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# Expression of Myostatin and Follistatin in *Mdx* Mice, an Animal Model for Muscular Dystrophy

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Follistatin is a functional antagonist of several members of the TGF- $\beta$  family of secreted signaling factors, including myostatin, the most powerful inhibitor of muscle growth characterized to date. Myostatin inhibition offers a novel therapeutic strategy for muscular dystrophy by restoring skeletal muscle mass and suppressing the progression of muscle degeneration. To assess the potential benefits of follistatin in treating muscle degenerative diseases, we examined the expression of myostatin and follistatin in *Mdx* mice, a model for Duchenne muscular dystrophy, and in B10 mice as a control. Our results demonstrated a temporary and coincident expression of follistatin and myostatin in both mouse strains, but this expression was significantly higher in *Mdx* mice than in B10 mice. The maximum expression of follistatin and myostatin in the presence of restoring necrotic muscle was detected 4 weeks after birth in *Mdx* mice. Interestingly, during the stage of complete regeneration, the absence of myostatin and follistatin proteins and a marked decrease in the expression of both genes were observed 9 weeks after birth in both mouse strains. These findings suggest that follistatin not only blocks myostatin but also allows other activators to function in muscle development, emphasizing that follistatin could be a very potent molecule in combating muscle loss during dystrophies and muscle ageing, disuse, or denervation.

**Key words:** follistatin, myostatin, dystrophin, muscular dystrophy, *Mdx* mice

## INTRODUCTION

Duchenne muscular dystrophy (DMD) is an eventually fatal disorder characterized by rapidly progressive muscle weakness and atrophy of muscle tissue. DMD is the most common form of muscular dystrophy. It is caused by a mutation in the gene encoding dystrophin, an essential cell membrane protein in muscle cells. There is no available cure at this time, although the activation of satellite cells could be a way to replace the damaged muscle tissue.

Muscle satellite cells are a distinct lineage of myogenic progenitors responsible for postnatal muscle growth and repair. Satellite cells are mitotically quiescent and express several myogenic markers. When stimulated by damage to the muscle, satellite cells are activated to reenter the cell cycle and express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to form new, replacement myofibers (Bischoff, 1989).

The extracellular matrix (ECM) is a complex alloy of

macromolecules, including collagen, proteoglycans, and glycoproteins. This matrix is not only the scaffold for cells in a given tissue but also serves as a reservoir for growth factors and modulates the cells' activation status (Kresse and Schönherr, 2001), and is essential for normal myogenesis (Melo et al., 1996). Myostatin, a member of the transforming growth factor-beta (TGF- $\beta$ ) family, is a negative regulator of skeletal muscle growth (McPherron et al., 1997; Thomas et al., 2000) expressed predominantly in muscle and in the blood (McPherron et al., 1997; Zimmers et al., 2002). The dramatic effect of myostatin on postnatal growth is due to its negative regulation of satellite cell activation, proliferation, and self-renewal (McCroskery et al., 2003), and also to its inhibition of myoblast proliferation and differentiation (Thomas et al., 2000; Langley et al., 2002). Interestingly, inhibition of myostatin activity is capable of increasing muscle mass and strength in the postnatal period and even in adults. These observations suggest that targeting myostatin would be suitable as a therapy for degenerative muscle diseases such as muscular dystrophy and cachexia, as well as for preventing muscle wasting due to aging (Whittemore et al., 2003; Grobet et al., 2003; Khurana and Davies, 2003). In fact, antibody-mediated myostatin blockage in *Mdx* mice, a model for Duchenne muscular dystrophy, was found to

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ameliorate the pathophysiology and muscle weakness (Bogdanovich et al., 2002). Myostatin propeptide-mediated amelioration of the symptoms in *Mdx* mice has also been reported (Bogdanovich et al., 2005). Moreover, we have observed high levels of decorin expression during the regenerative stage in *Mdx* mice (Kado, unpublished), which enhanced the proliferation and differentiation of myogenic cells through suppressing myostatin activity (Kishioka et al., 2008). Decorin has also been demonstrated to increase the activity of follistatin, a myostatin propeptide that blocks myostatin activity (Hill et al., 2002). Therefore, decorin may also have an indirect inhibitory effect on myostatin (Hill et al., 2002).

