Gene expression of myostatin during development and regeneration of skeletal muscle in Japanese Black Cattle

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Gene expression of myostatin during development and regeneration of skeletal muscle in Japanese Black Cattle[1]

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ABSTRACT: Myostatin is a specific negative regulator of skeletal muscle growth and is regarded as one of the most important factors for myogenesis. The aim of the current study was to analyze the developmental change in the gene expression of myostatin and an adipogenic transcription factor (peroxisome proliferator-activated receptor γ 2) in the semitendinosus muscle of Japanese Black Cattle throughout the whole life cycle. An additional aim was to compare the temporal expression patterns of myostatin and relevant myogenic regulatory factors (MRF) mRNA during muscle regeneration after frostbite injury at 16 mo of age. The developmental pattern of myostatin gene expression exhibited 2 peaks: the greatest expression occurred in utero ($P < 0.05$) and the second greatest occurred at 16 mo of age ($P < 0.05$). The greatest level of peroxisome proliferator-activated receptor γ 2 expression was observed at 16 mo of age ($P < 0.05$), which paralleled myostatin expression. During frostbite-induced muscle regeneration, gene expression for myostatin and 4 MRF; i.e., Myf5, MyoD, myogenin and MRF4, showed contrasting responses. Myostatin mRNA dramatically declined by 68.1 and 82.6% at 3 and 5 d after injury ($P < 0.05$), respectively, which paralleled its protein expression, and was restored at 10 d. In contrast, the expressions of all 4 MRF mRNA were low initially but increased by 5 d after injury ($P < 0.05$) and then remained constant or decreased slightly. These results suggest that myostatin may play a role in muscle marbling in the fattening period by decreasing myogenesis and increasing adipogenesis, and that the interaction between myostatin and MRF genes may take place at an early stage of skeletal muscle regeneration.

Key words: beef cattle, muscle, myogenic regulatory factor, myostatin, peroxisome proliferator-activated receptor γ 2, regeneration

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INTRODUCTION

Myostatin is classified as a member of the transforming growth factor-β superfamily and identified as a specific negative regulator of skeletal muscle growth. McPherron et al. (1997) discovered by using gene null mice that myostatin plays a critical role for the regulation of skeletal muscle mass. Natural mutation of myostatin in cattle has been shown to be associated with a double-muscling phenotype (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Myostatin was also shown to stimulate body fat accretion (McPherron et al., 1997; McPherron and Lee, 2002), probably through adipogenic transcription factors including peroxisome proliferator-activated receptor γ (**PPAR**γ; Lin et al., 2002). Although gene expression of myostatin in fetal and calf skeletal muscles has been reported (Jeanplong et al., 2001; Oldham et al., 2001; Shibata et al., 2003), detailed developmental changes throughout the whole life cycle have not been characterized yet in cattle.

The myogenic regulatory factors (**MRF**), including Myf5, MyoD, myogenin, and MRF4, are a family of basic helix-loop-helix transcription factors that have been shown to regulate muscle development (Megeney and Rudnicki, 1995). Whereas myostatin expression during muscle regeneration has been analyzed (Mendler et al., 2000; Sakuma et al., 2000; Armand et al., 2003), the interrelationship between myostatin and these relevant MRF has not directly been compared in cattle.

The aim of the current study was to investigate the expression pattern of the myostatin gene in relation to muscle development in Japanese Black Cattle, the most

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popular cattle breed in Japan. The specific aims were 2-fold—a long-term developmental study and a short-term regeneration study after a frostbite-induced injury. Our hypothesis was that the concomitant regulation of myostatin and adipogenic transcription factors would synchronize in the fattening period, thereby stimulating fat accretion and leading to muscle marbling in Japanese Black Cattle. In a regeneration process from a frostbite injury, we hypothesized that the expression of myostatin would have an inverse relationship with MRF.

