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**MYONUCLEUS-RELATED PROPERTIES IN SOLEUS MUSCLE  
FIBERS OF *MDX* MICE**

(*mdx* マウスヒラメ筋線維における筋核関連特性)

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## ABSTRACT

Distribution and total number of myonuclei in single soleus muscle fibers, sampled from tendon-to-tendon, were analyzed in *mdx* and wild type (WT) mice. Apoptotic myonuclei and microscopic structure around myonuclei were also analyzed. Three types of muscle fibers of *mdx* mice with myonuclear distribution at either central, peripheral, or both central and peripheral region were observed. All of the myonuclei were located at the peripheral region in WT mice. The total number of myonuclei in whole fiber was identical between *mdx* and WT mice and between fibers with different distribution of myonuclei in *mdx* mice and peripheral nucleus was noted where the central nucleus was missing. Fiber size, sarcomere number, myonuclear size, myosin heavy chain expression, satellite cell number, and neuromuscular junction were identical between each type of fiber. Apoptosis was not detected in any myonuclei located either central or peripheral region of fibers. Thus, it was suggested that apoptosis-related loss of central myonuclei and regeneration-related new accretion at the peripheral region is not the cause of the different distribution of myonuclei seen in muscle fibers in *mdx* mice. But it was speculated that cross-sectional translocation of myonuclei may be induced in response to regeneration from central to peripheral region, although microscopic evidence of any structures were not observed around myonuclei.

**Key words:** *mdx* mice, soleus muscle fibers, central and/or peripheral myonuclei, apoptosis

## INTRODUCTION

Dystrophin-deficient (*mdx*) mouse is a well-known animal model for Duchenne muscular dystrophy (DMD). The muscles of *mdx* mice are characterized by cycles of muscle fiber necrosis followed by regeneration (5). Further, the regenerating fibers can be easily identified, because they contain conspicuous internal (central) myonuclei (3). Williams *et al.* (24) reported that fibers with either a predominantly centrally located string of myonuclei, or a combination of central nuclei and peripheral pattern of nuclei appeared in soleus muscle fibers of *mdx* mice. However, it is unclear how the change of localization of myonuclei within a fiber is induced. It is not known whether the total number of myonuclei in a whole single muscle fiber response to necrosis/regeneration cycle, either.

It was reported that loss of myonuclei was associated with the atrophy of soleus muscle fibers (1, 2, 14). Oishi *et al.* (16) also reported that hindlimb unloading caused a significant decrease of myonuclear number in atrophied soleus muscle fibers of rats. They also observed many fibers with central nuclei at the early phase (day 3) of ambulation recovery period. However, the mechanism responsible for the relationship between the number and localization of myonuclei is still unclear.

Muscle satellite cells are myonuclear precursors lying between the sarcolemma and the basal lamina of myofiber (9), and myonuclear accretion occurs through the incorporation of satellite cell nuclei into the growing myofibers (13). Satellite cells also play one of the essential roles in the regeneration from injury (20) and growth of skeletal muscle (6). Kawano *et al.* (6) recently reported that the growth-associated increase of myonuclear number in a single fiber of rat soleus was less than the age-matched cage controls due to an inhibited elevation of satellite cell number,

suggesting a close relationship between satellite cells and myonuclei. However, role of satellite cells in the regulation of necrosis/regeneration cycle and translocation of myonuclei in muscle fibers of *mdx* mice is unclear. Therefore, the total numbers of both quiescent and mitotic active satellite cells, as well as the myonuclear number, were counted in whole fibers with various distribution of myonuclei in the present study.

Waerhaug & Lomo (22) reported that fast motoneurons form large ectopic junctions, whereas slow motoneurons form small ectopic junctions, concluding that the type of motoneuron, not muscle fiber, determines the fast or slow character of the neuromuscular junction. Changes in the distribution of acetylcholine receptors were also observed in muscle fibers of *mdx* mice (8, 11, 17, 21). However, it is not known whether the distribution or number of acetylcholine receptors is different between fibers with different localization of myonuclei. Therefore, the numbers and size of neuromuscular junctions (NMJs) were compared between fibers with different location of myonuclei in this study.

It is essential to develop a suitable method for stimulation of regeneration of damaged muscle. Therefore, the current study was performed to investigate the mechanism responsible for the cycle of necrosis and regeneration of muscle in *mdx* mice. The major purposes were to test the hypotheses that myonuclei in regenerating fibers translocate cross-sectionally from central to peripheral region, or that central myonuclei are lost due to apoptosis and new myonuclei are accreted at the peripheral region when the regeneration of fiber is advanced. The total number and location of myonuclei in single soleus muscle fibers, sampled from tendon-to-tendon, were measured. Apoptotic myonuclei were checked by using the terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) method. Further, the

existence of any evidence, which may help the translocation of myonuclei, was also investigated by analyses using electron microscopy.

## MATERIALS AND METHODS

All experimental procedures were conducted in accordance with the Japanese Physiological Society *Guide for the Care and Use of Laboratory Animals* and also followed the guiding principles of the American Physiological Society. The study was also approved by the Animal Use Committee at the university.

### *Animal care and experimental procedures*

Nine-week-old male C57BL/10-*mdx* Jic (dystrophin-deficient, *mdx*, n=10) and C57BL/6J Jcl (wild type: WT, n=10) mice were used in the present study. All mice were housed in a controlled environment with 12:12 hr of light:dark cycle and the temperature and humidity were maintained at ~23 °C and ~55%, respectively. Two to three mice were housed in a cage with 11 × 20 cm and 11 cm height. Solid food (CE-2, Nihon CLEA, Tokyo) and water were supplied *ad libitum*.

A single injection of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, 100 mg/kg body weight) in phosphate-buffered saline (PBS) was performed *i.p.* 2 days prior to the sampling. The mice were anesthetized by injection of sodium pentobarbital (5 mg/100 g body weight, *i.p.*). The soleus muscles were sampled bilaterally and cleaned of excess fat and connective tissue. For the longitudinal analyses of single fibers, the left muscles sampled from *mdx* and WT mice (n=5 each) were stored in cellbanker (Nihon Zenyaku, Tokyo) at -80 °C. The right muscles were stretched at an optimal length and pinned on a cork and frozen in isopentane cooled with liquid nitrogen. And

the mid-portion of the frozen muscle was mounted on a cork by using optimum cutting temperature (OCT) compound (Miles, IN, U.S.A) and frozen in liquid nitrogen for analyses of the expression of myosin heavy chain (MHC) and myonuclei with or without apoptosis, location of myonuclei in cross-sectional samples.

