Gene silencing of myostatin in differentiation of chicken embryonic myoblasts by small interfering RNA

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Sato, Fuminori, Masatoshi Kurokawa, Nobuhiko Yamauchi, and Masa-aki Hattori. Gene silencing of myostatin in differentiation of chicken embryonic myoblasts by small interfering RNA. Am J Physiol Cell Physiol 291: C538–C545, 2006.—Myostatin (GDF-8) is known to negatively regulate skeletal muscle mass in myogenesis, but few studies have been conducted on the function of endogenous GDF-8 in primary myoblasts. The present study was performed to assess the function of GDF-8 by RNA interference using primary culture of chicken embryonic myoblasts in which myoblasts were differentiated into myotubes. An active form of small interfering RNA (siRNA-1) targeting GDF-8 mRNA was introduced into myoblasts, and an inactive form of siRNA (siRNA-2) was used as a negative control. GDF-8 transcript level was significantly reduced 24 h after the introduction of siRNA-1 to 25% of the control, whereas a 52-kDa GDF-8 precursor was reduced to 45% of the control at 48 h. However, siRNA-2 did not decrease GDF-8 transcript level. When GDF-8-mediated promoter activity was measured chronologically by means of a pGL(CAGA)10-constructed luciferase reporter assay, a concomitant change in activity was initiated after 24 h. The activity rapidly decreased 30 h after siRNA-1 introduction, whereas high activity was maintained at 30–42 h in the control and siRNA-2-treated myoblasts. Myogenic factors such as MyoD and p21, but not myogenin, were altered after 72 h. Cell fusion of the multinucleated myotubes was delayed by the siRNA-1 introduction, and myotubes with aggregated nuclei were shorter and wider. These results strongly suggest that deficiency of GDF-8 delays cell differentiation and causes great alterations in the cellular morphology of chicken embryonic myotubes.

GDF-8; GDF-8-mediated promoter activity; multinucleated myotubes

MYOGENESIS IS REGULATED by the proliferation and differentiation of myoblasts with expression of myogenesis-related transcription factors. Myostatin, referred to as GDF-8, belongs to the TGF-β superfamily. Initially, GDF-8 is expressed in the myotome compartment of developing somites, and the expression is maintained in adult axial and paraxial muscle (2, 22). GDF-8-null mice (22) and genetically GDF-8-defective cattle (11, 23) showed a drastic increase in skeletal muscle mass, indicating a function of GDF-8 as a strong negative regulator in myogenesis. The blockage of endogenously produced GDF-8 action in mdx mice greatly improved dystrophic muscle (4, 37, 38). More recently, GDF-8 mutation leading to gross muscle hypertrophy was reported in humans (31). Conversely, increased expression of GDF-8 is observed in patients with chronic illnesses (26), human immunodeficiency virus infection (10), and the aging process (16) as a contributor to muscle atrophy. These findings raise the possibility that a blockade of GDF-8 activity might have intrinsic effects on human therapeutics as well as meat production (20).

GDF-8 is a secretory protein, and its mature form binds to activin receptor type IIB (ActRIIB) to induce Smad signals (19, 21, 41). Transgenic mice overexpressing a dominant-negative form of ActRIIB also exhibit increased skeletal muscle mass (21). Binding of GDF-8 to ActRIIB can be inhibited by the activin-binding protein follistatin (21), follistatin-related gene, and the GDF-8 propeptide (14, 33). The resulting activation of a Smad signal may suppress the expression of MyoD and myogenin (19). In contrast, GDF-8 increases p21 expression, as it accumulates hypophosphorylated Rb leading to the arrest of myoblasts in the G1 phase of the cell cycle (34). Most of these studies have been performed with cell lines such as C2C12 myoblasts with overexpression of GDF-8 or its recombinant. However, it is also suggested that myoblasts exhibit a differential response to the overexpression of GDF-8 and its recombinant (29) and that endogenous GDF-8 localizes mostly in the nuclei of C2C12 myotubes, probably participating in transcription regulation (3). Consequently, it is difficult to determine the precise functions of GDF-8 by using cell lines with overexpression of GDF-8 or its recombinant. Therefore, the investigation should be performed in primary cultures of myoblasts corresponding to in vivo physiological states.