MATERIALS AND METHODS

Animals and Tissues

The management of cattle and all procedures in the current study were performed according to the Animal Experimental Guidelines of the National Agricultural Research Center for Western Region (**WeNARC**), Japan. Cattle were fed with combinations of concentrate and Italian ryegrass hay ad libitum throughout the whole period. The skeletal muscles were obtained at slaughter or at biopsy from Japanese Black Cattle that had been bred in the WeNARC. At 8 mo of gestation, donor cattle were slaughtered and their fetuses were obtained from the WeNARC abattoir. After birth, muscle tissues from the semitendinosus muscle were obtained by biopsy periodically from ages 2 to 28 mo. The biopsy procedure was as follows: the animal was locally anesthetized by an i.m. injection of 0.06 mg/kg of xylazine (Bayer, Tokyo, Japan) and an s.c. injection of 400 mg of lidocaine (AstraZeneca, Osaka, Japan); subsequently, a 3- to 5-cm incision was made in the skin overlying the semitendinosus muscle. All of the samples were rapidly frozen in liquid nitrogen and stored at –80°C until RNA extraction.

For the muscle regeneration study, at 16 mo of age, a peak period of myostatin expression, frostbite was induced by an exposure of the muscle to dry ice, using a procedure described by Pavlath et al. (1998) and Nara et al. (2001), with some modifications (Figure 1). The normal muscle before frostbite treatment and the untreated muscle (n = 3) on d 5 were employed as a control group. The tissues were collected by biopsy from the semitendinosus muscle, as described above, on d 0, 3, 5, and 10 after frostbite treatment.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from muscle tissues using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first strand complementary DNA (**cDNA**) was synthesized from 3 μg of total RNA using SuperScript II RNase H^- (Invitrogen) reverse transcriptase with oligo dT primer (Amersham Pharmacia Biotech, Piscataway, NJ). After reverse transcription, analysis of gene expression of myostatin, PPARγ2, MyoD, Myf5, myogenin, and MRF4 was performed by real-time PCR using an ABI 7700 detection system (Applied Biosystems, Foster City, CA). The first strand cDNA was diluted with de-ionized water and amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) with gene-specific TaqMan probe and primers (Table 1). Every pair of oligonucleotide primers was designed to amplify a region including at least 1 intron. The real-time PCR reaction was carried out initially for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, and 1 min at 60°C. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was used as a normalizing control. The TaqMan probe and the primers were designed using Primer Express software (Applied Biosystems).

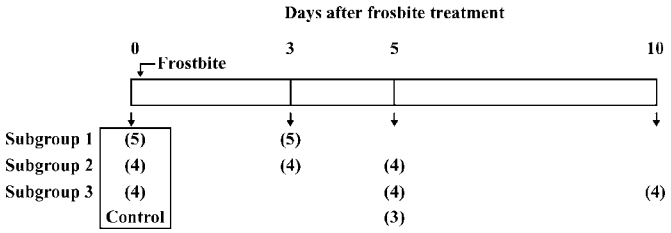

Figure 1. Experimental schedule of the regeneration study from a frostbite-induced injury of the semitendinosus muscle at 16 mo of age in Japanese Black Cattle. This study consisted of a series of subgroups (1, 2, and 3, with 5, 4, and 4 cattle, respectively). Because the data were combined from these 3 groups, total numbers of cattle were 13, 9, 8, and 4 on d 0, 3, 5, and 10, respectively. Because of large animal size, our studies were modified at 2 points from other studies with rats and mice to reduce the number of animals. Firstly, biopsy samples were collected serially from the same animals in each subgroup. Secondly, we made frostbite damage at 1 or 2 sites of the muscle on d 0 for each animal and took biopsy samples from different sites at different time intervals. Numbers of samples collected are represented in parentheses. The control group comprised normal muscle before frostbite treatment and the untreated muscle (n = 3) on d 5.

Preparation of Muscle Protein and Western Blot Analyses

Muscle tissues were pulverized in liquid nitrogen, and then they were homogenized in SDS-PAGE buffer containing 50 m*M* Tris-HCl (pH 6.8), 10% glycerol, 5% SDS, and 10 m*M* dithiothreitol (DTT). The muscle protein was boiled for 1 min and then centrifuged at 13,000 × *g* for 3 min at 4°C. The supernatant fraction of each sample was taken and used to determine protein content by RC-DC protein assay Kit (BioRad, Hercules, CA) according to the manufacturer's protocol. Total protein (40 μg) was separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech) by electroblotting. After blotting, the membranes were stained

Table 1. Sequence of real-time PCR primers used in this study[1]

