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Increased expression of decorin during regeneration stage of mdx mouse

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Short running title: Decorin expression in mdx mouse

Abstract

Satellite cells exist in postnatal muscle tissue and constitute the main source of muscle precursor cells for growth and repair. These cells carry out important roles for skeletal muscle formation postnatally during growth of muscle masses as well as damage-induced regenerative processes. Muscle regeneration supports muscle function in aging and have a role for functional impairment caused by progressive neuromuscular diseases. Major substances controlling this process are growth factors and extracellular matrix. Myostatin, a member of TGF-β family, was mainly expressed in muscle tissue. Decorin, a member of the small leucine-rich proteoglycan gene family, is composed of a core protein and a dermatan/chondroitin sulfate chain. Recent studies have shown that decorin enhanced the proliferation and differentiation of myogenic cells through suppressing myostatin activity. Thus, decorin appeared as a new molecule in myostatin signaling pathway and arose as a promising target for treatment of progressive neuromuscular diseases. Therefore in the current study, we examined localization of decorin as well as myostatin in a muscle dystrophy model mdx mice and B10 Scott Snells mice as a control to elucidate the differences of decorin and myostatin message as well as protein distribution. The current study revealed increased expression of decorin protein as well as mRNA at the regenerative stage of mdx mouse as compared to early stages, while only weak expression of decorin was detected at the control mice. Our study would contribute to

identify the relationship between decorin and myostatin as well as development of therapeutic strategy for progressive neuromuscular diseases.

Keywords: mdx mouse, myostatin, decorin, ECM, muscle regeneration, Duchenne muscular dystrophy

Introduction

Early embryonic development of skeletal muscle originates from the proliferation, differentiation, and then fusion of embryonic myoblasts within newly forming muscle beds. Quiescent myogenic cells, called as satellite cells, exist in postnatal muscle tissue between the basement membrane and the plasma membrane of the muscle fibers and constitute the main source of muscle precursor cells for growth and repair. These cells carry out important roles for skeletal muscle formation postnatally during growth of muscle masses as well as damage-induced regenerative processes (Hawke and Garry, 2001). Muscle regeneration supports muscle function in aging and has a role for functional impairment caused by progressive neuromuscular diseases such as Duchenne muscular dystrophy. Major substances controlling this process are growth factors as well as hormones such as growth hormone (GH), insulin-like growth factor-I (IGF-1) (Sara and Hall, 1990) and extracellular matrix (ECM). ECM regulates cell behavior by interacting with growth factors and through cellular signal transduction pathways (Velleman, 1999).

A muscle-specific growth-inhibiting factor, myostatin (growth / differentiation factor-8), has been identified in the last decade (McPherron et al., 1997). Myostatin was found to be a transforming growth factor (TGF)-β family member and was mainly expressed in muscle tissue (McPherron et al., 1997). Myostatin knocked-out mice showed a dramatic increase in muscle

mass due to both hypertrophy and hyperplasia of the muscle cells (McPherron et al., 1997). TGF-β1 has widely been known as a potent stimulator of fibrosis in various tissues and is closely associated with skeletal muscle fibrosis as well (Border and Noble, 1994; Lijnen et al., 2000; Li et al., 2004). TGF-β1 levels are enhanced in both dystrophic and injured muscles (Bernasconi et al., 1995; Gosselin et al., 2004). In fact, a recent study has shown that myostatin has been involved in fibrosis formation within skeletal muscle similar to TGF-β1 (Wagner et al., 2002). Thus inhibition of myostatin has been arisen as a potential therapy for degenerating muscle diseases such as muscular dystrophy and cachexia as well as muscle wasting due to aging. In fact, antibody-mediated myostatin blockade in mdx mice was found to improve the pathophysiology and muscle regeneration (Bogdanovich et al., 2002). However, elimination of myostatin did not ameliorate the phenotype in a laminin-α2-deficient dy^w mouse (Li et al., 2005). Thus the situation is complicated and needs the identification of related molecules in its signaling pathway to reach a full scale of understanding of the mechanism.

The ECM is a complex combination of macromolecules including collagen, proteoglycans, and glycoproteins. The ECM is not only a supportive substitute for cells but also provides a reservoir for growth factors and molecules that influence on many signaling including muscle regeneration. Decorin, a member of the small leucine-rich proteoglycan gene family, is

composed of a core protein and a dermatan/chondroitin sulfate chain. Decorin has been shown to bind several types of collagen, and regulate and stabilize collagen fibril formation (Bidanset et al., 1992; Schonherr et al., 1995; Danielson et al., 1997). Decorin also binds to and modulates activity of TGF-β (Takeuchi et al., 1994; Schonherr et al., 1998). Regarding with the role of decorin in myogenic cells, only few reports exist. A recent study has shown a physical binding between myostatin and decorin (Miura et al., 2006). This binding inhibited myostatin activity. Another study has also reported that decorin enhanced the proliferation and differentiation of myogenic cells through suppressing myostatin activity (Kishioka et al., 2008). Thus, decorin appeared as a new molecule in myostatin signaling pathway and arose as a promising target for treatment of progressive neuromuscular diseases.

Regarding with analysis of decorin in a muscle dystrophy model, only few studies exist in the literature. One such study examined decorin expression in relatively older mice (Caceres et al., 2000). Therefore in the current study, we for the first time examined localization of decorin with a consideration of both the degeneration and regeneration stages of the muscle using a muscle dystrophy model mdx mice and B10 Scott Snells mice as a control to elucidate the differences of decorin message as well as protein distribution. Our study would contribute to identify the relationship between decorin and myostatin as well as development of therapeutic strategy for progressive neuromuscular diseases.