Further, soleus muscles were sampled bilaterally in 5 *mdx* and WT mice. The left muscles were frozen in liquid nitrogen for analyses of apoptotic myonuclei in longitudinal single fibers. For electron microscopic analyses, the right muscles of *mdx* mice were fixed with 2% glutaraldehyde in 0.1% cacodylate buffer at 4°C for 2 hr, rinsed with 0.1% cacodylate buffer solution, and postfixed in 2% osmium tetroxide with 0.1% cacodylate buffer solution at 4°C for 2 hr. After rinsing with cacodylate buffer solution, the specimens were dehydrated in graded concentrations of ethanol and embedded in epoxy resin.

#### *Longitudinal analyses*

Myonuclei, satellite cells, and neuromuscular junction: The muscles stored in the cellbanker were thawed instantly at 35 °C. The muscles were placed in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 0.2% type I collagenase, 1% antibiotics, and 10% new-born calf serum (NCS) for 3 hr at 35 °C to digest the collagens. The collagenase-treated samples were fixed in 4% buffered-formaldehyde for 10 min and rinsed in PBS. Entire single muscle fibers were isolated from tendon-to-tendon using fine needles. The fibers were collected carefully using pipette to avoid any damage. The collected fibers were separated into tubes and immersed in DMEM containing 10% NCS.

For immunohistochemistry, tube No. 1 and 2 were used to label M-cadherin and

BrdU as described previously (23). The single fibers in these tubes were permeabilized with 1% triton X-100 diluted with PBS for 10 min. These fibers were, then, blocked in 10% goat serum diluted with PBS for 15 min at room temperature. The primary polyclonal antibody specific to M-cadherin (Santa Cruz Biotechnology, Inc.) and monoclonal antibody specific to BrdU (Becton Dickinson) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin (BSA) was applied to tube No. 1 and 2, respectively. And the tubes were kept at 4 °C for at least 12 hr. The primary antibody was detected by secondary antibody reaction, which was performed for 2 hr at 4 °C, using goat anti-rabbit immunoglobulin G (IgG) conjugated to fluorescein (Chemicon International, Inc.) and goat anti-mouse IgG conjugated to fluorescein-isothiocyanate (Jackson Immuno Research) diluted at 1:100 with 0.5% Tween 20 and 0.5% BSA for tube No. 1 and 2, respectively. The nuclei (myonuclei + satellite cells) in the antibody-reacted fibers in the tube No. 1 and 2 were further counterstained with propidium iodide (PI, 10 µg/ml PBS) for 10 min. Fibers in tube No. 3 were incubated with  $\alpha$ -bungarotoxin in PBS (1:100) for 10 min. The myonuclei were stained by PI for 10 min. After staining, the single fibers were rinsed with PBS and stored in tube filled with PBS at 4 °C until analyses. Immediately before the analysis, the fibers were mounted on a slide glass in 50% glycerol with coverslips with “struts” of hardened nail polish on the corners to minimize fiber compression.

Apoptotic myonuclei: The soleus muscles were gradually thawed to room temperature in a low-calcium relaxing solution with 50% glycerol after the soleus muscles were adapted to the solution at -4 °C overnight, as described previously (15). Single fiber segments (n=30 from each muscle) were mechanically isolated by using

fine tweezers under a dissection microscope, placed on gelatin-coated glass slide, and air dried. Mechanical isolation of single fibers has been shown to strip off the basal lamina of muscle fiber including satellite cells (15). Immediately, the apoptotic myonuclei were labeled using TUNEL method as described previously (1). Briefly, the fibers were fixed with 4% buffered formaldehyde for 1 hr and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 5 min. Subsequently, the fibers were incubated in the reaction buffer containing terminal deoxynucleotidyl transferase (Roche Diagnostic) for 1.5 hr at 37°C. After the reaction, the fibers were rinsed with PBS and mounted in the medium containing 4', 6-diamidino-2-phenylindole (DAPI). The DAPI-labeled total myonuclei and TUNEL-positive apoptotic myonuclei in the double-stained section were observed under a confocal microscope.

#### *Cross-sectional analyses*

Myosin heavy chain expression and myonuclei: Four serial cross-sections (10  $\mu\text{m}$  thickness) were cut in a cryostat maintained at  $-20\text{ }^{\circ}\text{C}$ . Two sections were used for the analyses of MHC expression. The expression of MHC was analyzed by using monoclonal antibodies specific for slow (type I) or fast (type II) MHC isoform, i.e., primary antibody, NCL-MHCs and NCL-MHCf (Novocastra Laboratories, UK), as described previously (7). Briefly, the avidin-biotin immunohistochemical procedure was used for the localization of primary antibody binding according to the instructions for ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.). The PBS was used as a buffer for all IgG primary antibodies. The visualization for primary antibody binding site was performed with diaminobenzidine tetrahydrochloride. The stained images were incorporated into a computer. Number of primary antibody positive fibers was

counted in the whole muscle cross-section stained with antibodies specific for slow or fast MHC. Fiber phenotypes were classified as type I, I+II, or II. The third section was stained for myonuclei using haematoxylin for 5 min at room temperature. The muscle fibers were matched with those in the first and second sections and MHC expression in fibers with or without central nuclei was determined.

Apoptotic myonuclei: The fourth cross-sections were incubated in the reaction buffer added anti-laminin antibody (Sigma) diluted 1:200 with reaction buffer. Anti-laminin antibody was visualized with donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Invitrogen) diluted 1:200 with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin (BSA). After the reaction, the section was rinsed with PBS and mounted in the medium containing DAPI. The apoptotic myonuclei were labeled using the same TUNEL method as mentioned above. The DAPI-labeled myonuclei and TUNEL-positive apoptotic myonuclei in the triple-stained section were observed under a confocal microscope.

#### *Confocal microscopy*

A FV-300 confocal microscope with an argon laser (488 nm of peak wavelength, Olympus) was used to analyze the fiber length, sarcomere number, fiber cross-sectional area (CSA), the number and distribution of PI- and TUNEL-positive myonuclei, and CSA of PI-positive myonuclei in the whole single fibers. The fibers with various distribution of PI-positive myonuclei were classified into three types as described previously (24); type C in which more than 80% of myonuclei were arranged as a straight chain in the central region of fiber, type P in which all myonuclei were

distributed at the peripheral region of fiber, and type C+P in which less than 80% of myonuclei were located at the central region and the residual myonuclei were located at the peripheral region (Fig. 1).

