 Resident stem cells are not required for exercise-induced fiber-type switching and angiogenesis but are necessary for activity-dependent muscle growth

Ping Li, Takayuki Akimoto, Mei Zhang, R. Sanders Williams, and Zhen Yan

Department of Medicine, Duke University Medical Center, Durham, North Carolina

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Li, Ping, Takayuki Akimoto, Mei Zhang, R. Sanders Williams, and Zhen Yan. Resident stem cells are not required for exercise-induced fiber-type switching and angiogenesis but are necessary for activity-dependent muscle growth. Am J Physiol Cell Physiol 290: C1461–C1468, 2006. First published January 11, 2006; doi:10.1152/ajpcell.00532.2005.—Skeletal muscle undergoes active remodeling in response to endurance exercise training, and the underlying mechanisms of this remodeling remain to be defined fully. We have recently obtained evidence that voluntary running induces cell cycle gene expression and cell proliferation in mouse plantaris muscle that undergo fast-to-slow fiber-type switching and angiogenesis after long-term exercise. To ascertain the functional role of cell proliferation in skeletal muscle adaptation, we performed in vivo 5-bromo-2′-deoxyuridine (BrdU) pulse labeling (a single intraperitoneal injection), which demonstrated a phasic increase (5- to 10-fold) in BrdU-positive cells in plantaris muscle between days 3 and 14 during 4 wk of voluntary running. Daily intraperitoneal injection of BrdU for 4 wk labeled 2.0% and 15.4% of the nuclei in plantaris muscle in sedentary and trained mice, respectively, and revealed the myogenic and angiogenic fates of the majority of proliferative cells. Ablation of resident stem cell activity by X-ray irradiation did not prevent voluntary running-induced increases of type IIa myofibers and CD31-positive endothelial cells but completely blocked the increase in muscle mass. These findings suggest that resident stem cell proliferation is not required for exercise-induced type IIb-to-IIa fiber-type switching and angiogenesis but is required for activity-dependent muscle growth. The origin of the angiogenic cells in this physiological exercise model remains to be determined.

endurance exercise; adaptation

ADULT SKELETAL MUSCLE FIBERS are terminally differentiated and no longer have the ability to proliferate, but they contain a pool of myogenic stem cells, also called satellite cells, that reside between the basal lamina and sarcolemma of myofibers (19). Upon stimulation, such as injury and increased work overload, satellite cells are activated and undergo proliferation and differentiation to participate in muscle regeneration and hypertrophy (1, 28, 34). It is well known that endurance exercise, defined as repetitive, prolonged exercise of submaximal intensity, induces phenotypic changes in skeletal muscles that lead to enhanced exercise capacity and improved metabolic homeostasis (6, 32). Recent advances in molecular biology have significantly improved the understanding of exercise-induced skeletal muscle adaptation, as reported in previous studies of the skeletal muscle signaling-transcription network (2, 7, 17, 22, 30, 33). However, little is known about cellular processes such as satellite cell proliferation and differentiation in skeletal muscle adaptation induced by endurance exercise.

Considering that satellite cell and other types of stem cells reside in adult skeletal muscle and could migrate from other peripheral tissues (5, 16, 19, 23), one might expect that these stem cells participate in skeletal muscle adaptation in response to exercise training if they undergo proliferation and differentiation. Indeed, increased satellite cell (muscle precursor) proliferation has been reported in various animal models of endurance exercise, including treadmill running, voluntary running, and chronic motor nerve stimulation (12, 14, 20, 25), as well as under conditions in which satellite cell proliferation induced by muscle injury and fiber-type transformation evoked by motor nerve stimulation was enhanced (18, 29). Accordingly, it has been hypothesized that myogenic stem cell proliferation is directly involved in or prerequisite to contractile activity-induced fiber-type switching. We recently obtained evidence that voluntary running stimulates cell cycle gene expression and promotes cell proliferation in mouse skeletal muscle (8) before the onset of fiber-type switching and angiogenesis (2, 3, 31). These findings prompted us to ascertain the functional role of cell proliferation in skeletal muscle adaptation in this physiological exercise model.