Materials and methods

Muscle biopsy specimen

Three groups of 10 mdx male (C57BL/10ScSn) at the ages of 2-4 weeks, respectively, and 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 TA muscle from all mice was used for morphological examination. The right TA muscle from all mice was used for examination at the transcriptional levels.

Immunohistochemical analysis

Myostatin and decorin stainings were performed using formalin-fixed, paraffin-embedded muscle serial sections. To find out the optimum condition, antigen retrieval method (thermal treatment, enzyme treatment or no treatment) as well as antibody concentration (0.4 μg/ml. 2 μg/ml, 10 μg/ml) were evaluated for each of the antibodies using 16.5day mouse cryostat sections. Sections (4-μm thick) were mounted on silanized slides (DAKO Japan Co., Tokyo, Japan), de-paraffinized in xylene for 20 min and rehydrated in graded ethanol solutions. Endogenous peroxidase was blocked by incubating the sections in 0.3% H_2O_2 in methanol for 15 min.

For myostatin staining, antigen retrieval in paraffin sections was performed using thermal method by autoclaving at 121 °C for 15 minutes.

For decorin staining, antigen retrieval in paraffin sections was done using enzyme treatment method (proteinase K at 37 °C for 10 minutes). After blocking of non-specific reactivity with DAKO protein block for 10 min at room temperature, the sections were incubated overnight at 4°C with the anti-myostatin (CMN AB3239) or anti-decorin antibody (R&D Systems AF1060). Identification of the distribution of the primary antibody was achieved by subsequent application of a biotinylated anti-primary antibody for 30 minutes at room temperature (Anti-Goat Ig Biotin, DakoE0466 for myostatin and decorin stainings) and streptavidin-horseradish peroxidase (Nichirei 426062) for 5 minutes at room temperature.

Immunostaining was developed using DAB/H_2O_2 solution (Histofine DAB substrate kit; Nichirei), and the sections were counterstained with hematoxylin. As a negative control, the sections were subjected to normal goat IgG.

RT-PCR analysis

From mice in each age group, the muscle was removed and snap-frozen in liquid nitrogen. mRNA was extracted by using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and cDNA was prepared by using a Ready-To-Go Kit (Amersham Pharmacia Biotech UK Ltd.). After determination of the optimal PCR conditions for all primers, experiments were performed using a

LightCyclerTM (Roche Diagnostics, Mannheim, Germany), which allows RNA quantification. Primer set designed from the sequences of the decorin gene was used. Experiments were performed according to the standard protocol for the LightCyclerTM. Ready-to-use LC FastStart DNA Master SYBR Green I (Roche) was used as a hot-start PCR reaction mixture for the LightCyclerTM. A series of cDNA dilutions (4.0 ng/μl) including $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, and $1/10^9$ were prepared. PCR reactions for the diluted standards contained 10.2 μl of sterile water, 5 μl of diluted control cDNA product, 1.6 μl of 25 mM MgCl2, and 2 μl of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution). In addition, 0.6 μl of each of forward and reverse primers designed by the software (Biogene Ltd.) were added to reach a final reaction volume of 20 μl for each tube. Primers based on sequences of the decorin gene were designed from specific segments of the entire DNA sequence, and are shown in Table 1. Each PCR mixture (final reaction volume, 20 μl) contained 14.2 μl of sterile water, 1.6 μl of 25 mM MgCl2, SYBR Green I (1/60,000 dilution), 2 μl of DNA [5pg/μl] containing LC FastStart DNA Master SYBR Green I, 0.6 μl of forward primer (10 pmol/μl), 0.6 μl of reverse primer (10 pmol/μl), and 1 μl of target DNA. The PCR mixtures (20 μl each) prepared for myostatin and decorin were added to the glass portion of 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 decorin gene was obtained by dividing each of the myostatin and the decorin expression level by that of a housekeeping gene, GAPDH. The primer sequences for GAPDH are given in Table 1.

Statistical analysis

Student's t-test was used for statistical analysis and a p-value of <0.05 was designated as significant.

Results

Immunohistochemical analysis

We first stained tibialis anterior muscle with HE to see differences in histopathological appearance of the control B10 and the mdx mice at various stages. As shown in figure 1, at 2-week after birth, both the B10 and mdx mice demonstrated almost normal morphology without necrosis. However, at the 3-week, the mdx mouse revealed extensive necrosis and fibrosis, which were not detected in the B10 mouse. On the other hand, at 4-week after birth, many regenerative cells with central nuclei were noticed at the mdx mouse.

Since importance of a proteoglycan, decorin, has been recently identified specifically during regeneration of the muscle fibers, we examined decorin

localization and expression both at the B10 and the mdx mice at various stages. Weak cytoplasmic expression of decorin was shown in few cells of the B10 mice at all stages. On the other hand, decorin showed strong expression around cells of the mdx mouse. An increased expression of decorin was detected at 3- and 4-week stages as compared the mouse with 2-week-old. Decorin especially revealed strong expression around those regenerative cells with central nuclei. The control normal goat IgG demonstrated few reaction both in the B10 as well as mdx mice, suggesting specific staining of our antibody (Figure 2, arrows).

Since myostatin is an important molecule of the decorin signaling pathway, we examined its protein expression by immunohistochemistry. Myostatin expression was observed in the cell cytoplasm. At 2-week after birth, since the mice are in growth period, the slight protein expression was detected not only the mdx mice but also the control group (Figure 3). At 3-week after birth, a part of mdx mice cells, especially those supposed to be in regeneration process due to fusion, showed expression. At 4-week after birth, many regenerated muscle cells of the mdx mice with central nuclei demonstrated expression. mRNA expression showed significant difference. Expression area was marked with arrows (Figure 3).

mRNA expression of decorin and myostatin

mRNA expression of decorin was examined to identify whether the

increased protein expression was linked to an increase at the transcriptional level. As shown in figure 4, examination of decorin mRNA showed similar level of expression at any stage of B10 mouse, though decorin was expressed relatively lower level at 2 and 3-week-old of the mdx mouse than the corresponding age of B10 mouse without statistical difference. However, at 4-week after birth, decorin revealed a higher level of expression at the mdx mouse sections as compared to B10 mouse tissue with statistical significance. Moreover, decorin showed stronger expression at sections of 4-week of the mdx mouse as compared to each of 2-week-old and 3-week-old of the mdx mice with statistical significance (Figure 4).