Chickens have been used as a model vertebrate for studying embryonic development including myogenesis because the embryos can be easily manipulated compared with other higher vertebrates (5). Studies on chicken embryogenesis have attracted interest recently because of the accomplishments of the chicken genome sequencing project (6, 32). Double-stranded RNA (dsRNA) has been demonstrated to induce sequence-specific posttranscription gene silencing, a phenomenon known as RNA interference (RNAi) (12, 13). The introduction of shorter dsRNA (small interfering RNA, siRNA) into mammalian cells also induces the degradation of targeted mRNA with sequence specificity (1, 7, 9, 36). More recently, RNAi was established in chicken embryonic cells and whole tissues with the use of a dual-fluorescence reporter assay to assess the specific suppression of targeted gene expression (30).

To extend our knowledge on the function of GDF-8 during myogenesis, loss-of-function analyses of endogenously produced GDF-8 were performed with siRNA targeting on GDF-8 (GDF-8-siRNA) in a primary culture of chicken embryonic myoblasts that was established to possess the ability of GDF-8
expression involved in proliferation and differentiation. In this report, we prove that deficiency of GDF-8 greatly induces alterations in the cellular morphology of myotubes differentiated from myoblasts.

**MATERIALS AND METHODS**

**Preparation of chicken embryonic myoblasts.** Fertilized eggs were purchased from a commercial source and incubated at 37.5°C under a relative humidity of 60–70%. The pectoralis muscles were collected from 12-day chicken embryos and washed twice with phosphate-buffered saline (PBS). They were minced and digested with 0.1% collagenase type I (Invitrogen, Carlsbad, CA) in α-MEM (Invitrogen) at 37°C for 20 min. Digested cells were then dispersed by pipetting and filtered to remove aggregated cells and myotubes. The cell suspension was washed twice with PBS and subjected to density gradient centrifugation according to a previous report (38) with some modifications. This was performed in a discontinuous layer with 20%, 27.5%, and 40% Percoll (Sigma, St. Louis, MO). The cell suspension was layered on 20% Percoll and centrifuged for 5 min at 15,000 × g at 8°C with an angle rotor (R12A2; Hitachi, Tokyo, Japan). After centrifugation, myoblasts were recovered from the 27.5%/40% Percoll interface, washed twice with PBS, and resuspended in α-MEM.

**Culture of chicken embryonic myoblasts.** Myoblasts were seeded in a 35-mm collagen-coated dish (Iwaki, Tokyo, Japan) at 1 × 10^5 cells/cm² with α-MEM containing 10% chicken serum (CS), 1× insulin-transferrin-selenium X supplement (ITS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and 3 mM sodium butyrate (Sigma). They were cultured for 44 h at 37°C in humidified 95% CO₂ and then used as GDF-8-siRNA (Table 1). siRNA-2 was used as an independent synthesis and annealed (Dharmacon Research, Lafayette, CO) and then used as GDF-8-siRNA (Table 1). siRNA-2 was used as a negative control as described in RESULTS. The introduction of siRNA was carried out with Lipofectamine 2000 (Invitrogen) according to the instruction manual with minor modifications. GDF-8-siRNA (50 pmol) was mixed and incubated for 5 min with 50 μl of Opti-MEM (Invitrogen). One microliter of Lipofectamine 2000 (Invitrogen) was mixed and incubated for 5 min with forty-nine microliters of Opti-MEM. Both of the two solutions were combined, mixed gently, and incubated at room temperature for 30 min. After myoblasts were washed twice with Opti-MEM, 900 μl of Opti-MEM was added to each dish, and then the 100-μl siRNA solutions were added. Six hours later, 1 ml of Opti-MEM containing 15% KSR was added to each dish. The control was not transfected with siRNA.