First, a maximum-intensity projection rotated orthogonally to the long axis of the fiber was produced from the stack, and the fiber CSA was measured at three non-overlapping regions randomly chosen along the fiber length. The PI-labeled nuclei were counted in the entire single fiber. Myonuclear domain size (10, 15) was also calculated by multiplying the fiber CSA and the fiber length and then dividing by the myonuclear number per fiber. The largest diameter of the myonuclei (at least 30 myonuclei in each fiber) was also measured. The fiber length and the length of 10 consecutive sarcomeres were also measured in each fiber by Normarski optic scan and the fiber CSA and the length of the fiber were normalized at a 2.5  $\mu\text{m}$  sarcomere length. Sarcomere number in whole fiber was also calculated. The TUNEL-positive myonuclei in the entire single fiber and cross-section were also checked.

### *Electron microscopy*

In the right soleus muscles of *mdx* mice, both longitudinal and cross-sectional ultra-thin sections were made with an OMU4 Ultracut microtome (Reichert, Vienna, Austria) and stained with 5% uranyl acetate and 1% lead acetate, 1% lead nitrate and 1% lead citrate. Structure around each myonucleus was examined using H-7650 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

### *Statistical analyses*

Values were expressed as means  $\pm$  SEM. Significant differences among groups were determined by two-way ANOVA, followed by Scheffé's post hoc test using StatView packaging software (HULINKS, Tokyo, Japan). Differences were considered significant at the 0.05 level of confidence.

## RESULTS

### *Distribution of myonuclei, size and phenotype of fiber, sarcomere number, and NMJ.*

Fibers with central nuclei were observed in *mdx*, but not in WT, mice (Fig. 1). The percentages of type C, C+P, and P fibers in *mdx* mice were 20.7 and 50.1, and 29.2%, respectively (Fig. 1B). Fiber length and sarcomere number (Fig. 2) were identical between *mdx* and WT mice. And these levels were similar in any types of fibers of *mdx* mice. Fiber CSA was not different between two species and also between 3 types of fibers in *mdx* mice (Fig. 3).

The percent distribution of fibers expressing type I, I+II, and II MHC was 29.1, 4.7, and 66.2% in WT mice (Fig. 4). And that in *mdx* mice was 7.6, 38.5, and 53.9% in fibers without central nuclei and 3.7, 48.4, 47.9% in fibers with central nuclei, respectively. The percentage of type I+II fibers was less and that of type II fibers was greater than that of type I fibers in WT mice ( $p < 0.05$ ). As for *mdx* mice, the percentage was identical in fibers with or without central nuclei. The percentage of fibers expressing pure type I MHC in *mdx* mice was less than in WT mice ( $p < 0.05$ ), and that of fibers co-expressing type I and II MHC was greater than in WT mice ( $p < 0.05$ ).

All type P fibers in WT mice had a single innervation (Fig. 5A). The percent type P fiber in *mdx* mice was significantly less than WT mice. And ~20.1% of fibers had 2 NMJs and no NMJ was detected in ~6.6% fibers. These characteristics were

similar in any fibers with different distribution of myonuclei. The absolute and relative diameter, normalized with fiber CSA, of NMJs, which may have a close relationship with MHC phenotype, were not different between 2 species and was identical in fibers with or without central myonuclei in *mdx* mice (Figs. 5B and C).

*Number, size, and domain of myonuclei, and satellite cells.* The mean myonuclear number of type P fibers in WT and *mdx* mice was 541 and 449 ( $p < 0.05$ , Fig. 6A). Myonuclear CSA of type P fibers was also greater in WT than *mdx* mice (16%,  $p < 0.05$ , Fig. 6B). But the total number and size of myonuclei were identical in any types of fibers with different distribution of myonuclei in *mdx* mice. Myonuclear domain was similar in any types of fibers of *mdx* mice. However, the level of type P fibers in *mdx* mice were 23 % greater than WT mice ( $p < 0.05$ , Fig. 6C). The numbers of both mitotic active and quiescent satellite cells in whole single fiber, as well as the percent mitotic active cells, were identical between 2 species and fibers with various distribution of myonuclei in *mdx* mice (Fig. 7A-C).

*Apoptotic myonuclei.* In the longitudinal fibers, the TUNEL-positive apoptotic myonuclei were not detected in type P fibers of WT mice and in all types of fibers of *mdx* mice (Fig. 8). In contrast, TUNEL-positive myonuclei were slightly detected in the cross-sectional sample of *mdx*, not WT, mice. Those nuclei were located at the peripheral region. Further, there was no relationship in the distribution of TUNEL-positive apoptotic nuclei between the myonuclei located at the central or peripheral region.

*Electron microscopic images of central and peripheral myonuclei.* Electron microscopic analyses were performed in both cross- and longitudinal-sections of *mdx* mice (Fig. 9). However, typical differences in the morphological characteristics, which may help the translocation of myonuclei, were not detected in both central and peripheral myonuclei.

## DISCUSSION

A series of central myonuclei were observed in muscle fibers, sampled from tendon-to-tendon, in *mdx* mice (Figs. 1A and B), which is the first evidence to the author's knowledge. Most of the previous studies analyzed the location of myonuclei in muscle cross-section (12, 19) or in a portion of longitudinal fibers (24). The total number of myonuclei was identical between fibers with different distribution of myonuclei (type C, C+P, and P fibers). Further, the peripheral myonucleus was noted generally at the site where the central myonucleus was missing in type C+P fiber (Fig. 1A). Thus, these observations suggest that the cross-sectional translocation of the myonuclei from the center to periphery of the fiber may be induced. The author, therefore, speculated that the shift of the fiber properties, for example type C→C+P→P, may be induced during the regeneration process in muscle fibers of *mdx* mice.