Examination of myostatin mRNA expression showed similar amount both in the mdx and control mice at 2- and 3-week old, although a little higher expression was seen in the mdx mice as compared to the controls. The expression differences were also not associated with statistical significance. However, mRNA expression of the mdx mice was significantly higher than the control group at 4-week stage (Figure 5).

Discussion

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disorder caused by the lack of dystrophin, a 427-kDa protein located in the subsarcolemmal space of muscle fibers (Hoffman et al., 1987). The mdx mouse, a model of DMD, results from a mutation in the dystrophin gene in a

C57BL/10 mouse (Bulfield et al., 1984). Similar to DMD patients, the mdx mouse lacks dystrophin. Dystrophin is part of a multi-subunit transmembrane glycoprotein complex connecting sarcolemmal proteins with the underlying skeleton and the extracellular matrix (ECM) (Caceres et al., 2000). The absence of dystrophin leads to destabilization of this complex, resulting in weaker muscle fibers that undergo progressive degeneration followed by massive necrosis. It was reported that, in somite-derived muscles of the extremities, muscle necrosis began at 2-week after birth, immediately followed by regeneration, and the majority of necrotic muscles became regenerated at 4-week after birth (Dangain et al., 1984; DiMario et al., 1991; Attal et al., 2000). A similar phenomenon was noted in the branchial arch-derived mdx mouse masseter muscle (Lee et al. 2006), and cell degeneration in this muscular dystrophy was ascribed to the lack of dystrophin, resulting in damage to cell membrane stability, leading to excessive influx of calcium ions into muscle cells, a mechanism similar to that of necrosis. Thus, mdx mice have been used as a model to study the mechanism of muscle necrosis and regeneration (Gillis, 1996).

Whether originating from injury or progressive neuromuscular diseases, skeletal muscle injury and subsequent regeneration are initially characterized by neutrophil infiltration and inflammation, followed by phagocytosis of necrotic fibers debris by macrophages, which have invaded the damaged region (McLennan, 1996). Satellite cells, which exist in

undamaged muscle in quiescent state, then become activated in response to growth factors and undergo proliferation and migration (Allen and Rankin, 1990). The resultant satellite cells fuse to form myotubes, which then undergo maturation and hypertrophy to form normal, mature skeletal muscle fibers. Various molecules have been shown to have a role for these processes, including growth factors, caspases and ECM.

Decorin is a component of ECM belonging to member of the small leucine rich proteoglycan gene family, and composed of a core protein and dermatan/chondroitin sulfate chain (Schonherr et al., 1995). Decorin participates in regulation of collagen fibril formation and stabilizes it by forming physical complex with them, including collagen types 1 and 2 (Danielson et al., 1997). Decorin also plays an important role in cell growth through modulation of growth factor activities. In this respect, decorin binds to transforming growth factor-β and modulates its activity (Takeuchi et al., 1994; Schonherr et al., 1998). Forced expression of decorin in Chinese hamster ovary cells leads to suppression of cell proliferation (Yamaguchi et al., 1990). These studies suggest that decorin acts as a signaling molecule in some kinds of cells. However, act of decorin to myogenic cells and its role remained mostly unknown.

Thus in the current study, we examined localization and expression status of decorin by immunohistochemistry as well as quantitative real-time RT-PCR. First, we checked our model for muscular dystrophy, mdx mouse for

presence of degeneration and regeneration of the muscle fibers at the histopathological level. Histopathological staining of muscle tissues from the mdx and control mice showed almost normal appearance at the early stages. However, then the muscle tissues from the mdx mouse demonstrated extensive necrosis and followed by immediate regeneration. By using this model, we investigated an important ECM component molecule, decorin and discussed its possible role and relationship with other molecules during degeneration and regeneration of the muscle fibers. Our results demonstrated an increased protein expression of decorin at 3-week-old as well as 4-week-old in the mdx mice. Decorin was localized especially at interfibrillar ECM area. Myostatin, a growth and differentiation factor belonging to TGF-β superfamily has recently been shown to act as a negative regulator of skeletal muscle mass (McPherron et al., 1997). The deletion of myostatin in mice causes a dramatic and widespread increase in skeletal muscle. Interestingly, decorin has recently been shown to sequester myostatin in ECM by physically binding it (Kishioka et al., 2008). Decorin has also been shown to enhance proliferation and differentiation of myogenic cells through suppressing myostatin activity. Myostation localizes normally in cytoplasm of myogenic cells. We speculate that during necrotic 3-week-old of the mdx mouse, myostatin is released into ECM space, and increased in necrotic muscle tissue. In fact, increase of myostatin in necrotic muscle tissue but decrease expression in regenerative myogenic cells has recently

been shown (Kirk et al., 2000). We believe that decorin expression is enhanced as a reaction to balance myostatin collection in ECM. Increase of decorin in ECM sequesters myostatin. Thus inhibition of myostatin by decorin sequestration leads to cell proliferation and regeneration at the 4-week, the stage in which myostatin is not seen.