Follistatin, a secreted glycoprotein, is a functional antagonist of several members of the TGF- $\beta$  family, including myostatin. Myostatin inactivation by follistatin thus might offer a novel therapeutic strategy for muscular dystrophy by suppressing the progression of muscle degeneration and permitting skeletal muscle mass restoration. Benabdallah et al. (2008) reported that myostatin inhibition by follistatin in combination with myoblast transplantation is a promising novel therapeutic approach for treating muscle wasting in diseases such as Duchenne muscular dystrophy. To provide a background for understanding the function of myostatin and follistatin during muscle degeneration and regeneration in an animal model for DMD, we investigated the distribution and expression of these proteins in skeletal muscle of *Mdx* mice, using B10 Scott Snells mice as a positive control.

## MATERIALS AND METHODS

### Specimens

Four groups of 10 *Mdx* male mice (C57BL/10ScSn) at the ages of 2, 3, 4, and 9 weeks, respectively, and four groups of 10 control mice (B10 Scott Snells) at the corresponding ages were used in the study. The mice were anesthetized with pentobarbital and sacrificed according to the Guidelines for Animal Experiments of Tokyo Dental College. The left tibialis anterior muscle (TA) from all mice was used for morphological examination and immunohistochemistry. The right TA muscle from all mice was used for western blotting, and the left for RT-PCR analysis.

### Morphological examination

The TAs of *Mdx* and B10 mice were removed 2, 3, 4, and 9 weeks after birth. The selected tissue was fixed in 4% paraformaldehyde. The specimens were then dehydrated and embedded in paraffin by using a Tissue-Tek VIP M1500 rapid tissue processor (Sakura, Tokyo, Japan), and serial sections 4  $\mu$ m thick were cut with an IVS-410 sliding microtome (Sakura, Tokyo, Japan). After the removal of paraffin, the samples were rinsed with water and stained with standard hematoxylin-eosin (H-E).

### Immunohistochemistry

For dystrophin staining, antigen was retrieved in paraffin serial sections by autoclaving them at 121°C for 15 minutes. After non-specific reactivity was blocked with DAKO protein for 10 min at room temperature, the sections were incubated overnight at 4°C with 2  $\mu$ g/ml anti-dystrophin (Abcam ab15277). The distribution of the primary antibody was observed by subsequent application of a biotinylated anti-primary antibody for 30 minutes at room temperature (anti-rabbit IgG biotin, Dako E0432) and streptavidin-horseradish peroxidase (Nichirei 426062) for 5 minutes at room temperature.

The immunostain was developed with a DAB/H<sub>2</sub>O<sub>2</sub> solution (Histofine DAB Substrate Kit; Nichirei), and the sections were counterstained with hematoxylin. As a negative control, sections were

incubated with 2  $\mu$ g/ml normal rabbit Ig (Dako X0936).

### Western blotting analysis of myostatin and follistatin

Electrophoretically separated protein bands were analyzed by western blotting. Briefly, TAs from 2-, 3-, 4-, and 9-week-old mice were removed while the animals were anaesthetized. The muscles were weighed, frozen in liquid nitrogen, and stored at -80°C. Frozen muscles were minced with scissors in nine volumes of ice-cold homogenization buffer. The minced muscle samples were then sonicated in a 250D Sonifier (Branson Ultrasonic, Danbury, CT, USA). The products were used for the preparation of washed myofibers, which were then boiled in sample buffer for 2 min at a final protein concentration of 0.125 mg/ml. Total protein concentration was measured with the Bradford technique by using the Bio-Rad Protein Assay (Nippon Bio-Rad Laboratory) and a Gene Quantpro spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western blotting analyses were performed to detect myostatin and follistatin. An equal amount of total protein (0.5  $\mu$ g) for each group was separated on a 12.5% SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk and incubated with each of the primary antibodies, anti-myostatin (1:1000, CMN AB3239) and anti-follistatin (1:500, GWB 18-003-44287), and detected with a horseradish peroxidase-conjugated secondary anti-rabbit/mouse IgG antibody by using a VECTASTAIN ABC Rabbit IgG Kit.

