),(b)). E(3+5+7)-mutation decreased obviously the transcriptional regulation activity of the sheep *myostatin* promoter in C2C12 myoblasts (P<0.05). In addition, the transcriptional regulation activity of the 1.2 kb wild type sheep *myostatin* promoter increased significantly after the C2C12 cells were differentiated (P<0.01) but the differentiation had no obvious effect on the activity of the E(3+5+7)-mutant type *myostatin* promoter (Figure 6 (c)).

3 Discussion

The obvious transcription and expression of the *myostatin* gene in fish species occur also in a variety of tissues including brain, intestine, gills, tongue, eye, ovary, skin and so on, besides skeletal muscle[16,29,30]. In mammals, the *myostatin* gene appears to be transcribed and expressed predominantly in skeletal muscle according to the related studies. But there was no agreement on the transcription and expression of the *myostatin* gene in other tissues of mammals. McPherron et al.[1] did not find the *myostatin* mRNA in many tissues such as heart, lung, thymus, brain, kidney, seminal vesicle, pancreas, intestine, spleen, testis, liver, ovary, uterus beyond skeletal muscle, only a little in adipose tissue. Ji et al.[31] and Sharma et al.[32] detected a little *myostatin* mRNA or protein in heart, adipose, breast tissues of pig or cattle. Spiller et al.[24] showed that the 1.6 kb bovine *myostatin* promoter had significantly lower activity in fibroblasts (NIH3T3 or CHO) than in C2C12 myoblasts by about one fourth, but still had obvious activity compared with the control vector (pGL3B) in fibroblasts (NIH3T3 or CHO). Our results showed that the 1.2 kb sheep *myostatin* promoter could activate the transcription and expression of *EGFP* in C2C12 myoblasts but not in sheep fibroblasts, which might be because enough necessary transcriptional factors binding to the myostatin gene promoter were present in myoblasts but not in fibroblasts. These results above suggested that the skeletal muscle specificity of the *myostatin* gene expression was attributed to the specificity of the *myostatin* gene promoter transcriptional regulation,and the presence or absence of the necessary transcriptional factors binding to the *myostatin* gene promoter in myoblasts or in fibroblasts conferred this essential difference. The difference for the *myostatin* promoter activity in fibroblasts between two experiments of us and Spiller et al.[24]

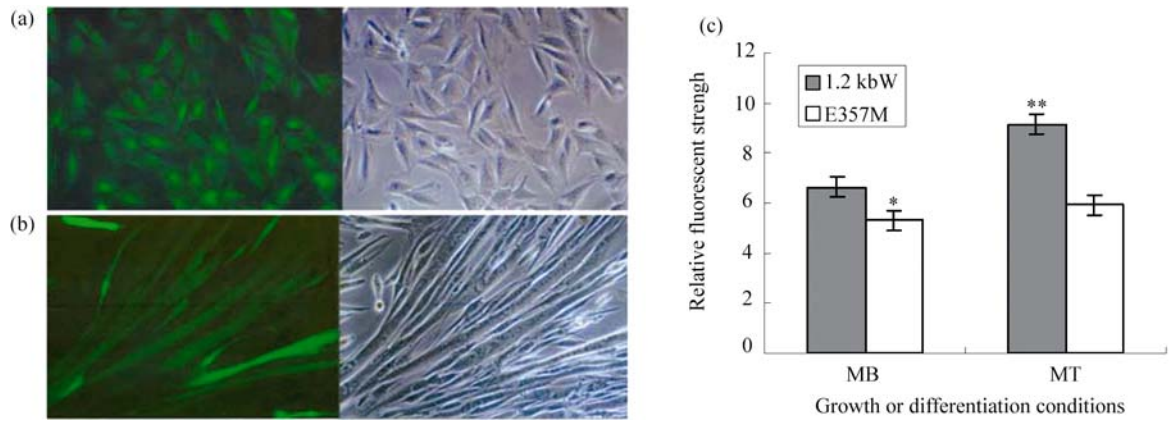

Figure 6 Effects of E(3+5+7) mutation and cells differentiation on the transcriptional regulation activity of the sheep *myostatin* promoter in C2C12 cells. (a) The C2C12 myoblasts (MB) transfected stably with 1.2 kb wild type *myostatin* promoter; (b) the C2C12 myoblasts (MB) transfected stably with 1.2 kb wild type *myostatin* promoter were differentiated into myotube (MT); (c) the fluorescent strength analysis of the MB or MT transfected stably with 1.2 kb wild type *myostatin* promoter or E(3+5+7) mutant type *myostatin* promoter. Bars indicate means ± standard deviations for six replicates. * or ** shows that there was obvious difference while comparing with MB transfected stably with the wild type *myostatin* promoter ($P<0.05$ or $P<0.01$).

