Macrophage deficiency in osteopetrotic (op/op) mice inhibits activation of satellite cells and prevents hypertrophy in single soleus fibers

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Ohira T, Wang XD, Ito T, Kawano F, Goto K, Izawa T, Ohno H, Kizaki T, Ohira Y. Macrophage deficiency in osteopetrotic (op/op) mice inhibits activation of satellite cells and prevents hypertrophy in single soleus fibers. Am J Physiol Cell Physiol 308: C848–C855, 2015. First published March 18, 2015; doi:10.1152/ajpcell.00348.2014.—Effects of macrophage on the responses of soleus fiber size to hind limb unloading and reloading were studied in osteopetrotic homozygous (op/op) mice with inactivated mutation of macrophage colony-stimulating factor (M-CSF) gene and in wild-type (+/+ and heterozygous (+/op) mice. The basal levels of mitotically active and quiescent satellite cell (−46 and −39% vs. +/+ and −40 and −30% vs. +/op) and myonuclear number (−29% vs. +/+ and −28% vs. +/op) in fibers of op/op mice were significantly less than controls. Fiber length and sarcomere number in op/op were also less than +/+ (−22%) and +/op (−21%) mice. Similar trend was noted in fiber cross-sectional area (CSA, −15% vs. +/+; P = 0.06, and −14% vs. +/op; P = 0.07). The sizes of myonuclear domain, cytoplasmic volume per myonucleus, were identical in all types of mice. The CSA, length, and the whole number of sarcomeres, myonuclei, and mitotically active and quiescent satellite cells, as well as myonuclear domain, in single muscle fibers were decreased after 10 days of unloading in all types of mice, although all of these parameters in +/+ and +/op mice were increased toward the control values after 10 days of reloading. However, none of these levels in op/op mice were recovered. Data suggest that M-CSF and/or macrophages are important to activate satellite cells, which cause increase of myonuclear number during fiber hypertrophy. However, it is unclear why their responses to general growth and reloading after unloading are different.

myonuclei; muscle fiber size; osteopetrotic mice; satellite cells; unloading and reloading

SKELETAL MUSCLES ARE COMPOSED OF MULTINUCLEATED MUSCLE FIBERS AND HAVE AN EXTENSIVE CAPACITY FOR MORPHOLOGICAL AND FUNCTIONAL ADAPTATION. FOR EXAMPLE, CHRONIC UNLOADING OF MUSCLES BY BEDREST IN HUMANS (45, 46, 64) AND HIND LIMB SUSPENSION AND ACTUAL SPACEFLIGHT IN ANIMALS (6, 40–42, 47, 59) RESULT IN MUSCLE FIBER ATROPHY AND A SHIFT TOWARD A FASTER MYOSIN HEAVY CHAIN PROFILE, PARTICULARLY IN ANTIGRAVITY MUSCLES COMPARED PRIMARILY OF SLOW-TWITCH FIBERS, SUCH AS THE SOLEUS (6, 42, 45–47) AND ADDUCTOR LONGUS (41, 59). MUSCLE FIBER ATROPHY AND THE SHIFT TOWARD A FASTER MYOSIN HEAVY CHAIN PROFILE ARE REVERSED IN RESPONSE TO MUSCLE RELOADING (41, 45, 46, 59). THE MUSCULAR RESPONSE TO RELOADING APPEARS TO BE CLOSELY RELATED TO INTRAMUSCULAR STIMULI THAT ARE ACTIVATED BY MECHANICAL LOADING (20, 21, 41, 47, 59) AND/OR MUSCLE ACTIVATION WITH RELOADING (1, 12, 20, 22, 41).

Macrophage colony-stimulating factor (M-CSF, CSF-1) is a cytokine that influences hematopoietic stem cells to differentiate into macrophages and other cell types (54). DEFICIENCY OF MACROPHAGES HAS BEEN WELL-REPORTED IN OSTEOPETROTIC (OP/OP) MUTANT MICE WITH INACTIVATING MUTATION OF M-CSF GENE, WHICH RESULTS IN THE ABSENCE OF CERTAIN MACROPHAGE AND IN OSTEOPTOSIS, FOLLOWING A LACK OF OSTEOCLASTS (60, 63). Tidball and Wehling-Henricks (58) reported important roles of macrophages in the repair of muscle fiber membrane, regeneration, and regrowth after unloading-related atrophy in mouse soleus muscle. Membrane repair during 4 days of reloading after 10-day hind limb suspension did not occur in mice that underwent an intraperitoneal injection of anti-F4/80 IgG, which binds to macrophage surface antigens and decreases the number of macrophages. Furthermore, regeneration and regrowth of muscle, as well as satellite cell differentiation, were inhibited in these mice. However, macrophages also can promote the tissue damage (56, 57). These results apparently suggest the divergent roles of macrophages in degeneration and regeneration of muscle.