In this study, we hypothesized that resident stem cell proliferation induced by voluntary running plays an essential role in fast-to-slow fiber-type switching, enhanced angiogenesis, and/or increased muscle mass. We first characterized temporal features of cell proliferation in mouse skeletal muscle during voluntary running using 5-bromo-2′-deoxyuridine (BrdU) labeling in vivo. We then determined the functional fates of the proliferative cells using long-term BrdU labeling. Finally, we used X-ray irradiation to ablate resident stem cell activity to ascertain the functional role of resident stem cell proliferation in skeletal muscle adaptation. In contrast to our hypothesis, our results have shown that only changes in muscle mass, not fiber-type switching or angiogenesis, are dependent on resident stem cell proliferation.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (8 wk of age) were obtained from The Jackson Laboratory (Bar Harbor, ME), housed in temperature-controlled (21°C) quarters, maintained on a 12:12-h light-dark cycle, and provided with water and chow ad libitum. All procedures involving these animals conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the Duke University Animal Use Committee.

Voluntary running model. Mice were randomly assigned to sedentary control and running groups. Mice in the running group were housed individually in cages equipped with locked running wheels for 3 days for acclimatization, and they were allowed to run by unlocking...
the wheels at the beginning of a dark cycle for 1, 3, 7, 14, or 28 days, followed by a 24-h resting period (n = 7 for each time point) as previously described (4, 31). Sedentary mice (n = 7) without access to a running wheel were used as controls. In the long-term BrdU labeling experiment, mice were randomly divided into sedentary (Sed; n = 7) and exercise groups (Ex; n = 7), and mice in the exercise group were allowed to run voluntarily for 4 wk. After single-hindlimb X-ray irradiation, mice were allowed to recover for 3 days before voluntary running for 2 or 4 wk (n = 5 for each time point). A separate group of irradiated mice with no running wheel access were used as sedentary controls (n = 5). X-ray irradiation of a single hindlimb did not have a significant impact on running activity (data not shown).

BrdU labeling in vivo. To pulse label proliferative cells in skeletal muscles, the mice were allowed to run voluntarily for 1, 3, 7, 14, or 28 days. A single dose of BrdU (500 mg/kg body wt) was injected intraperitoneally immediately after the last bout of running activity, followed by a 24-h resting period (by locking running wheels) before samples were harvested. For long-term labeling, the mice were allowed to run voluntarily for 28 days, and BrdU (500 mg/kg body wt) was injected intraperitoneally daily. Sedentary mice injected with BrdU were used as controls in both pulse-labeling and long-term labeling experiments.

Ablation of resident stem cells in hindlimb muscles by X-ray irradiation. Mice were anesthetized with pentobarbital sodium, and their right hindlimbs were exposed to a single dose of 2,500-rad ionizing irradiation for 23 min using an XRAD-320 orthovoltage unit (Precision X-ray, East Haven, CT). The rest of the animal was shielded from the radioactive source using a 2-cm-thick lead attenuator.

Immunohistochemistry. Muscles were harvested, saturated in 30% sucrose-PBS for ~2 h, placed into optimal cutting temperature (OCT) tissue-freezing medium (Miles Pharmaceuticals, West Haven, CT), and frozen in liquid nitrogen-cooled isopentane. Frozen sections (6 μm) were cut using a cryostat and placed onto microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Before being stained, the sections were fixed in 4% paraformaldehyde-PBS for 10 min at 4°C, permeabilized with 0.3% Triton X-100-PBS for 10 min at 4°C, and then blocked in 5% normal goat serum (NGS)-PBS for 30 min at room temperature, followed by incubation with a different primary antibody in 5% NGS-PBS at 4°C overnight. Rat anti-CD-31 antibody (1:25 dilution; Serotec, Raleigh, NC) and rat anti-laminin antibody (1:100 dilution; Chemicon International, Temecula, CA) were used for endothelial cells and basal lamina, respectively. Three consecutive washes with PBS for 5 min each were followed by sequential incubation with secondary antibodies (rhodamine or FITC conjugated, 1:25 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. The sections were then fixed in 4% paraformaldehyde-PBS for 5 min, and we continued to stain them for BrdU. In brief, each slice was treated in 1 N HCl at 37°C for 1 h, followed by incubation with either unconjugated (1:25 dilution) or Alexa Fluor-conjugated mouse anti-BrdU primary antibody (1:25 dilution; Molecular Probes, Eugene, OR) overnight at 4°C. If unconjugated primary antibody was used, we proceeded to use secondary antibody as mentioned above. Immunofluorescence was also performed as described previously (31) for type Ila plantaris muscle fibers. Images were captured under a confocal microscope (Olympus, Melville, NY) and further analyzed using Scion Image software (Scion, Frederick, MD). The percentage area comprising type Ila fibers was calculated in >100 fibers for each muscle. The capillary density was calculated by counting CD31-positive cells and measuring the total area for >100 fibers for each muscle.