Gene[2]	GenBank accession No.	Forward primer	Reverse primer	Product size, bp
Myostatin	AB076403	GGCCATGATCTTGCTGTAACCT	GCATCGAGATTCTGTGGAGTG	144
MyoD	AB257293	CGACTCGGACGCTTCCAGT	GATGCTGGACAGGCAGTCGA	180
Myf5	AB257294	ACCAGCCCCACCTCAAGTTG	GCAATCCAAGCTGGATAAGGAG	150
Myogenin	AB257560	GTGCCCAGTGAATGCAGCTC	GTCTGTAGGGTCCGCTGGGA	110
MRF4	AB257561	GGTGGACCCCTTCAGCTACAG	TGCTTGTCCCTCCTTCCTTGG	140
PPARγ2	Y12420	TGGGAGTCGTGGCAAATCC	CCAAAACGGCATCTCTGTGTC	151
GAPDH	U85042	TGACCCCTTCATTGACCTTCA	ACCCCAGTGGACTCCACTACAT	201

[1]All sequence data were from bovine, and all primers are shown 5′ to 3′.
[2]PPAR = peroxisome proliferator-activated receptor; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

with Ponceau S (Sigma, St. Louis, MO) to verify equal loading.

The membranes were blocked in Tris-buffered saline-Tween-20 (**TBS-T**), consisting of 20 m*M* Tris-HCl (pH 7.6), 137 m*M* NaCl, and 0.1% Tween-20 (Sigma), with 5% skimmed milk (Becton, Dickinson, Sparks, MD), for 1 h, and then incubated with a primary polyclonal antibody specific for myostatin (Hokkaido System Science, Sapporo, Japan) for 1 h at room temperature. The membrane was washed with TBS-T and further incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G secondary antibody (Amersham Pharmacia Biotech) for 30 min at room temperature. The HRP activity was detected by the enhanced chemiluminescence (ECL) plus detection kit (Amersham Pharmacia Biotech). The tissue distribution of myostatin protein was also examined. The extracts of skeletal muscle, liver, heart, spleen, lung, and fat (subcutaneous adipose tissue) were obtained, and the expression of myostatin was compared by Western blot analysis as described above.

Statistical Analyses

Gene expression data were represented as means ± SEM. The relationships between the relative mRNA levels of target genes and developmental stages or stages of the regeneration process were analyzed using 1-way ANOVA and a posthoc Scheffe test. $P < 0.05$ was considered to be statistically significant.

RESULTS

To view the whole developmental change in the myostatin gene expression in the semitendinosus muscle of Japanese Black Cattle from the fetal to fattening periods, real-time PCR was carried out using a myostatin gene specific primer set and TaqMan probe with GAPDH gene as an internal standard (Figure 2). The greatest abundance of myostatin mRNA was observed at 8 mo of gestation. After birth, mRNA abundance gradually declined until 10 mo but returned back to its second peak between 13 and 19 mo. Thereafter, mRNA decreased again up to 28 mo. This unexpected peak in myostatin expression at 16 mo led us to look for some physiological implications during development of Japanese Black Cattle. Because it has been known since its discovery that myostatin can affect not only myogenesis but also adipogenesis (McPherron et al., 1997), a gene expression related to fat metabolism was observed. One of the adipogenic transcription factors, PPARγ2, was measured at different stages of development by real-time PCR (Figure 3). The expression pattern was similar to myostatin, more marked showing a peak at 16 mo, which strongly indicated a coordinate regulation of these 2 genes at this age. Another adipogenic transcription factor CCAAT/enhancer binding protein α (C/EBPa) was also examined and showed a similar tendency, although not statistically significant (data not shown).

For short-term development regulation, skeletal muscle regeneration was tested using a frostbite-induced injury to the semitendinosus muscle. Here, myostatin gene expression was observed in relation to other relevant MRF. Regenerating skeletal muscles were obtained from the semitendinosus muscle of 16-mo-old Japanese Black Cattle on d 0, 3, 5, and 10 after a frostbite treatment. As shown in Figure 4, myostatin mRNA was dramatically decreased by 68.1 and 82.6% on 3 and 5 d after injury, respectively, and thereafter recovered to 74.5% of the initial on 10 d. At the same time, to confirm its protein expression, Western blot analysis with antimyostatin antibody was performed, and a single polypeptide of ~40 kDa was identified only in the skeletal muscle but not detected in other organs and tissues such as liver, heart, spleen, lung, or fat (Figure 5A). After a frostbite injury, band intensity decreased until d 5 (Figure 5B) in parallel with its mRNA (Figure 4). The expression patterns of 4 relevant MRF mRNA, however, showed opposite responses to a frostbite treatment compared with that of myostatin mRNA (Figure 6). In the normal muscle before injury, Myf5, MyoD, and myogenin mRNA was almost negligible or had very low abundance. Then Myf5 rapidly increased on d 3 and 5 during recovery and thereafter tended to decrease for 10 d (Figure 6A). The peak of MyoD gene expression