The mechanism for inhibition of myostatin activity by decorin remains unknown. Since physical binding of myostatin with decorin has been shown, sequestration of myostatin seems to be one reason for blocking its activity. However, decorin has also been demonstrated to increase activity of follistatin, a myostatin propeptide, which blocks myostatin activity. Therefore, decorin may also reveal an indirect inhibitory effect on myostatin (Hill et al., 2002). Further work is needed to elucidate the inhibitory mechanism of decorin on the molecules, which block myostatin function directly or indirectly.

In conclusion, we here show that decorin is overexpressed during degeneration and regeneration stages of the mdx mouse. Close relationship of myostatin with decorin suggests that decorin plays an important role in the proliferation, regeneration and differentiation of myoblasts by interfering with myostatin signaling in addition to by sequestering myostatin in the ECM. The current study would contribute to open a gate for identification of the relationship between decorin and myostatin as well as development of therapeutic strategy for progressive neuromuscular diseases.

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References

Allen RE, Rankin LL (1990) Regulation of satellite cells during skeletal muscle growth and development. Proc Soc Exp Biol Med 194, 81-6.

Attal P, Lambert F, Marchand-Adam S, Bobin S, Pourny JC, Chemla D, Lecarpentier Y, Coirault C (2000) Severe mechanical dysfunction in pharyngeal muscle from adult mdx mice. Am J Respir Crit Care Med **162**, 278-81.

Bernasconi P, Torchiana E, Confalonieri P, Brugnoni R, Barresi R, Mora M, Cornelio F, Morandi L, Mantegazza R (1995) Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with

fibrosis. Pathogenetic role of a fibrogenic cytokine. J Clin Invest **96**, 1137-44.

Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M (1992) Binding of the proteoglycan decorin to collagen type VI. J Biol Chem **267**, 5250-6.

Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS (2002) Functional improvement of dystrophic muscle by myostatin blockade. Nature **420**, 418-21.

Border WA, Noble NA (1994) Transforming growth factor beta in tissue fibrosis. N Engl J Med **331**, 1286-92.

Bulfield G, Siller WG, Wight PA, Moore KJ (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A **81**, 1189-92.

Cáceres S, Cuellar C, Casar JC, Garrido J, Schaefer L, Kresse H, Brandan E (2000) Synthesis of proteoglycans is augmented in dystrophic mdx mouse skeletal muscle. Eur J Cell Biol **79**, 173-81.

Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J Cell Biol **136**, 729-43.

Dangain J, Vrbova G (1984) Muscle development in mdx mutant mice. Muscle Nerve 7, 700-4.

DiMario JX, Uzman A, Strohman RC (1991) Fiber regeneration is not

persistent in dystrophic (MDX) mouse skeletal muscle. Dev Biol **148**, 314-21.

Gillis JM (1996) Membrane abnormalities and Ca homeostasis in muscles of the mdx mouse, an animal model of the Duchenne muscular dystrophy: a review. Acta Physiol Scand **156**, 397-406.

Gosselin LE, Williams JE, Deering M, Brazeau D, Koury S, Martinez DA (2004) Localization and early time course of TGF-beta 1 mRNA expression in dystrophic muscle. Muscle Nerve **30**, 645-53.

Hawke TJ, Garry DJ (2001) Myogenic satellite cells: physiology to molecular biology. J Appl Physiol **91**, 534-51.

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y (2002) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277, 40735-41.

Hoffman EP, Brown RH Jr, Kunkel LM (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51, 919-28.

Kirk S, Oldham J, Kambadur R, Sharma M, Dobbie P, Bass J (2000) Myostatin regulation during skeletal muscle regeneration. J Cell Physiol 184, 356-63.

Kishioka Y, Thomas M, Wakamatsu J, Hattori A, Sharma M, Kambadur R, Nishimura T (2008) Decorin enhances the proliferation and

differentiation of myogenic cells through suppressing myostatin activity. J Cell Physiol 215, 856-67.

Lee WH, Abe S, Kim HJ, Usami A, Honda A, Sakiyama K, Ide Y (2006) Characteristics of muscle fibers reconstituted in the regeneration process of masseter muscle in an mdx mouse model of muscular dystrophy. J Muscle Res Cell Motil 27, 235-40.

Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J (2004) Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. Am J Pathol 164, 1007-19.

Li ZF, Shelton GD, Engvall E (2005) Elimination of myostatin does not combat muscular dystrophy in dy mice but increases postnatal lethality. Am J Pathol **166**, 491-7.

Lijnen PJ, Petrov VV, Fagard RH (2000) Induction of cardiac fibrosis by transforming growth factor-beta (1). Mol Genet Metab **71**, 418-435.

McLennan IS (1996) Degenerating and regenerating skeletal muscles contain several subpopulations of macrophages with distinct spatial and temporal distributions. J Anat **188** (Pt 1), 17-28.

McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature **387**, 83-90.

Miura T, Kishioka Y, Wakamatsu J, Hattori A, Hennebry A, Berry CJ, Sharma M, Kambadur R, Nishimura T (2006) Decorin binds myostatin and modulates its activity to muscle cells. Biochem Biophys Res Commun **340**, 675-8.

Sara VR, Hall K (1990) Insulin-like growth factors and their binding proteins. Physiol Rev **70**, 591-614.

Schönherr E, Hausser H, Beavan L, Kresse H (1995) Decorin-type I collagen interaction. Presence of separate core protein-binding domains. J Biol Chem **270**, 8877-83.

Schönherr E, Broszat M, Brandan E, Bruckner P, Kresse H (1998) Decorin core protein fragment Leu155-Val260 interacts with TGF-beta but does not compete for decorin binding to type I collagen. Arch Biochem Biophys **355**, 241-8.

Takeuchi Y, Kodama Y, Matsumoto T (1994) Bone matrix decorin binds transforming growth factor-beta and enhances its bioactivity. J Biol Chem **269**, 32634-8.