**GDF-8-mediated promoter activity by luciferase reporter assay.** GDF-8 activity was estimated by using the pGL(CAGA)10-luciferase reporter assay following a previous report (33) with some modifications. A reporter vector containing 10 CAGA boxes [pGL(CAGA)10] was constructed with a pGL3-promoter vector (Promega, Madison, WI). Oligonucleotides containing 10 repeats of the CAGA boxes (sense: 5′-AGC CAG ACA-3′; antisense: 5′-TGT CTG GCT-3′) with MluI and BgII sites at respective 5′ upstream sites were synthesized, annealed and inserted between MluI and BgII sites of the pGL3-promoter vector. pGL3(CAGA)10 was prepared from transformed JM109 Escherichia coli (32) and used for transfection into myoblasts. pGL3(CAGA)10 was cotransfected with or without GDF-8-siRNA (siRNA-1 or siRNA-2) into myoblasts in 1 ml of Opti-MEM. One milliliter of Opti-MEM containing 15% KSR and 0.2 mM d-luciferin was added to each well. Twenty hours later luciferase activity was chronologically monitored at 37°C with an AB-2500 Kronos (Atto, Tokyo, Japan). Data were normalized at the starting time of determination (20 h after siRNA introduction) and expressed as relative activity.

**Semiquantitative RT-PCR.** Total RNA was extracted from the cultured cells with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the instruction manual. Reverse transcription (RT) was performed at 37°C for 1 h in 20 μl of 1× Moloney murine leukemia virus (MMLV) reaction buffer, 300 ng of oligo(dT)12–18, 20 U of RNA guard, 200 U of MMLV reverse transcriptase (Amersham Biosciences, Piscataway, NJ), dNTPs each at 0.5 mM (Applied Biosystems, Foster City, CA), and 1 μg of total RNA. The PCR was performed in 10 μl of 1× PCR buffer, dNTPs each at 0.2 mM, 0.25 U of AmpliTaq Gold (Applied Biosystems), synthetic primer sets (each at 0.2 μM; Table 2), and 0.1 μl of the RT reaction. After an initial denaturation step (95°C for 10 min), the amplification was performed in 25–40 cycles, according to the level of various transcripts, under a thermal profile of denaturation (95°C for 45 s), annealing, and extension. Table 2 shows the primer sets used in the experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primers</th>
<th>Position</th>
<th>Product Sizes</th>
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<tr>
<td>GDF-8</td>
<td>AF019621</td>
<td>Sense: 5′-GGAGACACGAAGCCTAAGAC-3′&lt;br&gt;Antisense: 5′-GTCGAGCTGTTGGCTGCTGCA-3′</td>
<td>196–706</td>
<td>511</td>
</tr>
<tr>
<td>MyoD</td>
<td>L34006</td>
<td>Sense: 5′-CGCTGCGCTGTTGGCTGCTGCA-3′&lt;br&gt;Antisense: 5′-GGAGACACGAAGCCTAAGAC-3′</td>
<td>157–679</td>
<td>523</td>
</tr>
<tr>
<td>Myogenin</td>
<td>NM_204184</td>
<td>Sense: 5′-GGAGACACGAAGCCTAAGAC-3′&lt;br&gt;Antisense: 5′-GTCGAGCTGTTGGCTGCTGCA-3′</td>
<td>276–616</td>
<td>341</td>
</tr>
<tr>
<td>p21</td>
<td>NM_204396</td>
<td>Sense: 5′-GGAGACACGAAGCCTAAGAC-3′&lt;br&gt;Antisense: 5′-GTCGAGCTGTTGGCTGCTGCA-3′</td>
<td>51–296</td>
<td>246</td>
</tr>
<tr>
<td>GADPH</td>
<td>V00407</td>
<td>Sense: 5′-GTCGAGCTGTTGGCTGCTGCA-3′&lt;br&gt;Antisense: 5′-GGAGACACGAAGCCTAAGAC-3′</td>
<td>−15 to 1017</td>
<td>1032</td>
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</tbody>
</table>

MyoD, myogenic regulatory factor.
annealing (for 45 s), and extension reaction (72°C for 1 min). As an internal control, chicken glyceraldehyde-3-phosphate dehydrogenase expression was used. The resulting PCR products were analyzed by electrophoresis on 2% agarose gels. The intensities of the bands were quantified with a densitometry program (Scion Image).