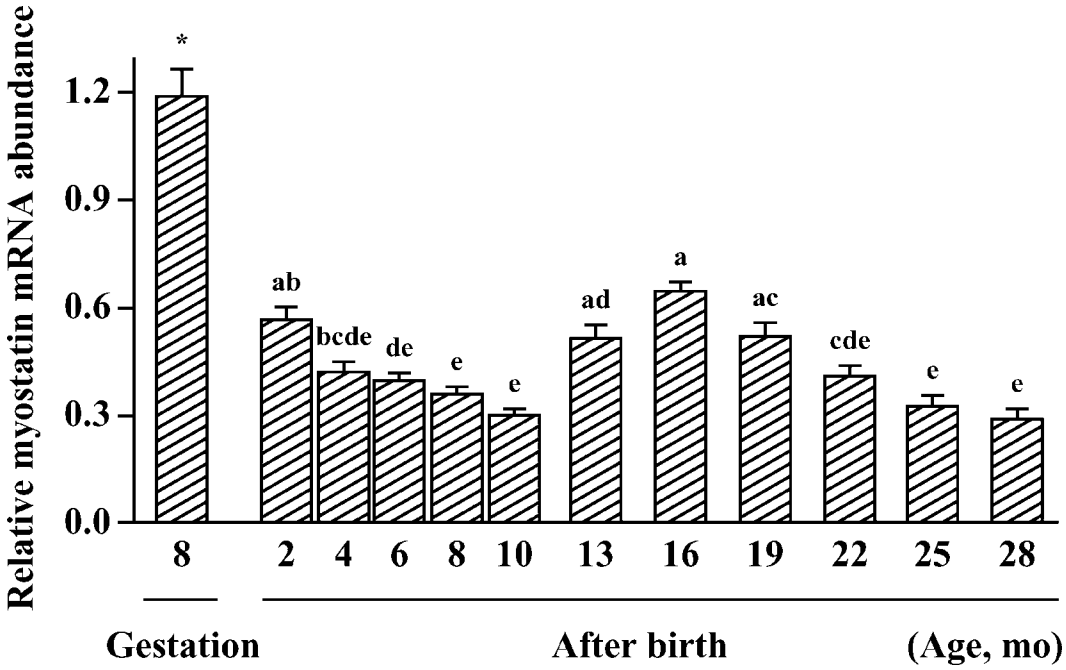

Figure 2. Developmental change in myostatin gene expression in the semitendinosus muscle of Japanese Black Cattle by real-time PCR. Relative myostatin mRNA abundance was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data represent means ± SEM for 8 to 10 cattle, except at 8 mo of gestation, at which time only 4 fetuses were obtained. Bars with different letters are significantly different ($P < 0.05$). Asterisk indicates that the means differ between gestation and all samples after birth ($P < 0.05$).

reached on d 5, and this high level was maintained (Figure 6B). Myogenin gene expression increased only on d 5 and thereafter tended to decrease (Figure 6C). The MRF4 exhibited a substantial basal level initially and showed a gradual increase during 10 d of regeneration (Figure 6D). All the target gene expressions seemed stable during this period because the data of the un-

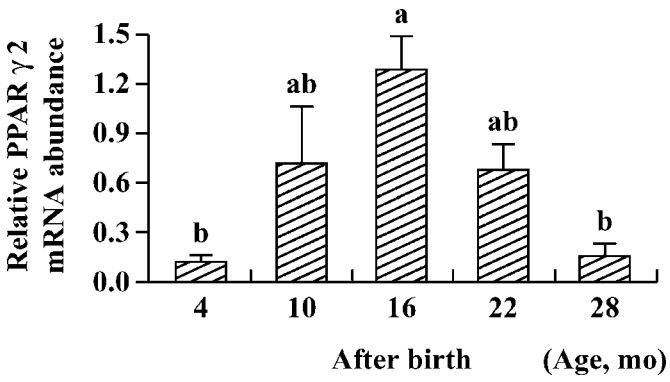

Figure 3. Developmental change in peroxisome proliferator-activated receptor γ 2 (PPARγ2) gene expression in the semitendinosus muscle of Japanese Black Cattle by real-time PCR. The mRNA abundance of PPARγ2 was normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA. Data represent means ± SEM for 4 cattle at each time point. [a,b]Bars with different letters are significantly different ($P < 0.05$).