It was reported that the fibers with mixed population of central and peripheral myonuclei were damaged (24). However, these central myonuclei were not always arranged like a chain, as was noted in the present study (type C fibers). Immature myogenic cells, such as myoblasts and myotubes, are centrally nucleated (18). Chain of central nuclei was observed in myotubes (4). The mean CSA and length of fibers were identical between *mdx* and WT mice in the present study. Furthermore, any

marked abnormalities were not noted between the fibers with different distribution of myonuclei in *mdx* mice. Many parameters, such as fiber CSA and length, sarcomere number, myonuclear number and CSA, MHC expression, and satellite cell number, were identical among the fibers with different distribution of myonuclei. Interestingly, myonuclear domain size was also similar between 3 types of fibers, even though the domain area of each myonucleus might be different between the myonuclei located at the central and peripheral region. The sarcomeres were also clearly seen throughout the fiber length even in the type C fibers, indicating that the intracellular myofibrils were formed normally. Furthermore, the type C fibers contained NMJ, suggesting the successful innervation of the motor axon even in the fibers containing central myonuclei. Therefore, the fibers with central nuclei may be not necessarily immature. It is also indicated that the cross-sectional migration of myonuclei in response to degeneration/regeneration cycle is not related to most of the characteristic of muscle fibers, including the morphologic, as well as contractile (24), properties. Meanwhile, apoptosis was not detected in any myonuclei located either central or peripheral region of fibers. Thus, it was suggested that apoptosis-related loss of central myonuclei and regeneration-related new accretion at the peripheral region is not the cause of the different distribution of myonuclei seen in muscle fibers in *mdx* mice.

In conclusion, the fibers containing the central nuclei were noted and the nuclei in these fibers were arranged like a single chain throughout the fiber length in the soleus muscle of *mdx* mice. The total number of myonuclei was the same in any fibers with different distribution of myonuclei. Further, peripheral nucleus was noted generally at the site where the central nucleus was missing. These results suggest that myonuclei migrate cross-sectionally, although microscopic evidence of any structures was not

observed around myonuclei.

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### FIGURE LEGENDS

Figure 1: (A) Various distribution of myonuclei in whole soleus muscle fibers, sampled from tendon-to-tendon, of *mdx* and wild type mice. Type C: With more than 80% of myonuclei at the central region. Type P: With all myonuclei at the peripheral region. Type C+P: With less than 80% of myonuclei at the central region. (B) Percentages of fibers with different distribution of myonuclei. All of the fibers in wild type mice were peripheral-nucleated. Mean  $\pm$  SEM. \* and †:  $p < 0.05$  vs. wild type mice and type C fibers of *mdx* mice, respectively.

- Figure 2: Fiber length and sarcomere number in whole fiber. Mean  $\pm$  SEM. See Figure 1 for the abbreviations.
- Figure3: Fiber cross-sectional area in whole fiber. Mean  $\pm$  SEM. See Figure 1 for the abbreviations.
- Figure 4: Percent distribution of fibers expressing slow (type I), fast (type II), or both slow and fast (type I+II) isoforms of myosin heavy chain. w/o: Fibers without central nuclei. w/: Fibers with central nuclei. Mean  $\pm$  SEM. \*, †, and §:  $p < 0.05$  vs. wild type mice and type I and I+II fibers in respective group. See Figure 1 for the abbreviations.
- Figure 5: (A) Percentage of fibers with different numbers of neural innervations in whole fiber. Absolute (B) and relative diameter, normalized with fiber cross-sectional area (CSA, C), of neuromuscular junctions are also shown. Mean  $\pm$  SEM. \*:  $p < 0.05$  vs. type P fibers in wild type mice. See Figure 1 for the abbreviations.
- Figure 6: Myonuclear number (A), myonuclear cross-sectional area (B), and myonuclear domain (C) analyzed in whole single fiber of wild type and *mdx* mice. Mean  $\pm$  SEM. \*:  $p < 0.05$  vs. type P fibers in wild type mice. See Figure 1 for the abbreviations.
- Figure 7: Number of mitotic active (A) and quiescent satellite cells per whole fiber

(B), and the percentage of mitotic active satellite cells relative to the total satellite cells (C). Mean  $\pm$  SEM. See Figure 1 for the abbreviations.

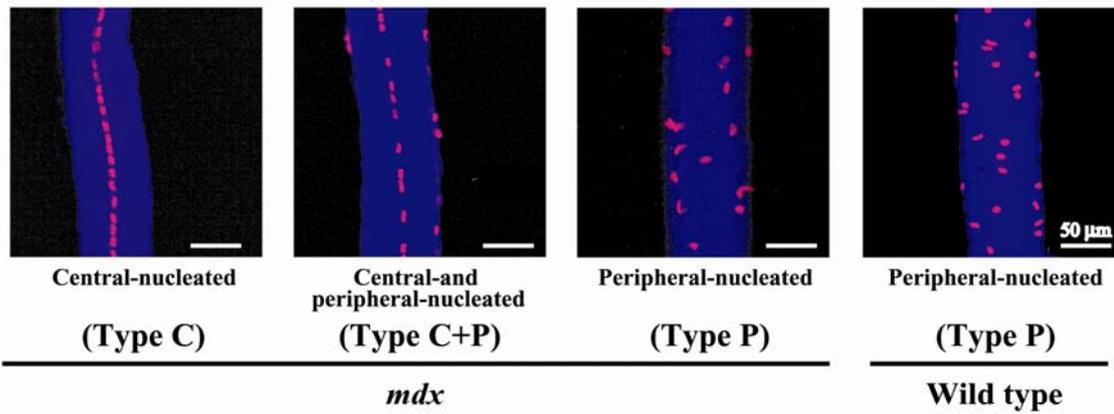
Figure 8: The distribution of myonuclei with or without {labeled using 4', 6-diamidino-2-phenylindole (DAPI)} apoptosis in muscle fibers of *mdx* and wild type (WT) mice. See Figure 1 for other abbreviations.

Figure 9: Electron micrographs of central and peripheral myonuclei in soleus muscle fibers of *mdx* mice.