### Reverse-transcription polymerase chain reaction analysis

From mice in each age group, the left TA muscle was removed and snap-frozen in liquid nitrogen. A QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech UK, Buckinghamshire, UK) was used to extract mRNA, and a Ready-To-Go kit (Amersham Pharmacia Biotech UK) to prepare cDNA. After determination of the optimal PCR conditions for all primers, experiments were performed by using a LightCycler (Roche Diagnostics, Mannheim, Germany), which allows RNA quantification. Two primer sets (Table 1) were designed from specific segments of the entire DNA sequences of the myostatin and follistatin genes by using OLIGO 5 software (Biogene, Huntingdon, UK). Experiments were performed according to the standard protocol for the LightCycler. Ready-to-use LC FastStart DNA Master SYBR Green I (Roche) was used as a hot-start PCR reaction mixture for the LightCycler. A series of cDNA (4.0 ng/ $\mu$ l) dilutions were prepared, including 1/10<sup>5</sup>, 1/10<sup>6</sup>, 1/10<sup>7</sup>, 1/10<sup>8</sup>, and 1/10<sup>9</sup>. PCR reactions for the diluted standards were conducted in 20- $\mu$ l volumes containing 10.2  $\mu$ l of sterile water, 5  $\mu$ l of diluted control cDNA product, 1.6  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2  $\mu$ l of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution), and 0.6  $\mu$ l of each of the forward and reverse primers.

**Table 1.** Primers used for the RT-PCR analysis.

myostatin	Forward	5'-CATCTTGTGCACCAAGCAAA-3'
	Reverse	5'-GGGAGACATTTTTGTCTGGAGT-3'
follistatin	Forward	5'-TGGATTAGCCTATGAGGGAAAG-3'
	Reverse	5'-TGGAATCCCATAGGCATTTT-3'
GAPDH	Forward	5'-TGAACGGGAAGCTCACTGG-3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'

The experimental PCR reactions each contained 14.2  $\mu$ l of sterile water, 1.6  $\mu$ l of 25 mM MgCl, 2  $\mu$ l of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution), 0.6  $\mu$ l of forward primer (10 pmol/ $\mu$ l), 0.6  $\mu$ l of reverse primer (10 pmol/ $\mu$ l), and 1  $\mu$ l of target DNA (5 pg/ $\mu$ l). The PCR mixtures (20  $\mu$ l each) prepared for myostatin and follistatin were added to the glass

portion of the capillaries. PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 80 s. Gene amplification was performed according to a melting program of 70°C for 15 s, and fluorescence was continuously monitored at a rate of 0.1°C/s.

The amount of final expression of each gene was obtained by dividing each gene expression level by that of the housekeeping gene GAPDH. Cycle threshold (Ct) values for the real-time data for each gene were found to be: GAPDH, Ct=19; myostatin, Ct=23; follistatin, Ct=23. The primers used for GAPDH are given in Table 1.

#### Statistical analysis

Student's t-test was used for statistical analysis, and *P* values < 0.05 were considered significant.

## RESULTS

### Histological examination

To observe histopathology, samples were stained with H-E (Fig. 1). At 2 weeks, *Mdx* mice showed normal skeletal muscle cells with the nuclei located on the periphery of each fiber. In contrast, large necrotic and fibrotic areas with many macrophages were evident at 3-weeks in the muscular dystrophic animal model. Interestingly, many regenerative skeletal muscle fibers with central nuclei were observed at 4 weeks, and complete muscle regeneration was noticed at 9 weeks in *Mdx* mice, with an absence of macrophages. The positive control, B10 mice, showed normal skeletal muscle without macrophages in all periods of time evaluated.