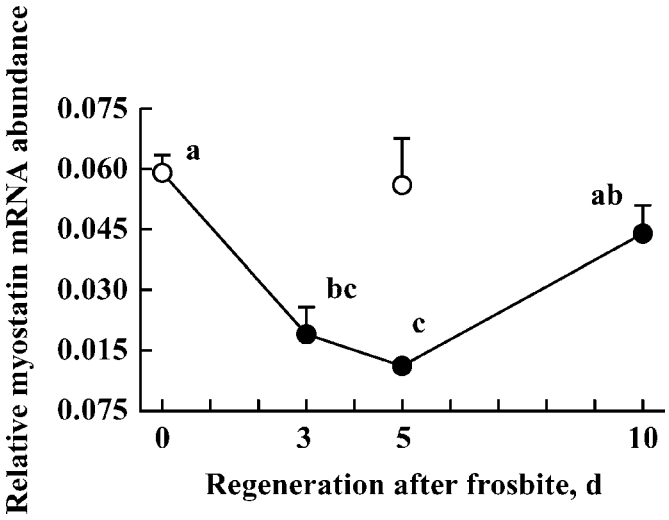

Figure 4. Pattern of myostatin mRNA abundance during regeneration from a frostbite-induced injury of the semitendinosus muscle at 16 mo of age. The mRNA abundance was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data represent means ± SEM for 4 to 13 animals (detailed number of samples at each point is shown in Figure 1). [a–c]Values with different letters are significantly different ($P < 0.05$). The samples from normal (control) and regenerated muscles are represented by open (○) and solid circles (●), respectively.

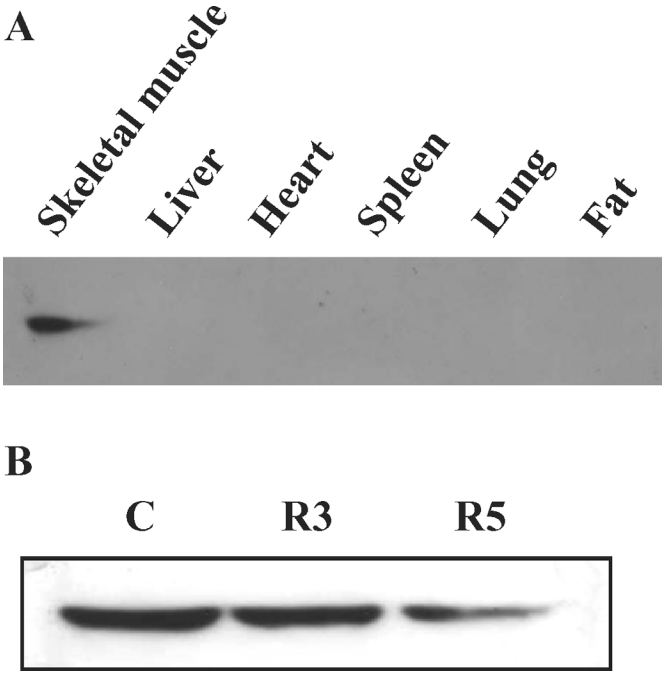

Figure 5. Myostatin protein in tissues of Japanese Black Cattle. (A) Tissue distribution of myostatin in the body. The myostatin protein was specifically expressed in skeletal muscle. (B) Change in myostatin protein level at 0 (C), 3 (R3), and 5 (R5) d during regeneration from a frostbite-induced injury of the semitendinosus muscle at 16 mo of age.

treated muscle on d 5 were not different from the data before injury (Figure 4 and 6).

DISCUSSION

Developmental Changes in Gene Expression of Myostatin and an Adipogenic Transcription Factor in the Semitendinosus Muscle of Japanese Black Cattle

Natural mutations of the myostatin gene are responsible for the double-muscled phenotype of some cattle breeds (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Boccard and Dumont (1974) reported that the hindlimb muscles including the semitendinosus muscle were increased in their weight in double-muscled cattle. In addition, the muscular hypertrophy characteristic of double-muscled cattle was more marked in hindlimbs than in forelimbs (Dumont, 1982). These findings indicate that the development of hindlimbs receives a more marked influence by myostatin. In fact, greater expression of the gene was always found in the semitendinosus muscle compared with the M. longissimus lumborum in Japanese Black Cattle (Shibata et al., 2003).