Velleman SG (1999) The role of the extracellular matrix in skeletal muscle development. Poult Sci **78**, 778-84.

Wagner KR, McPherron AC, Winik N, Lee SJ (2002) Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. Ann Neurol **52**, 832-6.

Yamaguchi Y, Mann DM, Ruoslahti E (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature **346**, 281-4.

Figurelegends

Figure 1

H&E staining of anterior tibialis muscle both in mdx and B10 mice. At 2-week-old stage, both the B10 and mdx mice demonstrated almost normal morphology without necrosis. However, at 3-week-old stage, the mdx mouse revealed extensive necrosis and fibrosis, which were not detected in the B10 mouse. On the other hand, at 4-week after birth, many regenerative cells with central nuclei were noticed at the mdx mouse. (Scale bars=50 μ m)

Figure 2

Immunohistochemical staining of decorin in anterior tibialis muscle of both mdx and B10 mice. Weak cytoplasmic expression of decorin was shown in few cells of the B10 mice at all stages. On the other hand, decorin showed strong expression around cells of the mdx mouse. An increased expression of decorin was detected at 3- and 4-week stages as compared the mouse with 2-week-old. Decorin especially revealed strong expression around those regenerative cells with central nuclei. The control normal goat IgG demonstrated no reaction both in the B10 as well as mdx mice, suggesting

specific staining of our antibody. Expression area was marked with arrows. (Scale bars=50 μ m).

Figure 3

Immunohistochemical staining of myostatin in anterior tibialis muscle of both mdx and B10 mice. At 2-week after birth, since the mice are in growth period, the slight protein expression was detected not only the mdx mice but also the control group. At 3-week after birth, a part of mdx mice cells, especially those supposed to be in regeneration process due to fusion, showed expression. At 4-week after birth, many regenerated muscle cells of the mdx mice with central nuclei demonstrated expression. Expression area was marked with arrows. (Scale bars=50 μ m)

Figure 4

mRNA expression of decorin in anterior tibialis muscle of both mdx and B10 mice. Examination of decorin mRNA showed similar level of expression at any stage of B10 mouse, though decorin was expressed relatively lower level at 2 and 3-week-old of the mdx mouse than the corresponding age of B10 mouse without statistical difference. However, at 4-week after birth, decorin revealed a higher level of expression at the mdx mouse sections as compared to B10 mouse tissue with statistical significance. Moreover, decorin showed stronger expression at sections of 4-week of the mdx mouse as compared to

each of 2-week-old and 3-week-old of the mdx mice with statistical significance.

Figure 5

mRNA expression of myostatin in anterior tibialis muscle of both mdx and B10 mice. Myostatin mRNA expression was similar both in the mdx and control mice at 2- and 3-week old. Although a little higher expression was seen in the mdx mice as compared to the controls at this stage, the expression differences were also not associated with statistical significance. However, mRNA expression of the mdx mice was significantly higher than the control group at 4-week stage.

Table 1 *The primers used for the RT-PCR analysis*

Decorin	Forward	5’- GAGGAGAAGTGAGGGGAGACA -3’
	Reverse	5’- GATTATCTCATGTATTTTCACGACCTT-3’
Myostatin	Forward	5’- CATCTTGTGCACCAAGCAAA -3’
	Reverse	5’- GGGAGACATTTTTGTCGGAGT -3’
GAPDH	Forward	5’-TGAACGGGAAGCTCACTGG-3’
	Reverse	5’-TCCACCACCCTGTTGCTGTA-3’