Muscle satellite cells are myogenic precursor cells that lie between the sarcolemma and the basal lamina of the myofiber (28), and myonuclear accretion occurs through the incorporation of satellite cells into the growing myofibers (33). Satellite cells have been shown to serve as a source of new myonuclei during regeneration after a muscle injury (31, 50, 52) and during functional overload (10, 15, 30, 53), although it was also reported that overload-related hypertrophy and/or regrowth of atrophied muscle were not inhibited by depletion of satellite cells following administration of tamoxifen (18, 29).

In contrast, hind limb unloading inhibited the growth-related increase of soleus muscle fiber size (23). This phenomenon was closely associated with the inhibited increases of satellite cells and myonuclear number. It was also reported that gravitational unloading downregulates the satellite cell mitotic activity (11, 35–37, 49). Furthermore, atrophy and regrowth of muscle fibers in soleus (59) and adductor longus (41) of Wistar Hannover rats were closely related to the number of satellite cells, which is also positively correlated with the distribution of
myonuclei. Unloading-related decrease or loss of satellite cells was noted in the central region of soleus fibers (59) and the caudal region of adductor longus (41), in which the sarcomere length was shortened in response to passive plantarflexion of ankle joints. Such decrease of satellite cell distribution was increased to the control level following stretching of muscle fibers during reloading in the cage. However, such region-specific responses of the distribution of satellite cells were not prominent in mouse soleus in the present study.

However, it is not known how the fiber size of soleus muscle in op/op mice responds to gravitational unloading and reloading. Therefore, the current study was performed to investigate the relationship between the distribution of satellite cells and myonuclei and cross-sectional area (CSA) in single fibers of soleus, sampled from tendon to tendon, in normal and op/op mice after hind limb suspension followed by ambulation recovery.

**METHODS**

**Experimental Design and Animal Care**

All experimental procedures were conducted in accordance with the Japanese Physiological Society and National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the Committee on Animal Care and Use at Osaka University.

Seventy-five 10- to 15-wk-old male wild-type (+/+) heterozygous (+/op) and homozygous (op/op) mice with mean ± SE body weight of 30.9 ± 1.2, 30.2 ± 1.4, and 26.3 ± 0.7 g (P < 0.05 vs. +/+ and +/op), respectively (n = 25 each; Laboratory Animal Center, Yamagata University School of Medicine), were used in the present study. The mice were separated randomly into preexperimental basal control (n = 15), cage control (n = 30) and hind limb-unloaded (n = 30) groups. Five control mice in each species were killed on the first day of the experiment and served as a preexperimental group. Hind limb suspension was performed in the hind limb-unloaded group as previously described (44). Initially, the tail of the mouse was washed and dried. Strips of sticky tape (~3 mm wide and 1.5 cm long) with good cushion were then placed longitudinally on the dorsal and ventral sides of the midportion of the mouse tail. These strips were anchored with tape wrapped loosely around the tail, so that the blood flow in the tail was not impeded. A string was inserted through the gap between the tail and the tape. The string was fastened to the roof of the cage (28 × 45 cm, and 20 cm height) at a height that allowed the forelimbs to support the weight, but prevented the hind limbs from touching the floor or the walls of the cage. The mice could reach food and water freely using their forelimbs. Two or three control mice were housed in identical cages as the unloaded mice. The amount of powdered diet (CE-2; Nihon CLEA), which was completely eaten within ~12 h, was supplied at ~10 A.M. daily (~3 g/day for each mouse). Water was supplied freely. The temperature and humidity in the animal room were maintained at 23°C and ~55%, respectively. The mice were also maintained under 12:12-h light-dark cycle conditions.