Immunoblot analysis. Samples were processed as previously described (3). Protein concentration was determined using the RC DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Total protein (60 μg) was resolved on 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and immunodetected using an advanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). The following primary antibodies were used for immunoblot analysis: rabbit anti-MyoD antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-γ-tubulin antibody (Zymed Laboratories, San Francisco, CA). The intensities of the bands were quantified using Scion Image software.

Statistical analysis. Data are presented as means ± SE. For the time course experiments, one-way ANOVA was used to compare mean values of each time point with those of the sedentary group. The Dunnett test was used to determine statistically significant differences. For comparison between the sedentary and trained mice, a two-tailed Student’s t-test was performed. Two-way ANOVA, followed by the Newman-Keuls test, was used to compare results in irradiated and contralateral nonirradiated plantaris muscles from sedentary and trained mice. P < 0.05 was accepted as statistically significant for all analyses.

RESULTS

Voluntary running induces phasic increase of cell proliferation in mouse skeletal muscle. To define the temporal features of voluntary running-induced cell proliferation in skeletal muscle, we performed in vivo BrdU pulse labeling in plantaris muscle. As shown by indirect immunofluorescence analysis (Fig. 1, A and B), voluntary running resulted in a significant increase in BrdU-positive cells between days 3 and 14 compared with the sedentary control mice (P < 0.01). The number of BrdU-positive cells returned to the basal level after 4 wk of training. This phasic increase in BrdU labeling occurred concurrently with induced mRNA expression of cell division control protein 6 Cdc6 (Fig. 1C), a cell cycle gene that is essential for cell proliferation and DNA replication (35).

Voluntary running induces cell proliferation in skeletal muscle with different functional fates. To better understand the functional role of cell proliferation in exercise-induced skeletal muscle adaptation, we traced the functional fate of the proliferative cells by labeling the proliferating cells and allowing them to differentiate into mature functional cells. The long-term BrdU labeling was achieved by daily intraperitoneal injection of BrdU during 4 wk of voluntary running. As shown in Fig. 2A, significantly more BrdU-positive nuclei were detected in plantaris muscles of sedentary control mice after long-term BrdU labeling (8.6 ± 0.8/mm2) compared with the pulse-labeling results (0.9 ± 0.2/mm2) (Fig. 1B). Voluntary running (4 wk) resulted in a 7.5-fold increase in BrdU-positive nuclei (102.6 ± 25.2 per mm2; P < 0.01 vs. sedentary control) in plantaris muscles (Fig. 2, A and B). The percentages of BrdU-positive nuclei in plantaris muscles were 2.0 ± 0.4% and 15.4 ± 1.6% in sedentary and exercise-trained mice, respectively. The number of BrdU-positive nuclei in soleus muscle was significantly higher than that in plantaris muscle (12.7 ± 1.8 per mm2; P < 0.001 vs. plantaris muscle) in sedentary control mice, and voluntary running resulted in a 12.5-fold increase in BrdU-positive nuclei (159.1 ± 26.5 per mm2; P < 0.001 vs. sedentary soleus muscles). The percentages of BrdU-positive nuclei in soleus muscles were 1.9 ± 0.4% and 20.0 ± 3.7% in sedentary and exercise-trained mice, respectively. Indirect immunofluorescence was used to ascertain the functional identities of these BrdU-positive cells. The BrdU-positive cells in exercised plantaris muscle were of multiple functional fates (Fig. 2C and Table 1). In general, 32.5 ± 3.8% of the BrdU-positive nuclei were confirmed to be muscle nuclei (according to relationship to laminin staining) and 28.4 ± 3.2%
Fig. 1. Voluntary running induces a phasic increase of cell proliferation in mouse plantaris muscle. A: indirect immunofluorescence staining of plantaris muscle sections from sedentary control (Sed) and 2-wk exercise-trained (Ex) mice. A section of the duodenum (Gut) was used as a positive control for cell proliferation. All sections were stained with anti-5-bromo-2'-deoxyuridine (anti-BrdU) antibody, followed by FITC-conjugated secondary antibody (green) and nuclear DNA with propidium iodide (PI; red). B: quantitative data for BrdU-positive nuclei in plantaris muscle (n = 7 for each time point). **P < 0.01, statistically significant difference vs. sedentary control muscle. C: semiquantitative RT-PCR for Cdc6 mRNA. Representative images (top) of RT-PCR products for Cdc6 mRNA and reference control of Gapdh mRNA are shown. Quantitative data (bottom) also are presented (n = 7 for each time point). *P < 0.05, statistically significant difference vs. sedentary control (running time = 0).
were angiogenic (i.e., CD31-positive) cells. The rest of the BrdU-positive nuclei were of unknown origin in the interstitial space outside the basal lamina sheath. The involvement of myogenic stem cells was also implicated by transiently induced expression of myogenic regulatory factor MyoD during long-term voluntary running, with peak expression occurring at 2 wk (Fig. 2D). Altogether, these findings provide direct evidence that the majority of the proliferative cells induced by voluntary running in skeletal muscle participate in myogenesis and angiogenesis.
Running at 2 wk. We observed a trend of muscle mass increase ($P = 0.06$) in nonirradiated plantaris muscles at 4 wk of running, which was completely abolished by irradiation. Irradiated soleus muscles showed a similar response to voluntary running, except that there also was decreased muscle mass in the sedentary mice (Fig. 3G).