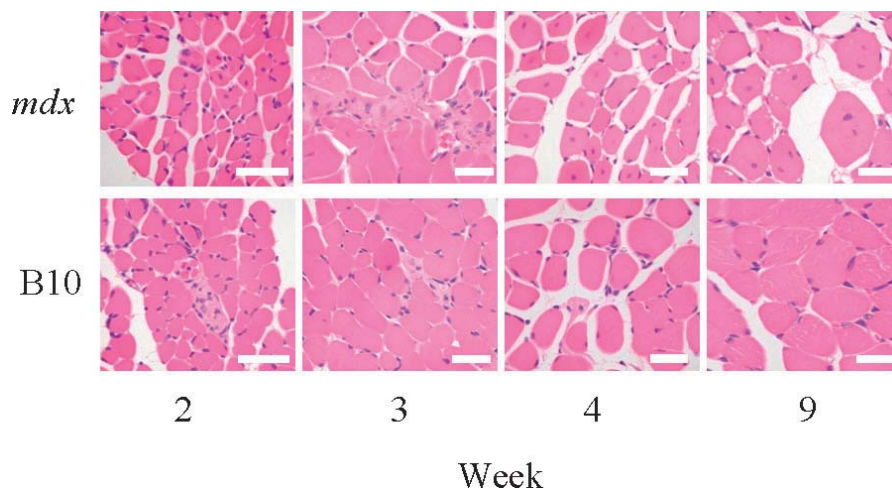
### Immunohistochemistry of dystrophin in TA muscle

To clarify the absence of dystrophin in *Mdx* mice, we observed the immunolocalization of this protein (Fig. 2). We found immunoreaction to dystrophin to be absent or weak in the tibialis anterior muscle of *Mdx* mice during the time course of evaluation. In contrast, dystrophin was strongly expressed in the same muscle of B10 mice at 2, 3, 4, and 9 weeks. Normal rabbit IgG used as a negative control in each group of the B10 and *Mdx* mice resulted in no immunoreaction.

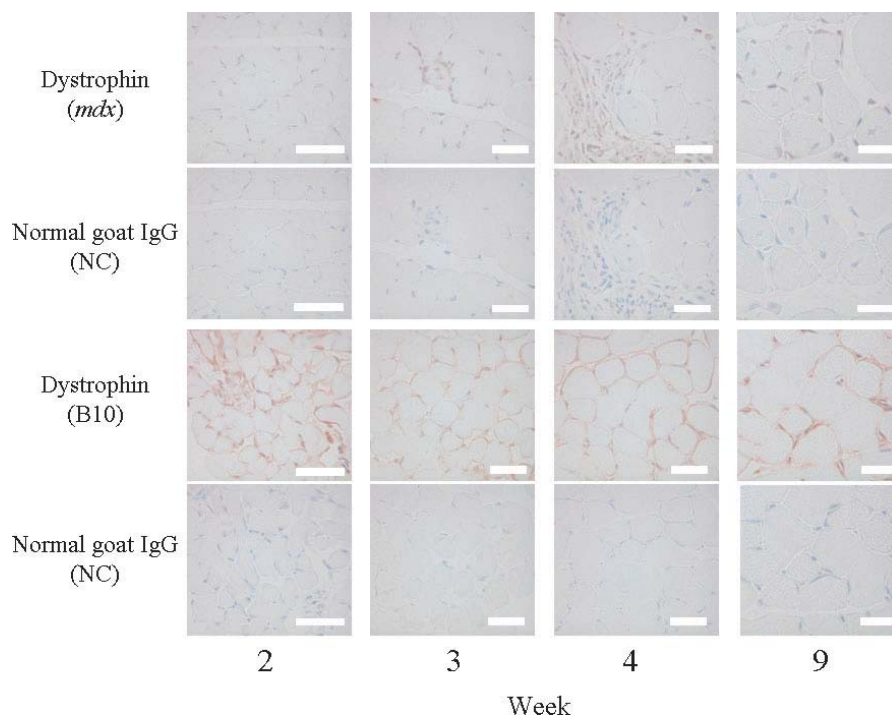
### Expression of myostatin and follistatin proteins