might be due to the difference of signal-background of reporter gene luciferase and EGFP, fibroblast origin, or other reasons. Various methods and more research were necessary for scientists to clear the problem "the transcription and expression of the *myostatin* gene in other non-skeletal muscle tissues of mammals".

The shortest length (272 bp) of the *myostatin* promoter deletion fragments in sheep was sufficient and the longest length (1211 bp) of the *myostatin* promoter deletion fragments in sheep was strongest for activating the transcription and expression of *EGFP* in C2C12 myoblasts in our experiment. From 272 bp to 1211 bp, the activities of the *myostatin* promoter regions did not exhibit regular ascending tendency with the increasing length of promoter fragments in sheep. This characteristic was similar to but different from the deletion studies of the *myostatin* promoter in cattle[24] or human[22]. This characteristic suggested that there were some important positive and negative regulatory motifs along the *myostatin* promoter.

Over-growth density can result in contact inhibition of cells and myostatin can inhibit the proliferation of skeletal muscle cells, which promoted us to investigate the unknown relationship between the growth density of skeletal muscle cells and the transcription and expression of the *myostatin* gene. In our experiment, the activity of the sheep *myostatin* promoter decreased obviously with increasing growth density of C2C12 myoblasts, which might involve a mechanism of feedback.

Ma et al.[22] showed that the transcriptional regulation activity of the human *myostatin* promoter was significantly higher in C2C12 or L6 myotubes than in myoblasts by transient-transfection analysis. Spiller et al.[24] showed that the transcriptional regulation activity of the bovine *myostatin* promoter was obviously lower in the undifferentiated quiescent reserve cells than in the differentiated myotubes. Similarly, in our experiment, the stable-transfection analysis showed that the transcriptional regulation activity of the sheep wild-type *myostatin* promoter was significantly higher in C2C12 myotubes than in myoblasts and the fluorescence of the undifferentiated quiescent reserve cells was too faint to observe. The results above evidenced by different methods suggested that the transcription of the *myostatin* gene in differentiated skeletal muscle cells appeared to be significantly higher than in growing skeletal muscle cells of mammals. Of our particular interest, the combinational mutations of E-box 3, 5 and 7 (three important E-boxes for the transcriptional regulation activity of the sheep *myostatin* promoter, which were verified by single-mutant analysis in our test, data not shown here) lessened the difference of the sheep *myostatin* promoter activity between C2C12 growing and differentiation conditions in our test. This result suggested that MyoD was responsible for the difference of the *myostatin* gene transcription-expression in skeletal muscle cells under growth and differentiation conditions, since MyoD (a major factor contributing to the myoblast differentiation) was obviously higher in differentiating myotubes than in growing myoblasts[10,24] and regulated the transcription

of its downstream target gene *myostatin* by binding to the E-box of *myostatin* promoter[24]. In fact, the relationship between MyoD and myostatin was complex because myostatin could also downregulate the *MyoD* expression[10,24]. The conclusive evidence requires to be investigated further.