After 10 days, one-half of the mice in both cage control and unloaded groups [n = 15 (5/type) in each group] were killed with intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The hind limb-unloaded mice were anesthetized while the hind limbs remained unloaded to avoid any effects of acute reloading. The left soleus muscles were sampled for the single fiber analyses. Normal 10-day ambulatory cage recovery was allowed for the remaining mice in both the control and unloaded group [n = 15 (5/type) in each group].

**Muscle Preparation and Fiber Dissection**

The soleus muscles were cleaned of excess fat and connective tissues immediately after removal. The muscles were stored in a cellbanker (Nihon Zenyaku, Tokyo, Japan) at −80°C until analyzed, as was reported previously (41, 55, 59). 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 20 μM 5′-bromo-2′-deoxyuridine (BrdU; Becton-Dickinson, Mountain View, CA), 0.2% type I collagenase, 1% antibiotics, and 10% newborn calf serum for 4 h at 35°C to digest the collagen. The working solution of type I collagenase was gel purified to remove the clostripain, which supposedly strips the basal lamina of the fibers (4). The collagenase-treated muscles were fixed in 4% buffered formaldehyde for 10 min. Entire single muscle fibers (Fig. 1), sampled from tendon to tendon, were isolated with the use of fine needles. The fibers were collected carefully using pipettes to avoid any damage. The collected fibers were separated into three tubes (30 fibers in each tube; tubes 1-3) and immersed in DMEM containing 10% newborn calf serum.

**Immunohistochemistry and Nuclear Labeling**

M-cadherin and BrdU were labeled immunohistochemically to stain the quiescent and mitotic active satellite cells, respectively, in the single fibers, as described previously (34, 36). The collected single fibers in the tubes were permeabilized with 1% Triton X-100 diluted with phosphate-buffered saline (PBS) for 10 min. Subsequently, the single fibers in the tubes were blocked with 10% normal goat serum diluted with PBS for 15 min.

Fibers in tube 1 were incubated overnight with primary polyclonal antibody specific to M-cadherin (Santa Cruz Biotechnology) diluted at 1:20 with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin (BSA). Overnight incubation with primary monoclonal antibody specific to BrdU diluted at 1:20 with PBS containing 0.5% Tween 20 and 0.5% BSA was performed for fibers in tube 2. Furthermore, the fibers in tube 3 were incubated overnight with both antibodies specific to M-cadherin and BrdU. The M-cadherin-positive (quiescent) satellite cells were detected with goat anti-rabbit IgG conjugated to either fluorescein isothiocyanate (tube 1; Chemicon International) or rhodamine (tube 3; Chemicon International) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA, respectively. The BrdU-positive nuclei (mitotic active) satellite cells were detected with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (tubes 2 and 3; Jackson Immunoresearch) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA, respectively. The BrdU-positive nuclei (mitotic active) satellite cells were detected with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (tubes 2 and 3; Jackson Immunoresearch) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA, respectively. The BrdU-positive nuclei (mitotic active) satellite cells were detected with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (tubes 2 and 3; Jackson Immunoresearch) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA, respectively. The BrdU-positive nuclei (mitotic active) satellite cells were detected with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (tubes 2 and 3; Jackson Immunoresearch) diluted at 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA, respectively. The BrdU-positive nuclei (mitotic active) satellite cells were detected with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (tubes 2 and 3; Jackson Immunoresearch).
Fig. 2. Length (axis on left) and total sarcomere number (axis on right) in single muscle fibers, sampled from tendon to tendon, before the initiation of experiment (pre-exp), at the end of 10 days of cage housing or unloading (R + 0), and of ambulation recovery (R + 10) in macrophage colony-stimulating factor-deficient osteopetrotic (+/lop), +/+ and heterozygous (+/lop) mice. Means ± SE; n = 5 mice in each bar. P < 0.05 vs. pre-exp (†), respective cage control at R + 0 (‡), respective unloaded group at R + 0 (¶), and +/+ (§) and +/lop (¶) in cage control and unloaded group at each stage.

Confocal Microscopy

A FV-300 confocal microscope with an argon laser (488 nm of peak wavelength) and a He-Ne laser (543 nm of peak wavelength) (Olympus) was used to analyze the length and CSA of fibers, and the numbers of the sarcomeres, myonuclei, and quiescent and mitotically active satellite cells.