Fig.1

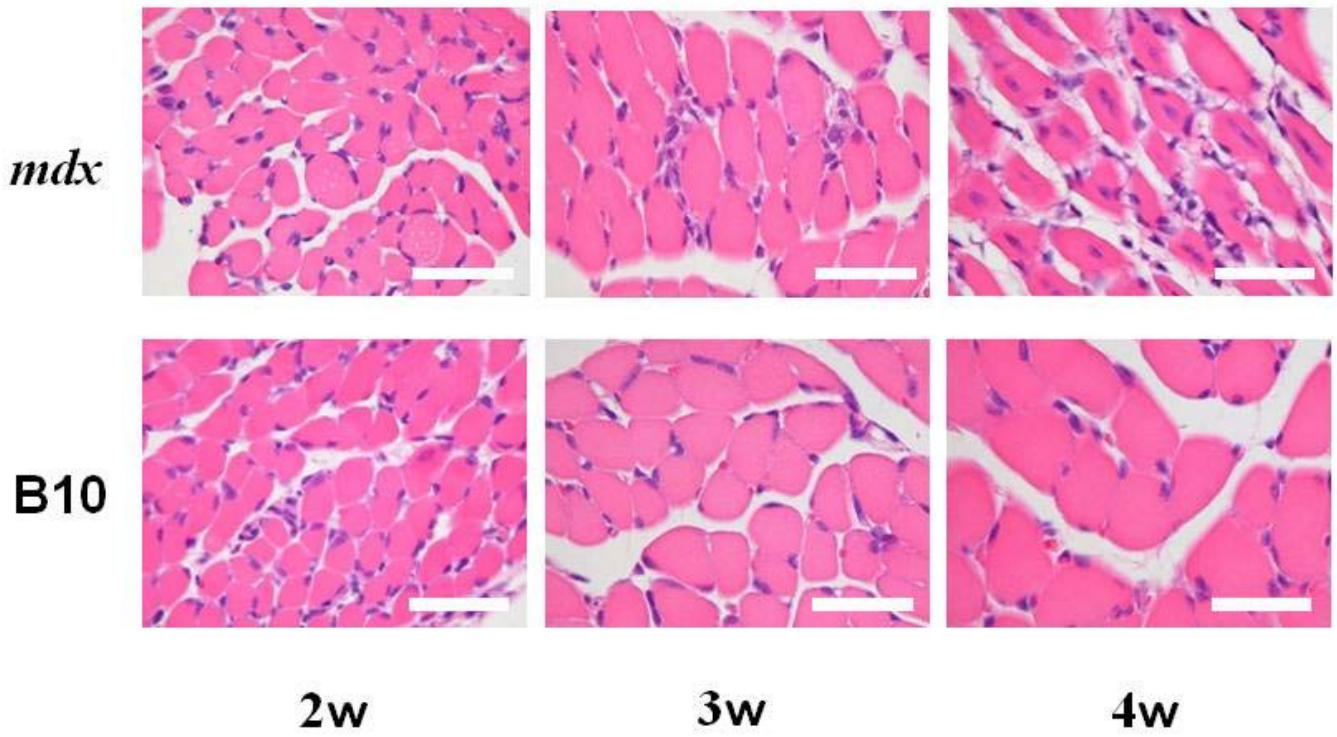


Fig.2

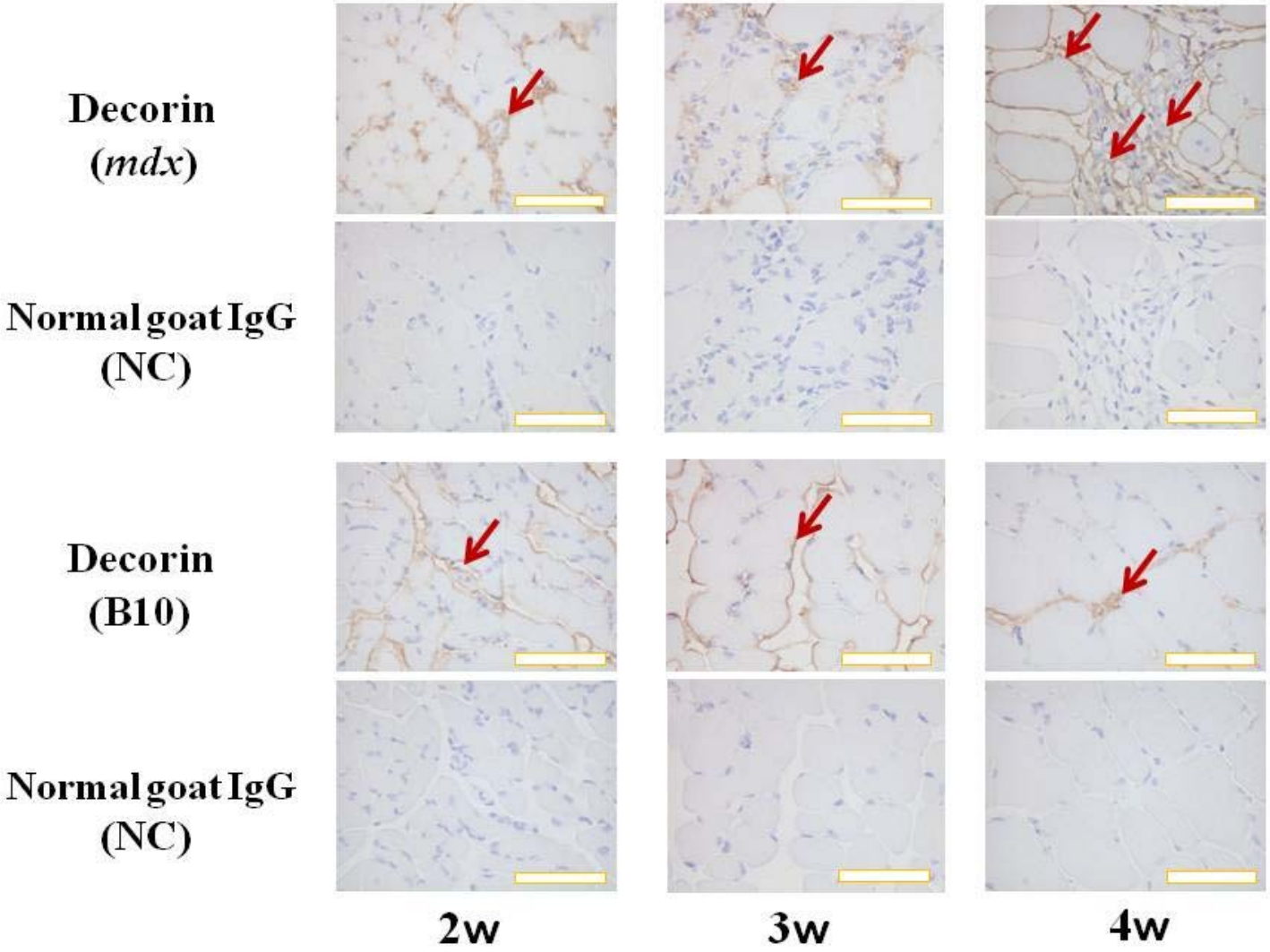

Decorin
(mdx)
Normal goat IgG
(NC)
Decorin
(B10)