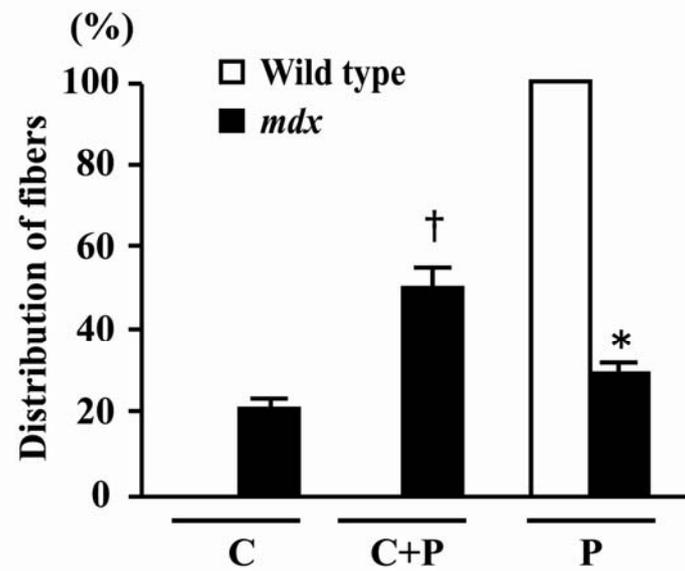
Fig. 1

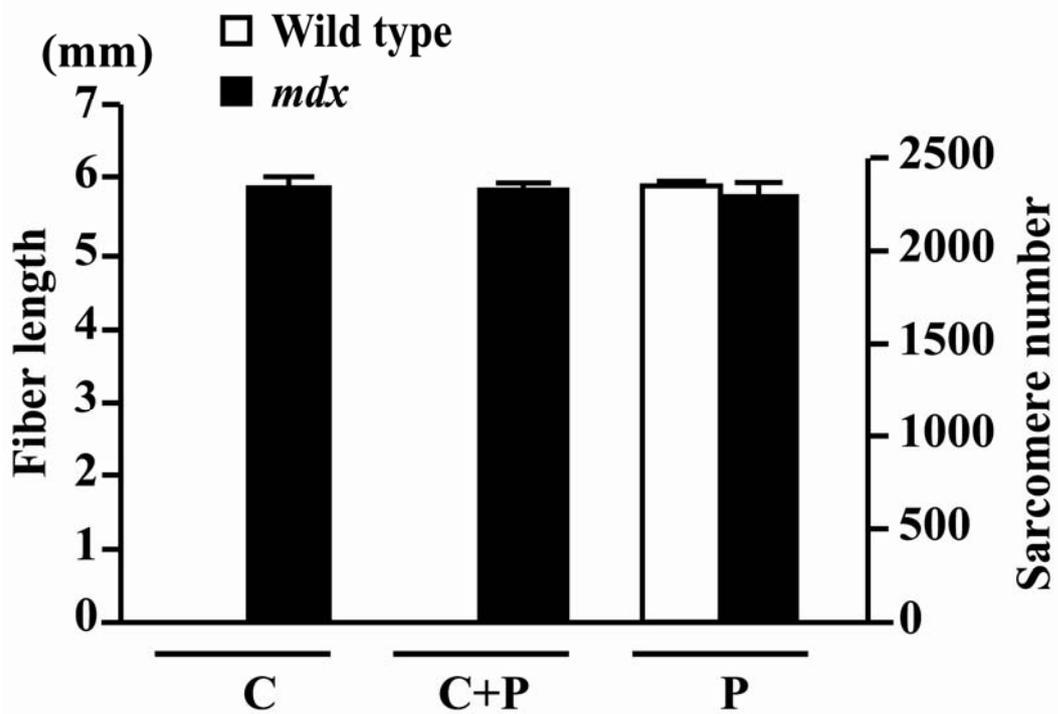
A

## Patterns of myonuclear distribution



B



**Fig. 2**

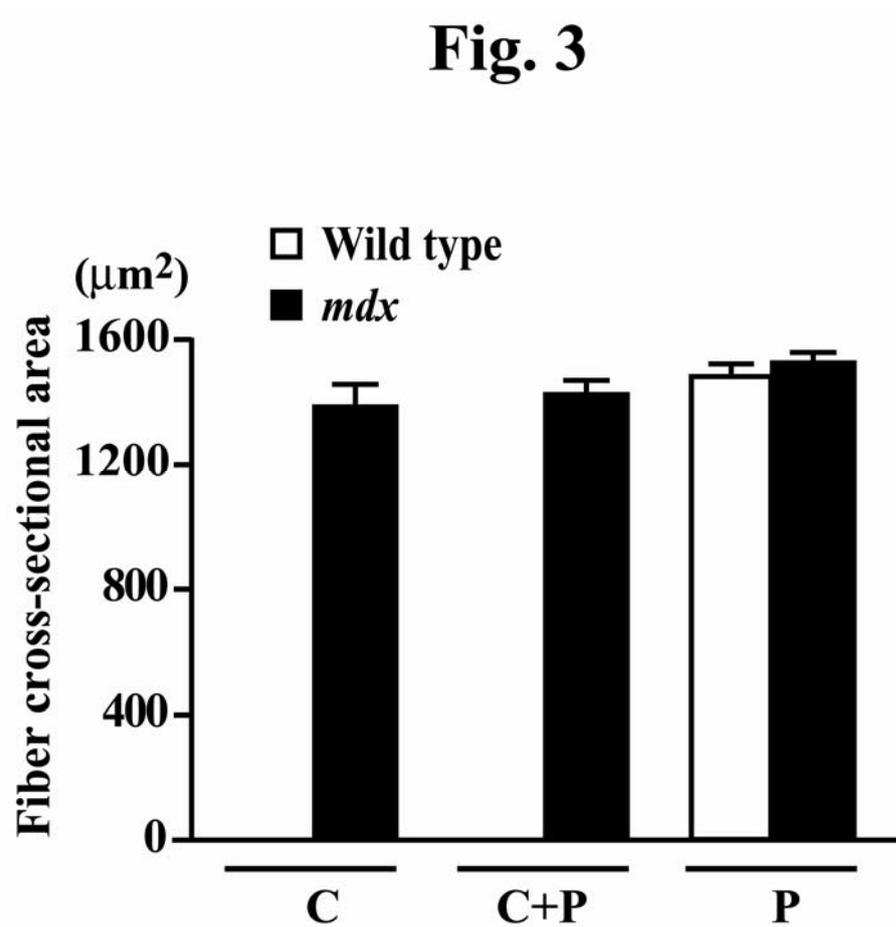