The total number of myonuclei labeled by PI was counted under a microscope. The numbers of M-cadherin-labeled and BrdU-labeled quiescent and mitotically active satellite cells, respectively. The total number of sarcomeres in whole muscle fibers was positively correlated with that of fiber length. The baseline total sarcomere number in +/+ and +/lop mice was ~2,300/fiber and that in oplop mice was ~1,800 (P < 0.05).

Reorganization of fibers and sarcomeres was induced following 10 days of unloading and reloading. The length of fibers and sarcomere number in +/+ (~9%), +/lop (~14%), and oplop (~9%) mice were decreased in response to unloading (P < 0.05, Fig. 2) and were also less than those in the respective age-matched cage controls (P < 0.05). Following 10 days of reloading, both fiber length and sarcomere number in +/+ and +/lop mice returned to the control levels (P < 0.05).

However, no recoveries were noted in oplop mice and were significantly less than the +/+ and +/lop mice, which were unloaded previously (P < 0.05).

The mean baseline fiber CSAs in +/+ and +/lop mice at the beginning of experiment were 1,230 and 1,211 μm², respectively (Fig. 3). Fiber CSA in oplop mice (1,041 μm²) was slightly less than +/+ (P = 0.06) and +/lop (P = 0.07) mice. The fiber CSAs in the cage controls were maintained throughout the experimental period. Significant atrophy vs. preexperimental levels and the age-matched cage controls were induced after 10 days of unloading (~38.5 and ~33.3% in +/+ and ~31.5% in +/lop, and ~34.6 and ~34.7% in oplop mice, respectively). The fiber sizes in +/+ and +/lop mice increased toward the levels in cage controls in response to reloading [+32.5 and +42.3% vs. the end of 10 days of unloading (R + 0), P < 0.05], even though the full recoveries were not noted. However, the atrophied fibers in oplop mice remained unchanged and were still significantly less than the age-matched cage controls (~36.4, ~33.9, and ~26.1% vs. +/+ and +/lop, and oplop mice, respectively) and the age-matched/unloaded +/+ and +/lop mice (~22.6 and ~26.1%).

Distribution of Myonuclei

The total myonuclear numbers in single muscle fibers, sampled from tendon to tendon, of +/+ and +/lop cage control mice were identical, (~560–570) and remained constant throughout the experimental period (Fig. 4), but the total myonuclear number in oplop mice was significantly less than +/+ and +/lop mice at any stages (~28.8 and ~27.0%, ~24.7 and ~18.5%, and ~19.2 and ~18.6% before experiment and at R + 0 and ambulation recovery (R + 10), respectively).

In response to 10 days of unloading, significant decrease vs. the preexperimental baselines, and also the age-matched cage
controls, were induced in all types of mice (−32.9 and −32.9% in +/+ and −28.9% in +/op, and −23.1 and −27.3% in op/op mice, respectively, Fig. 4). The myonuclear numbers in +/+ and +/op mice increased toward the levels in cage controls in response to reloading (+21.4 and +36.5% vs. R + 0, P < 0.05). However, the numbers of myonuclei in op/op mice remained unchanged, even after 10 days of reloading, and were still significantly less than the age-matched unloaded cage controls, was induced in all types of mice (−32.9 and −32.9% in +/+ and −28.9% in +/op, and −23.1 and −27.3% in op/op mice, respectively, Fig. 4). The myonuclear numbers in +/+ and +/op mice increased toward the levels in cage controls in response to reloading (+21.4 and +36.5% vs. R + 0, P < 0.05). However, the numbers of myonuclei in op/op mice remained unchanged, even after 10 days of reloading, and were still significantly less than the age-matched cage controls (−41.8, −41.4, and −28.1% vs. +/+ and +/op, respectively, and the age-matched/unloaded +/+ and +/op mice (−31.1 and −37.4%).

**Myonuclear Domain**

The baseline sizes of myonuclear domain (cytoplasmic volume/myonucleus) in all types of mice were identical (−13,000−14,500 μm³) (Fig. 5). Although the myonuclear domains in the cage controls were stable throughout the experimental period, significant decreases were induced after unloading (−25.0% in +/+ , −24.5% in +/op, and −23.4% in op/op mice), indicating that fiber atrophy advanced more than the decrease of myonuclear number. The myonuclear domain sizes in both +/+ and +/op mice were normalized after 10-day reloading (P < 0.05), but the myonuclear domain size of op/op mice did not recover and was still less than the previously unloaded age-matched +/+ and +/op mice (P < 0.05).