Myostatin and follistatin were identified in the skeletal muscle of

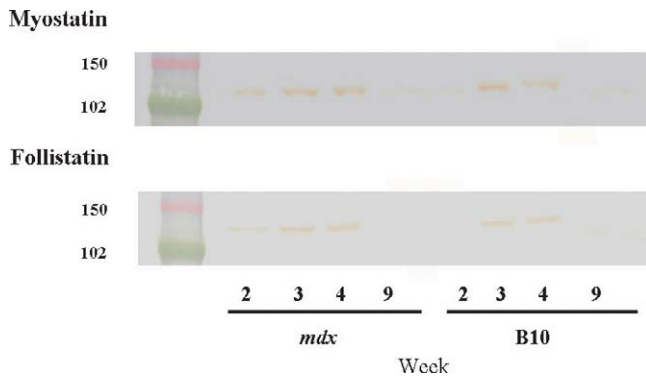
*Mdx* and B10 mice (Fig. 3). Myostatin and follistatin were detected in both animal strains at 2 weeks. Immunoreactive bands indicating these proteins were strongly expressed at 3 and 4 weeks. However, myostatin and follistatin were not observed in *Mdx* or B10 mice at 9 weeks.



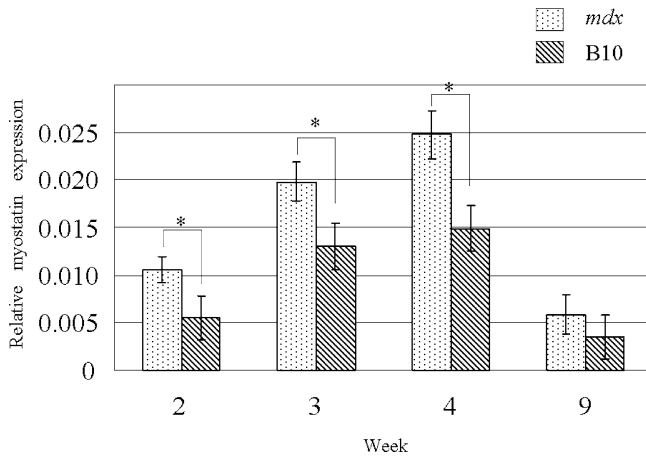
**Fig. 1.** Histology of the tibialis anterior muscle in *Mdx* and B10 mice; sections were stained with hematoxylin-eosin (H-E). At 2 weeks (2w) of age, normal muscle is evident in both animal strains; however, at 3 weeks (3w), extensive necrotic and fibrotic areas are evident in the *Mdx* mice. At 4 weeks (4w), some regenerative muscle cells are evident in the *Mdx* mice. At 8 weeks (8w), dystrophic muscle has completely replaced regenerative muscle cells. Note the normal skeletal muscle in B10 mice at all ages. Scale bar, 50  $\mu$ m.



**Fig. 2.** Immunohistochemical examination of dystrophin in the tibialis anterior muscle in *Mdx* and B10 mice. No immunostaining for dystrophin is evident in *Mdx* mice at any of the ages examined. In contrast, dystrophin is strongly immunostained in B10 mice at all ages. Normal rabbit IgG used as negative control (NC) in both animal strains showed no specific immunoreaction. 2w–9w, 2–9 weeks after birth. Scale bar, 50  $\mu$ m.