Western blot analysis. After 48 h of transfection, myoblasts and/or myotubes were washed with PBS, suspended in 62.5 mM Tris·HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 0.14 M β-mercaptoethanol, and then treated at 99°C for 3 min. SDS-PAGE was performed on 10% polyacrylamide gels for immunodetection of GDF-8 and electrotransferred onto Immobilon polyvinylidene difluoride (Millipore, Bedford, MA). Membranes were blocked in 10 mM Tris·HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 0.05% Tween 20 with 10% nonfat dry milk and incubated at 4°C with primary antibodies monoclonal anti-GDF-8 antibody MAB788 (R&D Systems, Minneapolis, MN) and monoclonal anti-α-tubulin antibody DM1A (Sigma). The anti-GDF-8 antibody MAB788 detects the processed and precursor forms of GDF-8. Thereafter, membranes were incubated with a peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA). After being washed several times, membranes were incubated with LumiGLO (Cell Signaling Technology) and exposed to BioMax MS films (Kodak, Tokyo, Japan) to visualize the bound proteins.

**RESULTS**

**GDF-8 expression in chicken embryonic myoblasts after induction of cell differentiation.** Chicken embryonic myoblasts were cultured in culture medium containing 10% CS and 3 mM sodium butyrate, and then cell differentiation was induced by replacement with serum-free medium. Total RNA was extracted from myoblasts and/or multinucleated myotubes cultured for 0–72 h after induction of cell differentiation, and RT-PCR was performed to assess GDF-8 expression. The levels of GDF-8 transcript gradually increased in myoblasts and newly formed myotubes from 24 h to 72 h (Fig. 1A). Cultured myoblasts were finally differentiated at 72 h into myotubes, which showed long multinucleated forms (Fig. 1B).

**Assessment of GDF-8-siRNA application.** RT-PCR and Western blotting were performed to assess the alteration of GDF-8 expression in the GDF-8-siRNA-introduced myoblasts. A 75% reduction in GDF-8 transcript level was observed by the introduction of siRNA-1 compared with the control and siRNA-2-introduced cells after 24 h (P < 0.05; Fig. 2A). The lower level of GDF-8 mRNA was maintained until 72 h (data not shown). Western blot analysis showed the 52-kDa GDF-8 precursor in cell extracts, although the 12-kDa processed form (monomer) was at undetectable levels. The 52-kDa GDF-8 precursor was significantly reduced to 45% of control values 48 h after the introduction of siRNA-1 (Fig. 2B).

**Statistical analysis.** Data are expressed as means ± SD, and the differences between them were evaluated with Student’s t-test after one-way ANOVA. P < 0.05 was considered significant.

**Fig. 1.** Expression of GDF-8 in chicken embryonic myoblasts during their differentiation into myotubes. Chicken embryonic myoblasts were cultured in culture medium containing 10% chicken serum, 3 mM sodium butyrate, and insulin-transferrin-selenium X, and then cell differentiation was induced by replacement with a serum-free medium as described in MATERIALS AND METHODS. A: total RNA extracted from cells was subjected to semiquantitative RT-PCR. Data are means ± SD from 3 independent experiments normalized to the values at 0 h. Values with different letters are significantly different (P < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B: after 72 h of induced differentiation, nuclei were stained with Hoechst 33252 (bottom). Top, phase contrast. Bars = 100 μm.
myotubes was induced by the introduction of siRNA-1. In the following experiments, expressions of myogenic factor genes such as MyoD, myogenin, and p21 were assessed by RT-PCR. Myogenin was not altered by siRNA-1 introduction until 72 h, although expression was decreasing in both cells at that time (Fig. 4). In contrast, MyoD and p21 were significantly altered at 72 h but not at 24 and 48 h, although MyoD and p21 were decreasing in the control cells until 72 h. MyoD was expressed 50% higher in siRNA-1-introduced cells than in control cells at 72 h. In contrast, p21 expression decreased at 72 h.