**Distribution of Satellite Cells**

The total number of mitotically active satellite cells in single muscle fibers of +/+ and +/op cage control mice was similar and remained constant throughout the experimental period (Fig. 6), but the total number of mitotically active satellite cells in control op/op mice was significantly less than +/+ and +/op mice at any stages (−46.1 and −40.2%, −23.3 and −24.0%, and −19.0 and −14.6% before experiment and at R + 0 and R + 10, respectively).

In response to 10 days of unloading, significant decrease vs. the preexperimential baselines, and also the age-matched cage controls, was induced in all types of mice (−61.5 and −58.3% in +/+ , −58.2 and −59.5% in +/op, and −40.0 and −54.4% in op/op mice, Fig. 6). Statistical significance between three unloaded groups at R + 0 was not observed. The total number of mitotically active satellite cells in +/+ and +/op, but not in op/op mice, increased toward the levels in cage controls in response to reloading (+80.0 and +128.6% vs. R + 0, P < 0.05). The mean total number in op/op mice was still significantly less than the age-matched cage controls (−57.8, −55.5, and −47.9% vs. +/+ , +/op, and op/op mice, respectively) and the age-matched/unloaded +/+ and +/op mice (−45.6 and −56.3%).

The total number of quiescent satellite cells in single muscle fibers of +/+ and +/op cage control mice was similar and remained constant throughout the experimental period (Fig. 7), but the total number of quiescent satellite cells in op/op mice
Although hind limb reloading increased fiber size and the number of myonuclei and satellite cells in both +/+ and +/lop mice, neither fiber size nor satellite cells were increased in olop mice. The data suggested that the inhibited upregulation of mitotically active satellite cells in response to reloading might be the main limiting factor for the blunted reloading-related recovery of fiber CSA, as well as the myonuclear distribution in olop mice.

Specific Phenomena in the Muscle Fibers of olop Mice

Deficiency of macrophages has been well-reported in olop mice due to the lack of functional M-CSF (38, 60, 62, 63). Cecchini et al. (7) reported that macrophage marker antigen F4/80+ cell densities in striated muscle of olop mice at the age of 3 mo were not detectable. Furthermore, Begg et al. (3) analyzed the time course changes of the structure, cellularity, hematopoietic progenitor cell, and macrophage content, and osteoclast activity in the hematopoietic organs of olop mice, and they showed that the percentage of olop mononuclear phagocytes, defined by F4/80 antigen expression, progressively increased to normal levels by 35 wk of age. The macrophage number was not analyzed in the present study, but the results of these studies suggest that macrophage number in muscles of olop mice with the age of 10–15 wk at the beginning of experiment in the present study may be lower than controls.

Growth-related gain of body weight is also inhibited in olop mice (2, 9, 61). It was also reported that growth-associated increase of fiber diameter in the anterior belly digastric muscle of olop mice tended to be less vs. that in normal littermate mice fed the same granulated diet (24). The baseline levels of most of the parameters (fiber length, the number of sarcomeres, myonuclei, and mitotically active and quiescent satellite cells in entire single muscle fibers) were less in olop mice than +/+ and +/lop mice in the present study. Similar trend was also noted in fiber CSA (P > 0.05). These phenomena were generally correlated with the growth inhibition indicated by body weight. However, the mean levels of myonuclear domain size, cytoplasmic volume per myonucleus, were identical in all types of mice (Fig. 5). Thus, it is indicated that the inhibited longitudinal and cross-sectional growth of muscle fiber mass was positively correlated to the number of

![Fig. 7. A: representative micrographs showing a single muscle fiber stained with M-cadherin and PI (red) for quiescent satellite cells and nuclei, respectively. M-cadherin-positive cells are shown by arrows. B: total number of quiescent satellite cells in whole single muscle fibers. Means ± SE; n = 5 in each bar. P < 0.05 vs. pre-exp (*), respective cage control at R + 0 (†), respective unloaded group at R + 0 (¶), and +/+ (‡) and +/lop (§) in cage control and unloaded group at each stage. See Fig. 2 for the abbreviations.](http://ajpcell.physiology.org/)

**DISCUSSION**

Responses of the distribution of satellite cells and myonuclei and mass in single fibers of soleus, sampled from tendon to tendon, in +/+ , +/lop, and olop mice to hind limb suspension followed by ambulation recovery were studied. Significant unloading-related decrease of fiber size and the number of myonuclei and satellite cells was noted in all types of mice.