Normal goat IgG
(NC)
2w
3w
4w

Fig.3

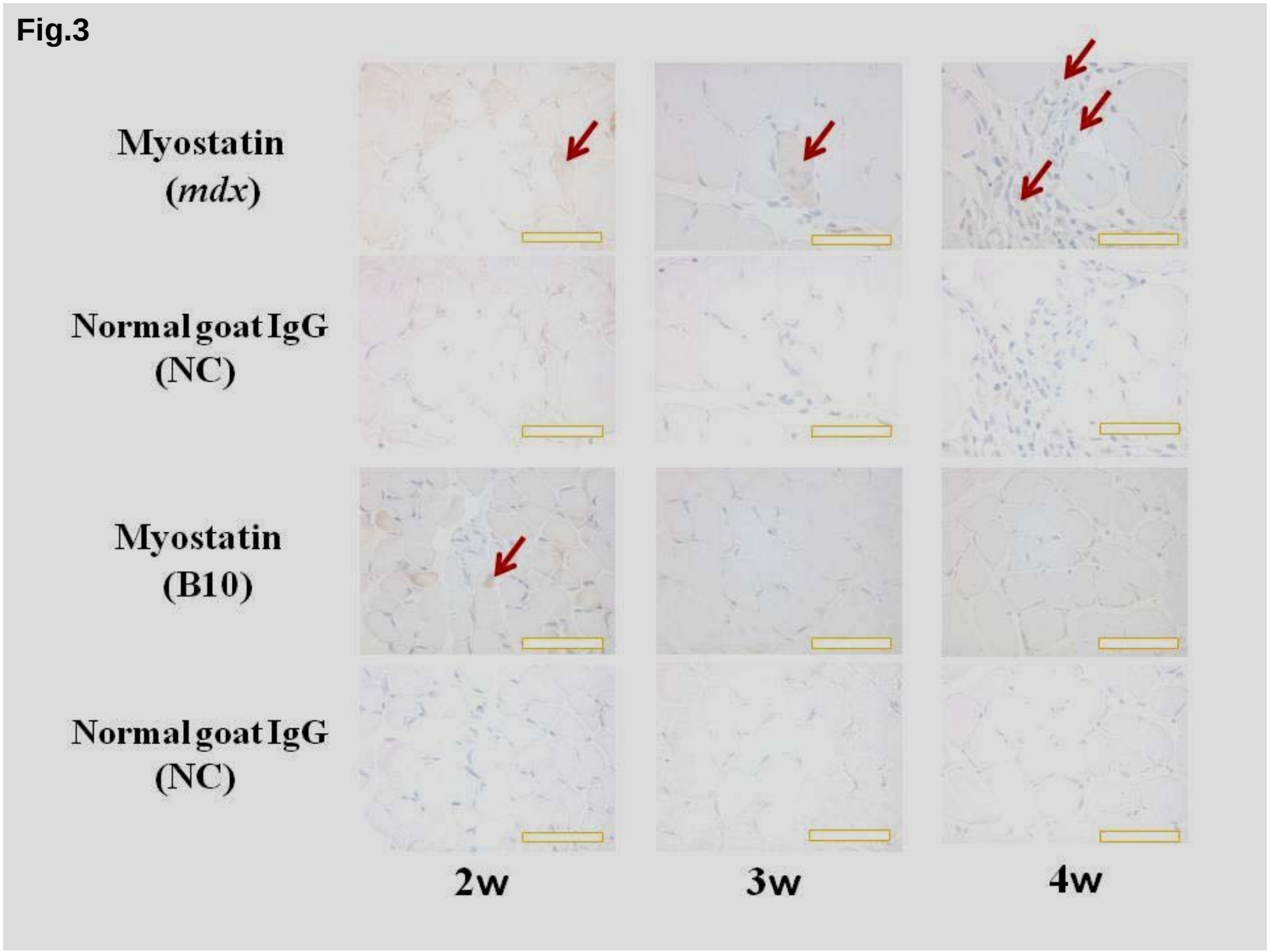

Myostatin
(mdx)
Normal goat IgG
(NC)
Myostatin
(B10)
Normal goat IgG
(NC)
2w
3w
4w