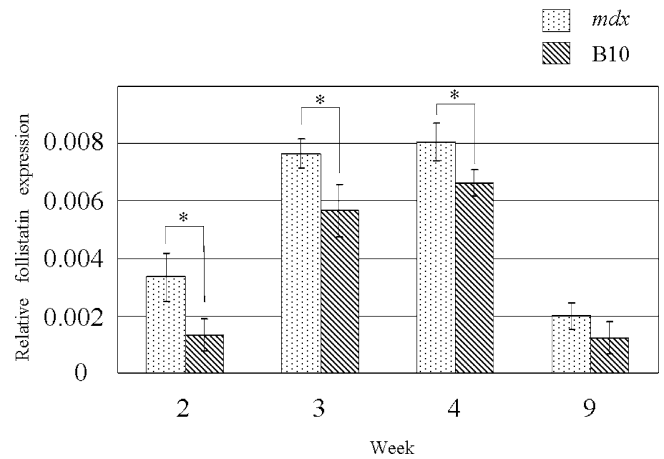
**Fig. 3.** Western blot analysis of myostatin and follistatin in the anterior tibialis muscle in *Mdx* and B10 mice. Note the similar protein expression in both animal strains, which show strong myostatin and follistatin expression at 3 and 4 weeks but lack both proteins at 9 weeks. The numbers at the left indicate the molecular mass of the proteins in kiloDaltons. 2w–9w, 2–9 weeks after birth.



**Fig. 4.** Expression of myostatin mRNA in the tibialis anterior muscle in *Mdx* and B10 mice. Numbers on the vertical axis represent the expression level relative to that of the housekeeping gene GAPDH. The pattern of myostatin mRNA expression is similar in *Mdx* and B10 mice, with a gradual increase from 2 weeks after birth, maximum expression at 4 weeks, and a marked decrease at 9 weeks. Interestingly, *Mdx* mice showed significantly higher myostatin expression than B10 mice at 2, 3, and 4 weeks after birth (\*,  $P < 0.05$ ).

#### Quantitative analysis of the myostatin and follistatin genes

To determine whether myostatin or follistatin mRNA is expressed in the skeletal muscle of *Mdx* and B10 mice, total RNA was isolated from the TA and evaluated by RT-PCR analysis. Figs. 4 and 5 summarize the quantification of myostatin and follistatin mRNA in the skeletal muscle of *Mdx* and B10 mice. Both genes began to show increased expression after 2 weeks, reaching maximum expression at 4 weeks and showing low expression at 9 weeks in both animal strains. Interestingly, *Mdx* mice showed significantly higher myostatin and follistatin expression than did B10 mice at 2, 3, and 4 weeks after birth ( $P < 0.05$ ).



**Fig. 5.** Expression of follistatin mRNA in the anterior tibialis muscle in *Mdx* and B10 mice. Numbers on the vertical axis represent the expression level relative to that of the housekeeping gene GAPDH. The pattern of follistatin mRNA expression is similar in *Mdx* and B10 mice, with a gradual increase from 2 weeks after birth, maximum expression at 4 weeks, and a marked decrease at 9 weeks. Interestingly, *Mdx* mice showed significantly higher follistatin expression than B10 mice at 2, 3, and 4 weeks after birth (\*,  $P < 0.05$ ).

#### DISCUSSION

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disease caused by the inability to synthesize dystrophin, which is involved with maintaining the integrity of the sarcolemma. Muscle fibers undergo a process of degeneration and regeneration. Until now, there has been no known cure for DMD, and treatment has aimed at controlling symptoms to maximize the quality of life; however, we believe that the activation of satellite cells could be a way to replace the damaged muscle tissue. Satellite cells represent a unique population of muscle precursor cells located between the basal lamina and sarcolemma of adult myofibers (Bischoff and Heintz, 1994; Grounds et al., 1993). In response to several stimuli, quiescent satellite cells activate, proliferate, and differentiate to repair damaged skeletal muscle (Bischoff, 1989).