Fig. 4

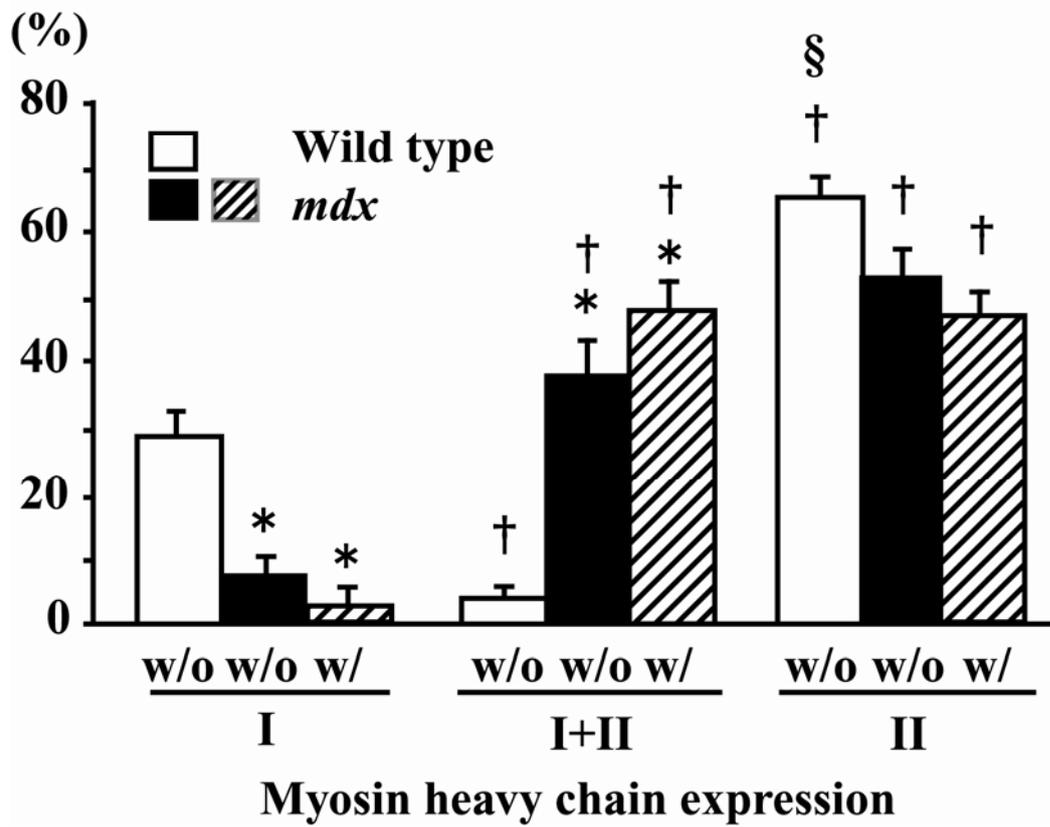


Fig. 5

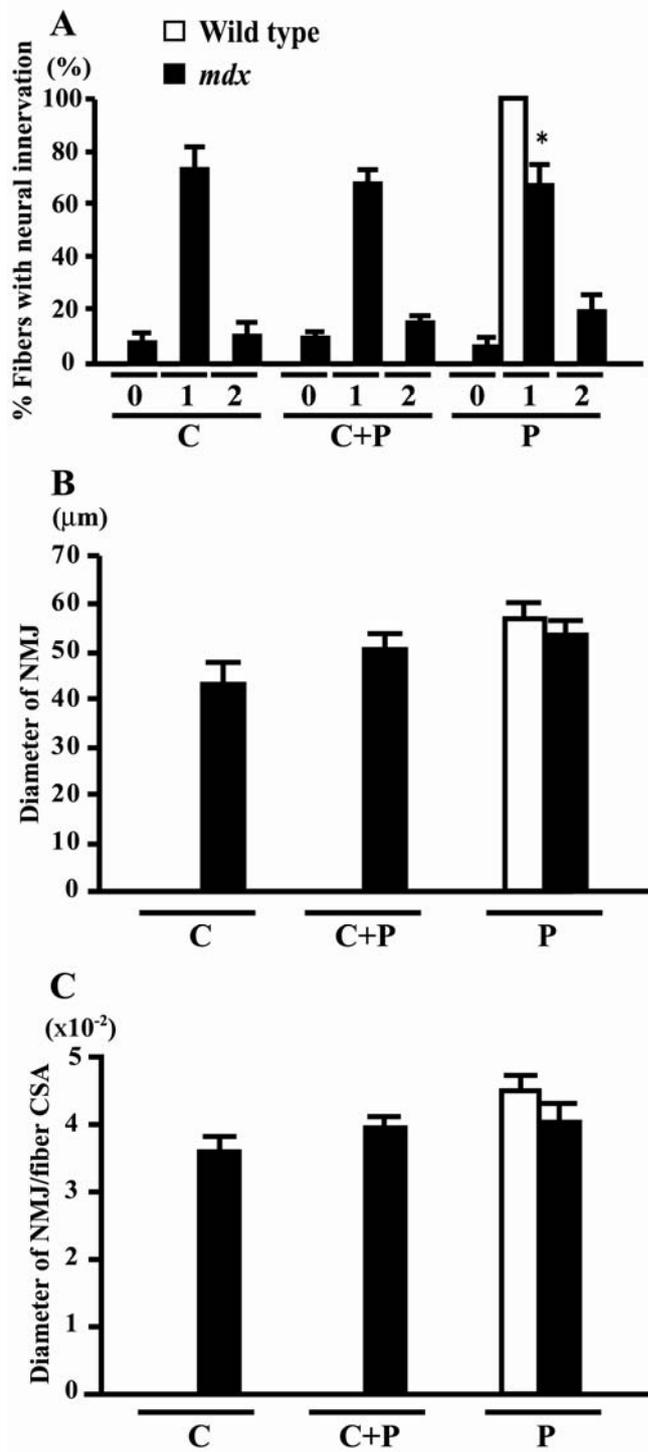


Fig. 6

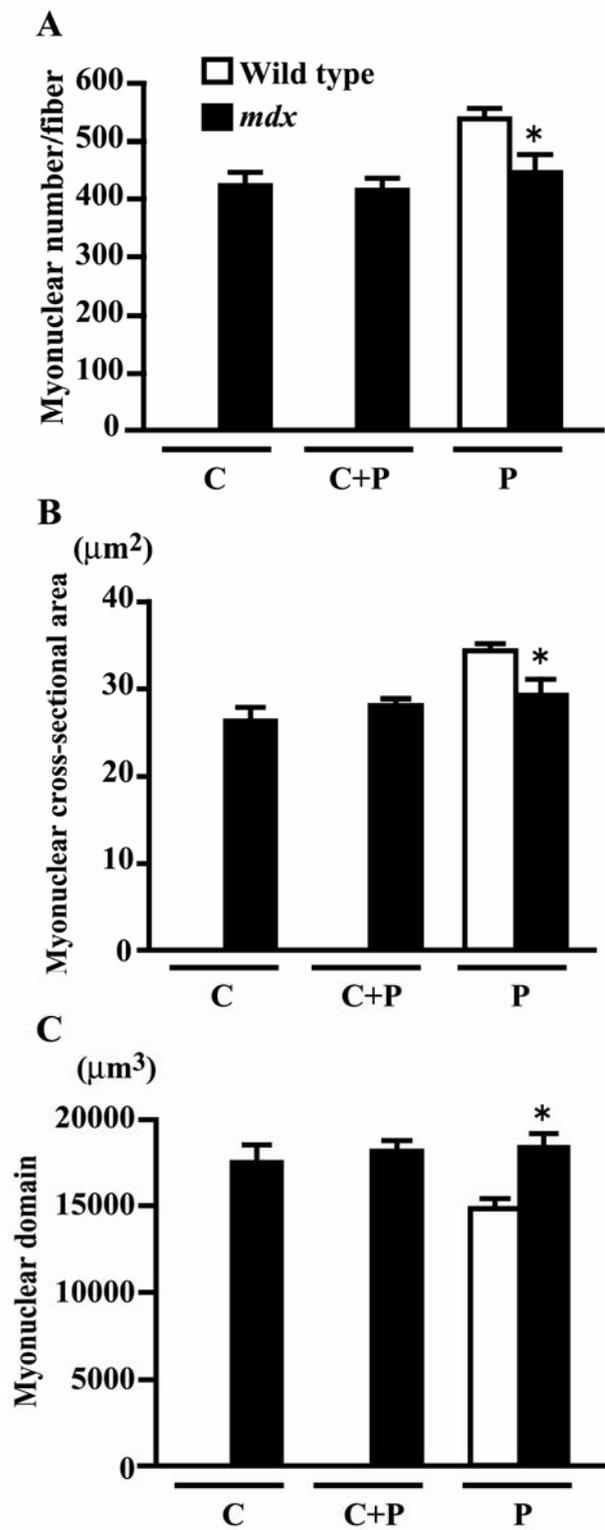
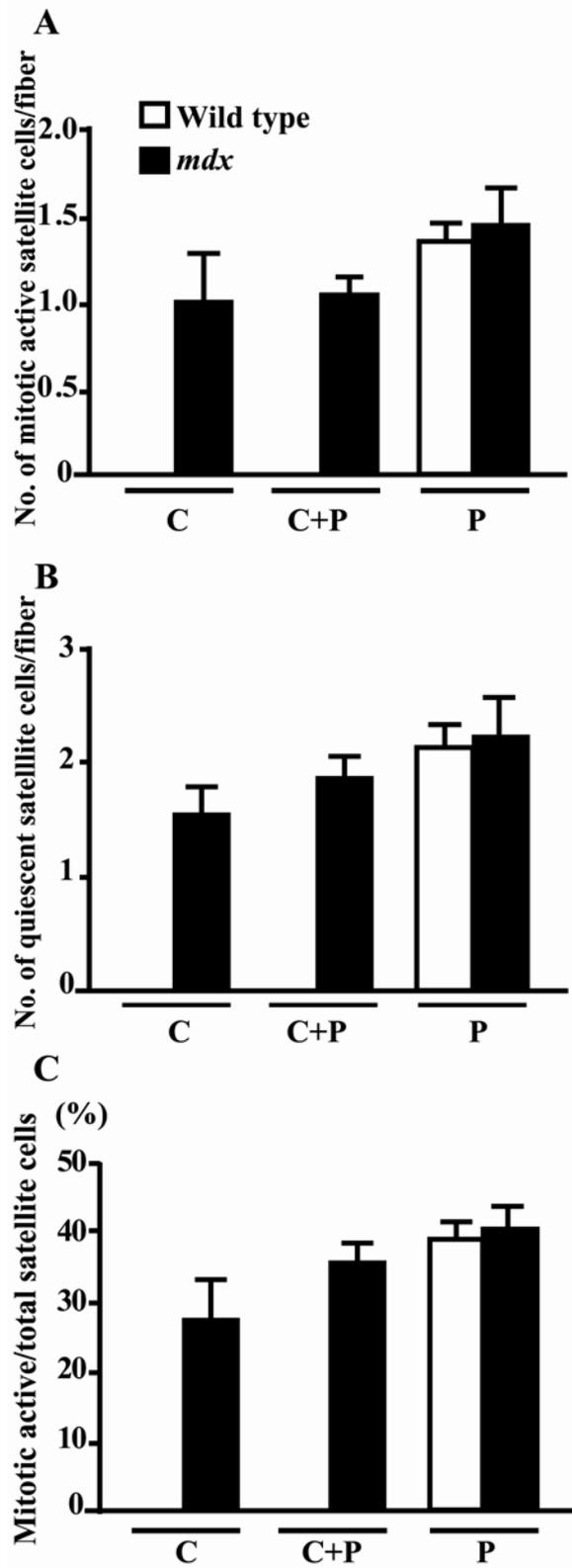