Fig.4

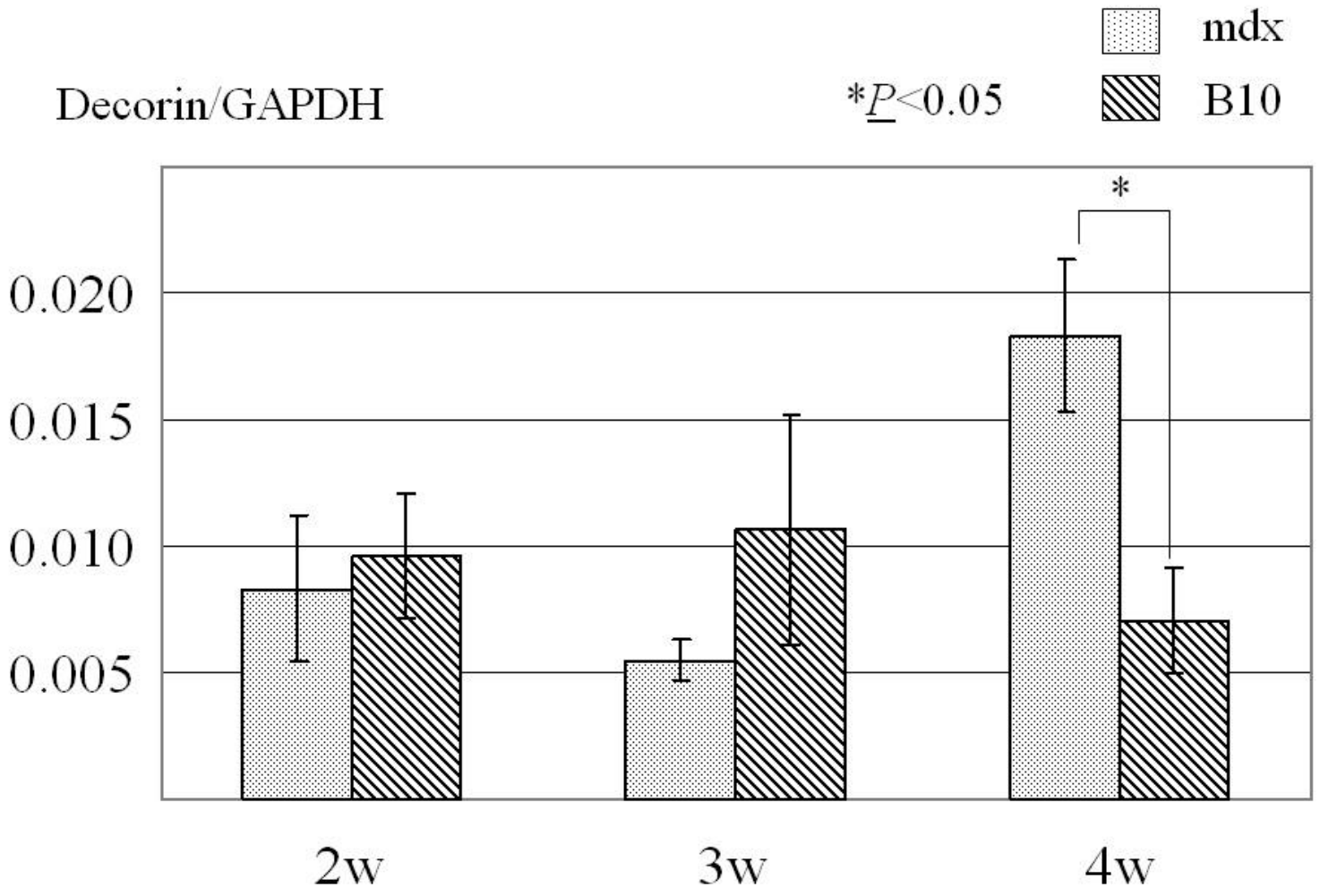
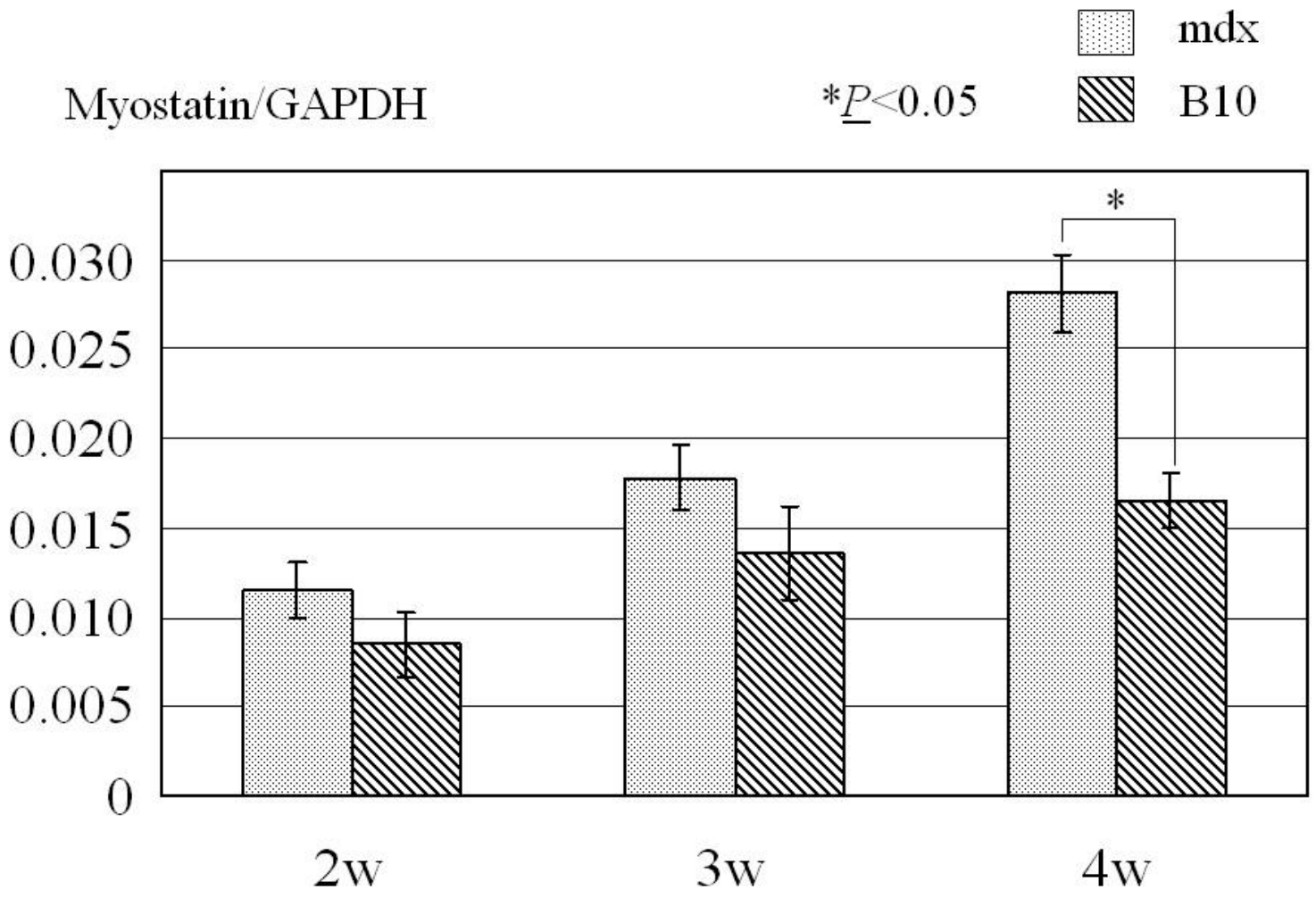


Fig.5


mdx
B10
Myostatin/GAPDH
*P<0.05
*
0.030
0.025
0.020
0.015
0.010
0.005
0
2w
3w
4w