In the current study, unexpectedly, the myostatin expression was enhanced between 13 and 19 mo of age. Considering from the role for negative regulation of

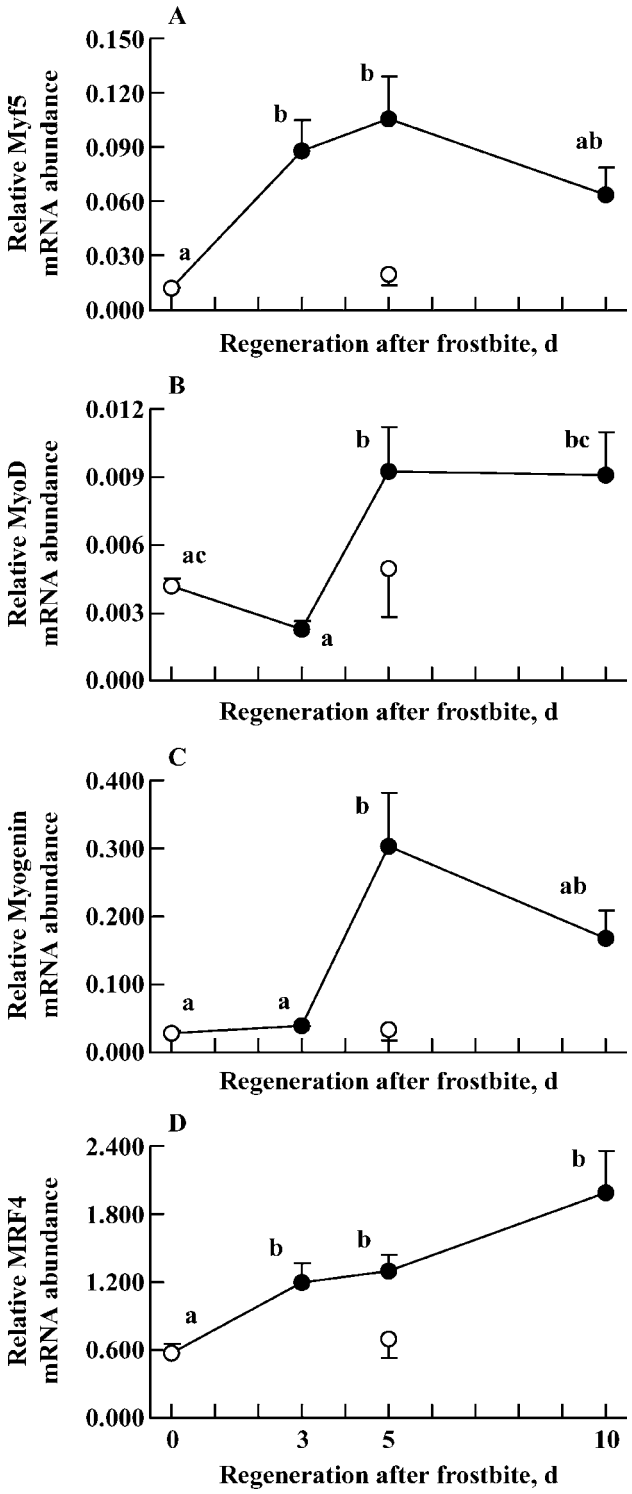

Figure 6. Patterns of 4 myogenic regulatory factor mRNA abundance during regeneration from a frostbite-induced injury of the semitendinosus muscle at 16 mo. The mRNA abundance of Myf5 (A), MyoD (B), myogenin (C), and MRF4 (D) was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data represent means ± SEM for 4 to 13 animals (detailed number of samples at each point is shown in Figure 1). [a–c]Values with different letters are significantly different ($P < 0.05$). The normal (control) and regenerated muscles are represented by open (○) and solid circles (●), respectively.