Proliferation and differentiation after GDF-8 suppression. Cell proliferation was assessed by a DNA fluorometric assay. However, there was no significant difference in cell proliferation between the cells for 24–72 h, although both cells proliferated (data not shown). In contrast, the fusion index of myoblasts as a differentiation marker significantly altered 24–72 h after introduction of siRNA-1. siRNA-1 introduction significantly delayed the fusion of myoblasts, which form myotubes, for 24–48 h (P < 0.05; Fig. 5). However, cell fusion was significantly accelerated after 72 h by siRNA-1 introduction.

The cellular morphology of myoblasts and myotubes was further analyzed during differentiation. Two types of myoblasts were first observed, those being stretched and spherical types, within 24 h after induction of differentiation (Fig. 6). Stretched types were predominant in the control cells, although ~50% of them initiated fusing to each other. In the siRNA-1-introduced cells, however, spherical types were still predominant after 24 h of introduction. At 48 h the stretched type of control cells almost fused to each other, and consequently long,
multinucleated myotubes were markedly formed. In the siRNA-1-introduced cells, there was a great decrease in spherical types and they partly differentiated to multinucleated myotubes. However, the multinucleated myotubes were very short and few in number. At 72 h, the control myotubes showed little morphological change. In contrast, the multinucleated myotubes differentiated from siRNA-1-introduced myoblasts grew longer. Finally, a large number of myotubes were formed from the siRNA-1-introduced myoblasts compared with the control cells, although cell sizes were significantly altered in the siRNA-1 introduction group. Aggregation of nuclei was detected in many myotubes introduced with siRNA-1 (Fig. 7). Myotubes with aggregated nuclei were very short and wide. The cell length of myotubes was significantly shortened by siRNA introduction, showing ~60% of control values, whereas the cell width was increased, showing 140% of control values (Table 3).

**DISCUSSION**

The present study was first performed to investigate the function of endogenously produced GDF-8 in a primary culture of chicken embryonic myoblasts by using RNAi that has been developed as a powerful tool in the analyses of gene function in various species. Recently, in avian species the application of RNAi was reported in chicken embryonic cells and tissues (25, 30, 35). Chicken embryos have been used widely as a model vertebrate species for studying embryogenesis including myogenesis and body planning (5) because of accomplishments in chicken genome sequencing (6). In the present study, first of all, a primary culture of chicken embryonic myoblasts was established to investigate myogenesis in vitro. The primary culture was assessed to exhibit the ability of cell differentiation of myoblasts into multinucleated myotubes with a constitutive expression of GDF-8. Since GDF-8-null mice showing a large and widespread increase in skeletal muscle mass were reported (22), there have been many reports that GDF-8 participates in proliferation and differentiation of myoblasts using its recombinant or overexpression in myoblast cell lines such as C2C12 (17, 19, 27, 28, 34). In contrast, in the present study loss-of-function analyses by RNAi were applied to primary chicken myoblasts cultured in a medium containing KSR (without serum component).

Myoblasts were generally cultured for proliferation in a medium containing a higher concentration of serum, but for differentiation they were cultured in a serum-reduced medium. Serum is not suitable for investigation of the function of GDF-8 because of a GDF-8 inhibitor or GDF-8 itself contained in the serum (14, 15, 42). A KSR-containing medium was used...
in the primary chicken myoblasts after GDF-8-siRNA introduction. Myoblasts principally differentiated into myotubes in the medium containing KSR, although parts of myoblasts were proliferated. GDF-8 expression constantly increased during cell differentiation of myoblasts, as previously reported (27). As revealed by Western blotting, the 52-kDa GDF-8 precursor form was detected, but the 26-kDa active protein was not detectable. In studies using primary myoblast cultures (34), the 26-kDa GDF-8 was also not detected in cell extracts. Because the 52-kDa GDF-8 precursor was significantly reduced by the introduction of siRNA-1, GDF-8-mediated promoter activity was measured for the endogenous activity of GDF-8 with a (CAGA)₁₀-constructed luciferase reporter assay (33), which contains the regulatory region of Smad binding element in the promoter (8). An increasing GDF-8 expression may support the activity of regulatory Smads (Smad2, Smad3, Smad4) (41). High activity was maintained at 30–42 h in control and siRNA-2 cells, and then the activity gradually decreased, probably resulting from a negative feedback mechanism through the Smad7 promoter (41). However, further study will be required to analyze the molecular mechanism of GDF-8-induced activity obtained from real-time monitoring. In contrast to control and siRNA-2 cells, introduction of siRNA-1 led to a rapid decrease in the activity 30 h later. These results suggest that endogenous GDF-8 activity was slight in siRNA-1-introduced myoblasts, even though myoblasts could differentiate into multinucleated myotubes. The differentiation into myotubes may not be surprising, because GDF-8-null mice (22) and genetically GDF-8-defective cattle (11, 23) have skeletal muscle mass differentiated from myoblasts.