1 McPherron A C, Lawler A M, Lee S J. Regulation of skeletal muscle mass in mice by a new TGF-ß superfamily member. Nature, 1997, 387: 83－90[DOI]
2 Rios R, Fernandez-Nocelos S, Carneiro I, et al. Differential response to exogenous and endogenous myostatin in myoblasts suggests myostatin acts as an autocrine factor *in vivo*. Endocrinology, 2004, 145(6): 2795－2803[DOI]
3 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[DOI]
4 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): 910－916
5 Grobet L, Martin L J, 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[DOI]
6 Grobet L, Poncelet D, Royo L J, et al. Molecular definition of an allele series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mamm Genome, 1998, 9(3): 210－213[DOI]
7 Bellinge R H S, Liberles D A, Laschi S P A, et al. Myostatin and its implications on animal breeding: A review. Anim Genet, 2005, 36(1): 1－6 [DOI]
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 [DOI]
9 Lee S J, McPherron A C. Regulation of Myostatin activity and muscle growth. Proc Natl Acad Sci USA, 2001, 98(16): 9306－9311[DOI]
10 Langley B, Thomas M, Bishop A, et al. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem, 2002, 277(51): 49831－49840[DOI]
11 Zhu X, Topouzis S, Liang L F, et al. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. Cytokine, 2004, 26(6): 262－272 [DOI]
12 Forbes D, Jackman M, Bishop A, et al. Myostatin auto-regulates its expression by feedback loop through Smad7 dependent mechanism. J Cell Physiol, 2006, 206(1): 264－272
13 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 [DOI]
14 Tellgren A, Berglund A C, Savolainen P, et al. Myostatin rapid sequence evolution in ruminants predates domestication. Mol Phylogenet Evol, 2004, 33: 782－790 [DOI]
15 Gu Z, Zhang Y, Shi P, et al. Comparison of avian myostatin genes. Anim Genet, 2004, 35(6): 470－472 [DOI]
16 Ostbye T K, Galloway T F, Nielsen C, et al. The two myostatin genes of Atlantic salmon (Salmo salar) are expressed in a variety of tissues. Eur J Biochem, 2001, 268 (20): 5249－5257[DOI]
17 Xu C, Wu G, Zohar Y, et al. Analysis of myostatin gene structure, expression and function in zebrafish. J Exp Biol, 2003, 206: 4067－4079 [DOI]
18 Amali A A, Lin C J F, Chen Y H, et al. Up-regulation of muscle-specific transcription factors during embryonic somitogenesis of zebrafish (*danio rerio*) by knock-down of myostatin-1. Dev Dyn, 2004, 229(4): 847－856 [DOI]
19 Martin C I, Johnston I A. The role of myostatin and the calcineurin-signalling pathway in regulating muscle mass in response to exercise training in the rainbow trout *Oncorhynchus mykiss* Walbaum. J Exp Biol, 2005, 208: 2083－2090[DOI]
20 Kerr T, Roalson E H, Rodgers B D. Phylogenetic analysis of the myostatin gene sub-family and the differential expression of a novel member in zebrafish. Evol Dev, 2005, 7(5): 390－400 [DOI]
21 Oldham J M, Martyn J A, Sharma M, et al. Molecular expression of myostatin and MyoD is greater in double-muscled than normal-muscled cattle fetuses. Am J Physiol Regul Integr Comp Physiol, 2001, 280(5): R1488－R1493
22 Ma K, Mallidis C, Artaza J, et al. Characterization of 5'-regulatory region of human myostatin gene: Regulation by dexamethasone *in vitro*. Am J Physiol Endocrinol Metab, 2001, 281(6): E1128－E1136
23 Allen D L, Unterman T G. Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. Am J Physiol Cell Physiol, 2007, 292: C188－C199 [DOI]
24 Spiller M P, Kambadur R, Jeanplong F, et al. The myostatin gene is a downstream target gene of basic helix-loop-helix transcription factor MyoD. Mol Cell Biol, 2002, 22(20): 7066－7082 [DOI]
25 Crisa A, Marchitelli C, Savarese M C, et al. Sequence analysis of myostatin promoter in cattle. Cytogenet Genome Res, 2003, 102(1-4): 48－52 [DOI]
26 Salerno M S, Thomas M, Forbes D, et al. Molecular analysis of fiber type-specific expression of murine myostatin promoter. Am J Physiol Cell Physiol, 2004, 287(4): C1031－C1040[DOI]
27 Du R, Chen Y F, An X R, et al. Cloning and sequence analysis of myostatin promoter in sheep. DNA Seq, 2005, 16(6): 412－417
28 Yu Z Q, Li Y, Meng Q Y, et al. Comparative analysis of the pig BAC sequence involved in the regulation of myostatin gene. Sci China Ser C-Life Sci, 2005, 48(2): 168－180
29 Radaelli G, Rowlerson A, Mascarello F, et al. Myostatin precursor is present in several tissues in teleost fish: A comparative immunolocalization study. Cell Tissue Res, 2003, 311(2): 239－250
30 Roberts S B, Goetz F W. Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. FEBS Lett, 2001, 491(3): 212－216[DOI]
31 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(4): R1265－1273
32 Sharma M, Kambadur R, Matthews K G, et al. Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol, 1999, 180(1): 1－9[DOI]