![Fig. 8. The percent distribution of mitotically active satellite cells in whole single muscle fibers. Means ± SE; n = 5 in each bar. See Fig. 2 for the abbreviations.](http://ajpcell.physiology.org/)
myonuclei, probably not the function of each nucleus. The lower number of satellite cells in the whole muscle fibers in op/op mice was also proportional to the short length of fibers. Thus, it is speculated that lower distribution of satellite cells and myonuclei, which inhibit protein synthesis, play some important role(s). However, it is not clear how the inhibited distribution of M-CSF, macrophages, or both is involved for the limited growth of muscle fibers, since the levels of these parameters were not determined in the present study.

**Responses to Unloading**

**Morphological properties.** Unloading-related fiber atrophy was noted, as was reported elsewhere (6, 40–42, 47, 59). Exposure to microgravity or hind limb unloading of rodents causes passive shortening of soleus muscle due to ankle plantarflexion (20, 39, 59), which reduces the mechanical stress (20) and neural activity (19, 20, 39, 43). Even though it is well-reported that muscles composed predominantly of slow-twitch fibers are more susceptible to unloading-related atrophy (6, 41, 42, 45–47, 59), atrophy in soleus muscle of mice composed of fast-twitch fibers mainly was also observed in all types of mice, as was reported elsewhere (40, 48, 55). The unloading-related decreases in fiber length and sarcomere number may be associated with the remodeling of muscle fibers and sarcomeres, as was reported before (20, 59).

**Myonuclei and satellite cells.** The numbers of myonuclei and both mitotically active and quiescent satellite cells in entire single muscle fibers (Figs. 3, 5, and 6) in all types of mice were decreased following 10 days of unloading and agree with the previous reports (41, 59). Such responses may be closely related to decreased mechanical stress caused by ankle plantarflexion-related passive shortening of muscle fibers, as was reported elsewhere (20, 59).

**Responses to Reloading**

**Morphological properties.** In +/+ and +/op mice, the unloading-related decreases in the CSA and length of fibers and sarcomere number were normalized following 10 days of reloading, as was reported before (59). However, these parameters in op/op mice remained unchanged. To our knowledge, this is a unique phenomenon observed in skeletal muscle of op/op mice. It was reported that suppression of macrophage function by intraperitoneal injection of anti-M-CSF receptor IgG and anti-CD4 IgG impaired skeletal muscle regeneration, accompanied with severe fibrosis, in mice (51). It was also reported that slower regeneration was noted due to delayed infiltration of macrophages into the soleus muscle with damage caused by injection of cardiotxin, if the hind limbs were unloaded in mice (26). Matsuba et al. (27) also observed that regeneration of atrophied soleus muscle is inhibited due to hind limb unloading in mice. Tidball and Wehling-Henricks (58) studied the role of macrophages in the recovery of atrophied soleus muscle in mice. Macrophages were depleted by a series of intraperitoneal injections of anti-F4/80 IgG. Regeneration and growth of soleus during increased use were inhibited in these mice.

In addition, intramuscular injection of M-CSF into atrophied mouse soleus caused a 1.6-fold increase in macrophage density and faster recovery of fiber size and force after 7 days of reloading (13). Treatment with M-CSF also caused a shift of bone marrow-derived macrophage phenotype toward a wound-healing phenotype (anti-inflammatory macrophages), thereby promoting the release of myogenic factors that decrease the activity of the ubiquitin-proteasome pathway, which suppressed protein degradation in growing myotubes (13). Furthermore, it was reported that insulin-like growth factor-I, released by macrophages, plays important roles during the recovery of muscle from atrophy (14). Keeling et al. (25) further reported that M-CSF-induced macrophage differentiation stimulated the increase of nuclear number and myotube elongation in C2C12 cells. These results clearly indicate the beneficial effects of macrophages during the response to muscle injury, although detrimental actions of macrophages are also reported (5, 56, 57), suggesting the divergent roles of macrophages.