*Mdx* mice, which lack the muscle protein dystrophin and show histological lesions similar to those in human muscular dystrophy, are a suitable animal model for Duchenne muscular dystrophy (DMD). To clarify the histology and the absence of dystrophin corresponding to DMD, H-E staining and immunolocalization of dystrophin were performed in *Mdx* mice. Histologically, *Mdx* mice showed large areas with degenerative, necrotic, and regenerative muscle fibers (Fig. 1) but no immunoreaction to dystrophin (Fig. 2), corresponding to DMD. In contrast, B10 mice showed normal skeletal muscle and immunolocalization of dystrophin. It has been reported that in somite-derived muscles of the extremities, muscle necrosis begins 2 weeks after birth, immediately followed by regeneration, and the majority of necrotic muscles are regenerated by 4 weeks after birth (Dangain and Vrbova, 1984; DiMario et al., 1991; Attal et al., 2000; Honda et al., 2007; Abe et al., 2007). We observed a similar pattern in *Mdx* mice, though over a different period of time, with complete muscle regeneration at 9 weeks.

Myostatin, a member of the transforming growth factor- $\beta$  family, is the most powerful inhibitor of muscle growth characterized to date. Mice lacking myostatin (*Mstn*  $-/-$ ) exhibit a dramatic and widespread increase in muscle mass resulting from a combination of hyperplasia and hypertrophy (McPherron, 1997). In fact, Kirk et al. (2000) found an increase in myostatin in necrotic muscle tissue but decreased expression in regenerative myogenic cells. We made similar observations in the current study. We observed strong expression of myostatin protein and a high level of myostatin mRNA in the presence of necrotic tissue formation after 3 weeks of birth. Interestingly, in both *Mdx* and B10 mice after 9 weeks of birth, we observed a complete regenerative muscle mass without evidence of myostatin. These findings raise the possibility that blocking myostatin activity may be an effective therapeutic strategy for increasing muscle strength in patients with muscle degenerative diseases, including the muscular dystrophies.

Follistatin, a secreted glycoprotein under study due its role in the regulation of muscle growth in mice, is an antagonist to myostatin and rescues muscle differentiation (Amthor et al., 2004). Although follistatin shows potent myostatin-inhibiting effects, it also acts as an effective inhibitor of activins (Nakatami et al., 2008, Tsuchida et al., 2008). Because activins are involved in multiple functions in various organs, their blockage by follistatin would affect multiple tissues other than skeletal muscle (Nakatami et al., 2008, Tsuchida et al., 2008). For this reason a myostatin inhibitor derived from follistatin was developed that does not affect activin signaling (Nakatami et al., 2008, Tsuchida et al., 2008).

In the present study, we examined the expression of myostatin and follistatin in *Mdx* mice, and in B10 mice as a control. We detected temporary and coincident expression of the follistatin and myostatin proteins in both groups (Fig. 3). The time course of follistatin and myostatin gene expression pattern was similar in both groups, but significantly higher in *Mdx* mice than in B10 mice 2, 3, and 4 weeks after birth (Figs. 4, 5). Maximum follistatin and myostatin gene expression occurred at 4 weeks in the presence of restoring necrotic muscle. On the other hand, during the stage of complete regeneration, an absence of the myostatin and follistatin proteins and a marked decrease in the expression of both genes were observed 9 weeks after birth in both mouse strains. Interestingly, the myostatin mRNA level significantly increases in C2C12 cells treated with rh-follistatin at an early stage, but then sharply decreases at a late stage (Kocamis et al., 2004). This suggests that the beneficial effect of follistatin would occur in the presence of degenerative and necrotic muscle fibers.

We recently observed high levels of decorin expression during the regenerative stage of *Mdx* mice (Kado, unpublished observations). Decorin, a small leucine-rich proteoglycan gene family member composed of a core protein and a dermatan/chondroitin sulfate chain (Schönherr et al., 1995) enhances the proliferation and differentiation of myogenic cells by suppressing myostatin activity (Kishioka et al., 2008). Decorin has also been demonstrated to increase follistatin activity (Hill et al., 2002). Therefore, we believe that follistatin not only blocks myostatin but also allows decorin and other activators of muscle development to function.

Moreover, it is important to mention that the expression of both myostatin and follistatin could have been influenced by the presence of macrophages in *Mdx* mice, and by growing skeletal muscle in the positive control mice. Our results emphasize that follistatin could be a very potent molecule for combating muscle loss during dystrophies and muscle aging, disuse, or denervation.

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