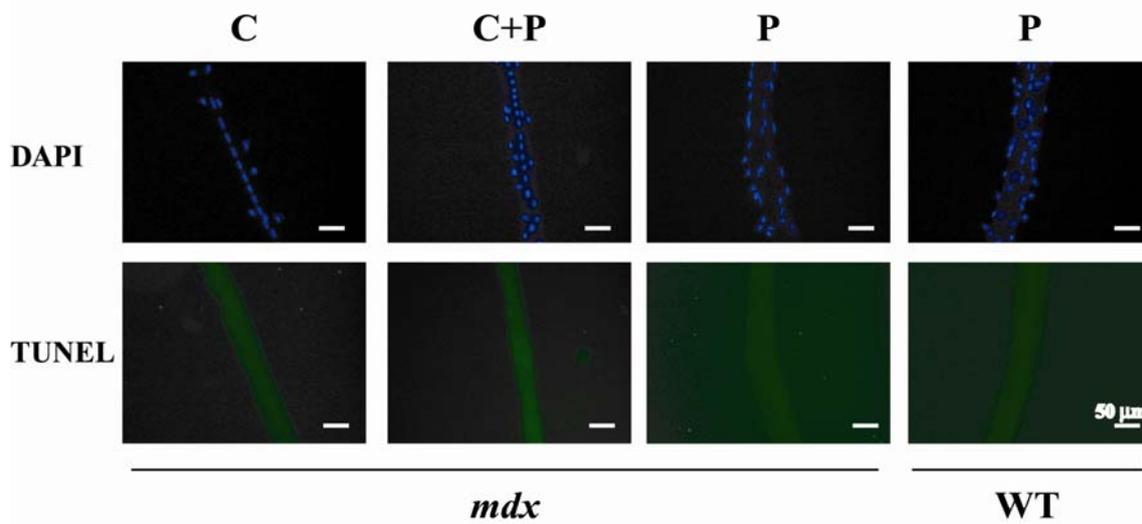
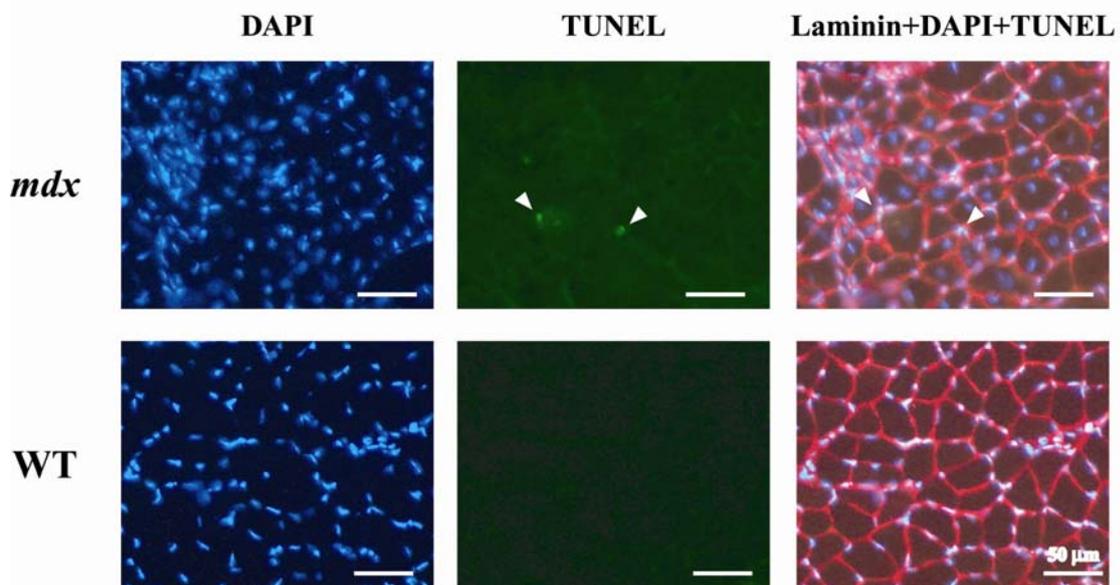
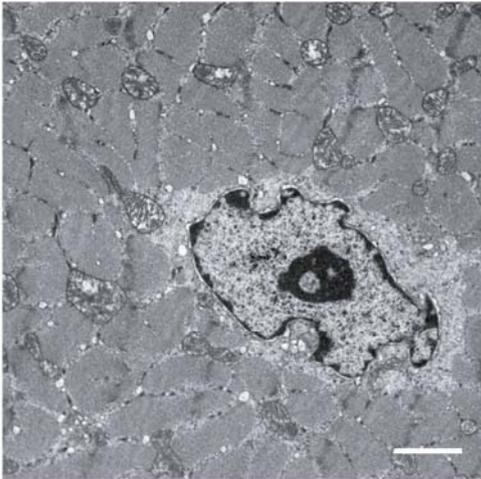
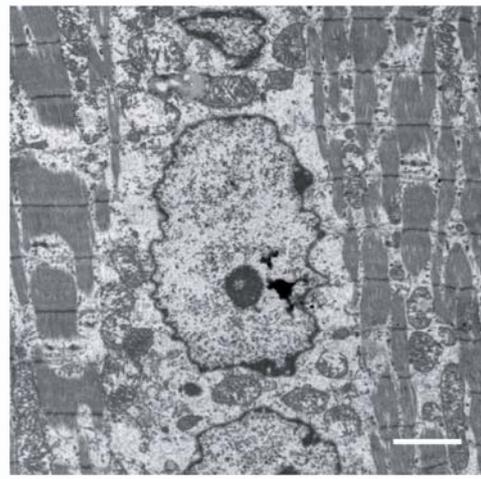