muscle growth, the result clearly suggested that a greater expression of this gene leads to suppression of muscle development during this period. However, the relevant information suggesting suppression of muscle growth at this age in cattle development could not be obtained. Apart from a direct role for myogenesis, several reports indicated that the fat content of double-muscled cattle is less than that of normal cattle (Butterfield, 1966; Lohman et al., 1971; Shahin and Berg, 1985). Recently, studies with myostatin null mice revealed that a decrease in body fat accumulation results from a reduction of adipogenesis, and accordingly leptin secretion was decreased (Lin et al., 2002; McPherron and Lee, 2002). They suggested that myostatin is involved in the regulation of adiposity as well as myogenesis. In this sense, it is quite interesting that a greater expression of myostatin and PPARγ2 genes was observed at 16 mo in Japanese Black Cattle. These results strongly support their hypothesis that myostatin may regulate fat metabolism through activation of PPARγ2. The response of PPARγ2 might have been more distinct by using Japanese Black Cattle because of the sensitivity of adipogenesis. The American Wagyu, a breed similar to Japanese Black Cattle, was reported to have greater ability to accumulate intramuscular adipose than Angus cattle (Lunt et al., 1993). In Japanese Black Cattle, a breed known for its ability to accumulate intramuscular adipose tissue, intramuscular adipose accretion was reported to be slow until 15 mo age and then increase dramatically at 15–16 mo of age (Fukuhara et al., 1970). Mitsuhashi et al. (1987) also observed an increase in fat accumulation at 15 to 20 mo in skeletal muscle of Japanese Black Cattle. Therefore, all these findings strongly support the hypothesis that myostatin mainly contributes to effective marbling formation in Japanese Black Cattle by suppression of myogenesis and stimulation of fat accumulation during this period.

Temporal Analyses of Myostatin and MRF Gene Expressions During Muscle Regeneration

To determine gene expression of myostatin in the myogenesis of short-term development, real-time PCR analyses of myostatin in combination with relevant MRF genes were carried out using regenerating skeletal muscle. The change in myostatin gene expression after a frostbite injury in the current study supported previous studies. For example, Mendler et al. (2000) showed that myostatin mRNA abundance decreased in rat extensor digitorum longus muscle during d 1 to 5 after notexin injection and were restored to control levels after 7 d. Similar findings were also obtained by Kirk et al. (2000) and Armand et al. (2003). In addition, a rapid decrease in myostatin protein was observed after bupivacaine injection in rat tibialis anterior muscle (Sakuma et al., 2000). In contrast, Yamanouchi et al. (2000) reported that myostatin transcripts are expressed in myogenic and nonmyogenic mononucleate cells 48 h after regeneration of rat skeletal muscle.

Compared with myostatin, the 4 MRF mRNA responded in an opposite way to frostbite injury. The expression of myogenin and MRF4 gene, well known as differentiated myotube markers (Megeney and Rudnicki, 1995), increased after injury. It is conceivable that these proteins assist with new myotube formation beginning within a few days after injury. These results suggest that the maturation of newly formed myotubes does not occur in bovine regenerating skeletal muscle until 5 d after injury and that the process of muscle regeneration after injury may be slower in cattle than in rats. Cornelison and Wold (1997) found that activated single satellite cells from myofibers, isolated or cultured, initially begin to express MyoD or Myf5, and subsequently express MyoD and Myf5. Beauchamp et al. (2000) reported using isolated myofibers that quiescent satellite cells express Myf5. These data revealed that, at least, Myf5 is expressed in quiescent satellite cells at an early stage in myogenesis. Furthermore, McCroskery et al. (2003) suggested that myostatin blocks activation of satellite cells and also signals their quiescence. Expression pattern of Myf5 and myostatin gene shown in this study suggest that skeletal muscle regeneration may occur, not only due to the activation and proliferation of satellite cells by Myf5, but also due to the release from proliferation inhibition by myostatin and that the interaction of these 2 genes results in early-stage regulation of skeletal muscle formation.

In summary, long-term developmental changes in myostatin gene expression in semitendinosus muscle of Japanese Black Cattle up to the fattening period, age of 10 to 28 mo, were observed. Increased expression of the myostatin gene was found at 13 to 19 mo, and the PPARγ2 expression was also enhanced at 16 mo. These parallel increases suggested a possible contribution to marbling formation during the fattening period in Japanese Black Cattle. Gene expressions for myostatin and 4 relevant MRF during muscle regeneration following a frostbite injury were compared. Myostatin and MRF showed contrasting expression patterns. Early stage of skeletal muscle formation may be regulated by the interaction between myostatin and MRF gene expression.

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