According to some previous studies using C₂C₁₂ (17, 27, 28), MyoD and myogenin are downregulated by GDF-8, whereas p21 is upregulated. In primary chicken embryonic myoblasts, however, MyoD, myogenin, and p21 gradually decreased during exposure to endogenously produced GDF-8. The introduction of GDF-8-siRNA (siRNA-1) led to significant alterations of MyoD and p21. After 72 h, increased expression of MyoD and decreased expression of p21 were observed compared with control myotubes, consistent with previous reports (17, 27, 28). However, these alterations may not influence the cellular morphology of GDF-8-siRNA-introduced myoblasts, because the morphological changes were clearly observed 24 h after introduction. The fusion index was ~20% of total myoblasts 24 h after GDF-8-siRNA introduction, whereas it was 50% in control myoblasts. In control myoblasts, the fusion index attained its peak level (~80%) at 48 h and decreased at 72 h. The decreased fusion index may indicate that differentiation of newly proliferating myoblasts into myotubes is inhibited. On the other hand, in GDF-8-siRNA-introduced myoblasts, the fusion index time-dependently increased 24–72 h after introduction. Finally, at 72 h, the fusion index was significantly higher in GDF-8-siRNA-introduced myoblasts than in control cells, although myotubes were significantly shortened and widened. Such changed cell sizes may be consistent with hypertrophy or hyperplasia observed in the skeletal muscle of GDF-8-null mice (22).
alterations in cellular morphology in chicken embryonic myo-

Chicken embryonic myoblasts and that the deficiency of GDF-8

Morphological changes of newly formed myotubes may result

GDF-8 caused either hypertrophy or hyperplasia (24, 40).

ent signaling pathways for regulation in the number and size of

was detected in a large number of myotubes introduced with

GDF-8-siRNA introduction. Chicken embryonic myoblasts were cultured as described

166796).

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GRANTS

Interestingly, morphological changes of myotubes were in-

duced after the GDF-8-siRNA introduction: the aggregated

nuclei probably have a causal relationship with cell length and

width. This is the first evidence that the aggregation of nuclei

was detected in a large number of myotubes introduced with

GDF-8-siRNA, and these myotubes with the aggregated nuclei

were very short and wide. At present, we do not know exactly

why the aggregated nuclei were induced during the differenti-

ation of myoblasts into myotubes. GDF-8 may mediate differ-

eration and differentiation by myostatin.

In conclusion, the present study provides evidence that

GDF-8 is expressed and functions in the primary culture of

myogenesis.

Table 3. Morphological changes of chicken embryonic

myotubes after GDF-8-siRNA introduction

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>siRNA-1</th>
</tr>
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<tbody>
<tr>
<td>Cell Length (μm)</td>
<td>582.5 ± 256.5</td>
<td>340.8 ± 196.0*</td>
</tr>
<tr>
<td></td>
<td>6.26 ± 1.79</td>
<td>8.70 ± 3.17*</td>
</tr>
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</table>

Data are means ± SD for n myotubes. Cell length and width were measured in 10 and 20 myotubes per square, respectively, and for cell width 5 evenly spaced points per myotube were measured and averaged. Branched myotubes were considered as being the fusion of several myotubes, and they were not included for cell length. *Statistical significance (P < 0.001) vs. control.

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Myostatin function assessed by siRNA

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