Morioka et al. (32) reported that functional overloading by amputation of synergists, gastrocnemius and plantaralis, facilitated the regeneration of soleus muscle injured by injection of cardiotxin in mice. These results may suggest a beneficial role of inflammation reaction caused by injury or acute increase of mechanical stress in the promotion of muscle regeneration. Therefore, we also studied recently how the immune system works in muscle regeneration (16). Regeneration of mouse tibialis anterior muscle with injury caused by intramuscular injection of cardiotxin was accelerated by administration of rat anti-mouse interleukin-6 receptor antibody (MR16-1). It was suggested that MR16-1, which was found primarily in infiltrated macrophages in the damaged region, might facilitate muscle regeneration via immune modulation.

**Distribution of myonuclei and satellite cells.** The unloading-related decreases of the number of myonuclei (Fig. 3) and satellite cells in +/+ and +/op mice (Figs. 5 and 6) were reversible in response to reloading. However, such recovery was not seen in op/op mice. Suppression of macrophages, which impaired muscle regeneration, delayed the proliferation and differentiation of muscle satellite cells in vivo (51). Depletion of macrophages, due to intraperitoneal injection of anti-F4/80 IgG, also inhibited satellite cell differentiation (indicated by reduction in MyoD-expressing satellite cells), and regeneration of fibers (indicated by central nucleation) and regrowth of atrophied soleus were not observed in mice, as was stated above (58).

However, Jackson et al. (18) reported that myonuclear number in transgenic Pax7-DTA mice with depletion of satellite cells (>90%) following administration of tamoxifen did not change in response to hind limb unloading and reloading, but atrophy and regrowth of soleus were induced following 14 days of unloading and reloading. Therefore, muscle atrophy caused by hind limb unloading may be recovered because the basal myonuclear number was maintained. However, the unloading-related decreases of myonuclear number and muscle fiber size in op/op mice were not recovered following reloading in the present study. These phenomena could be one of the causes for the discrepancy between these results. It was speculated that the different effects on regrowth of atrophied muscle between op/op mice and Pax7-DTA mice with depleted satellite cells following administration of tamoxifen may be related to some unknown factor(s) that may be involved in the accretion of myonuclei, rather than the number of satellite cells, caused by either M-CSF mutation or tamoxifen treatment. However, it is still unclear why the regrowth of atrophied

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muscle was induced in satellite cell-depleted mice following unloading, even though the myonuclear distribution was maintained.

It is further unclear why the effects of tamoxifen treatment on induction of compensatory hypertrophy in response to the ablation of synergist muscles are not constant. The same group as Jackson et al. (18) studied the responses of muscle fiber size to functional unloading in transgenic Pax7-DTA mice with depletion of satellite cells following administration of tamoxifen (15, 29). In response to 2-wk unloading, hypertrophy was induced in plantaris, although myonuclear number did not change (29). However, the hypertrophy of plantaris was attenuated following 8 wk of unloading (15).

In conclusion, responses of morphological properties and the number of myonuclei and satellite cells in single fibers of soleus muscle to gravitational unloading and reloading were studied in op/op, +/+ and +/op mice. Changes in muscle fiber size were closely related to the number of satellite cells and myonuclei. It was indicated that such phenomena were closely related to the limited number of myonuclei, probably not the function of each myonucleus, because the myonuclear domain size in op/op mice was identical to the levels in +/+ and +/op mice. The unloading-related fiber atrophy was closely related to the decrease in the number of satellite cells and myonuclei in +/+ and +/op mice, suggesting that these responses were induced irrespective of M-CSF deficiency. However, the regrowth of atrophied muscle fibers was completely inhibited only in op/op mice, being associated with the irreversible responses of satellite cell and myonuclear distribution. Important roles of M-CSF, macrophages, or both in the regulation of muscle fiber size were suggested especially during the process of regrowth from atrophy, although the effects on the general growth and development were minor.

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DISCLOSURES
The funders had no roles in the study design, data collection and analyses, decision to publish, or preparation of the manuscript. Authors have no financial interest related to this project.

AUTHOR CONTRIBUTIONS

REFERENCES
ROLE(S) OF SATELLITE CELLS IN FIBER SIZE REGULATION

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