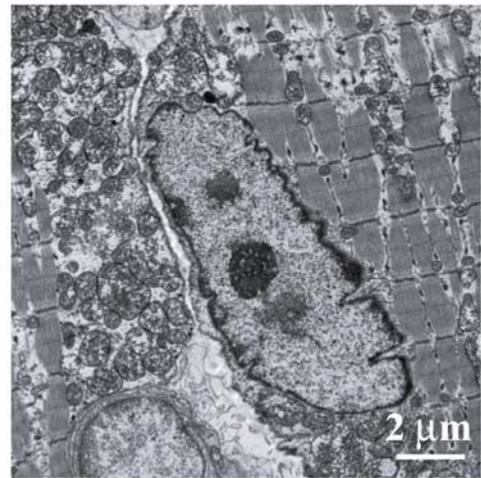
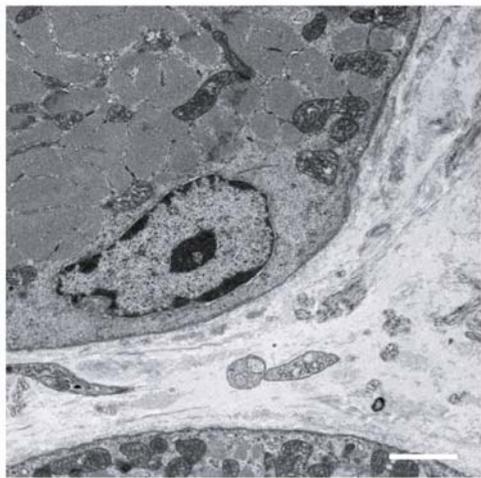
Fig. 7



**Fig. 8****Longitudinal analyses****Cross-sectional analyses**

**Fig. 9****Cross sectional analyses****Longitudinal analyses**

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**Central nuclei**

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**Peripheral nuclei**

## 研究業績目録

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## 講演

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## 受賞

1. 第11回研究奨励賞、日本航空環境医学会第54回大会、2008年11月14日。
2. Young Investigator Award 研究奨励賞、第16回日本運動生理学会大会、2008年8月3日。
3. Travel award ( \$ 250), American Society for Gravitational and Space Biology, 23th Annual Meeting, 25-28 Oct, 2007.
4. Special Student Poster Presentation and Young Researcher Sponsorship (Euro 350), 26<sup>th</sup> Annual International Gravitational Physiology Meeting, 26 June - 1 July, 2005.