**DISCUSSION**

In the present study, we have defined a robust, phasic increase of cell proliferation in mouse plantaris muscle during long-term voluntary running. The increase was associated with fiber-type transformation, enhanced angiogenesis, and muscle growth. These findings confirmed and extended our previous observations in the same exercise model that voluntary running induces cell cycle gene expression and cell proliferation in the recruited skeletal muscles. Consistent with these findings, long-term in vivo BrdU labeling revealed that voluntary running-induced cell proliferation contributed to myogenesis and angiogenesis in plantaris muscles. Most important, we have demonstrated that ablation of resident stem cell activity blocks activity-induced muscle growth but has no impact on exercise-induced fiber-type switching and angiogenesis.

Endurance exercise training has been characterized by the adaptive changes in fiber-type composition and microvascularity (6, 32), and less attention has been paid to the effect on muscle mass. It was recently shown that voluntary running with resistance induces increases in recruited skeletal muscle mass in mice and rats (13, 15), supporting the notion that workload is a potent determinant of muscle mass in endurance exercise. However, it is worth noting that in these studies, animals that ran with little or no load also showed a trend or a significant increase in plantaris muscle mass after long-term voluntary running (7–8 wk) compared with sedentary control mice. These findings raised an issue of activity-dependent muscle growth, which is also supported by the finding that treadmill running induces moderate muscle growth in rats (26). Our findings that voluntary running promotes muscle growth in plantaris muscles are in complete agreement with these previous findings. Importantly, we have extended the findings to confirm that the muscle growth induced by voluntary running in mice is dependent on resident myogenic stem cell function. X-ray irradiation not only may elicit DNA damage and cell cycle checkpoint to block satellite cell proliferation but also may result in damage to the microenvironment of the muscle, and collectively these effects may impair the ability of satellite cells to fulfill their normal function in promoting muscle growth in response to exercise.

Several other features of cell proliferation in skeletal muscle have been revealed in this study. First, we have found that in sedentary mice, soleus muscle, a muscle that is frequently recruited soleus muscle, but not in plantaris muscle,
even in sedentary mice (Fig. 3G). This treatment blocked voluntary running-induced increases in muscle mass in both soleus and plantaris muscles. Collectively, these findings strongly support the notion that contractile activity promotes muscle growth through myogenic cell proliferation. We have also confirmed that voluntary running is associated with muscle injury and regeneration, as evidenced by the detection of central nuclei (Fig. 2C) and induction of MyoD protein expression (Fig. 2D). MyoD plays an essential role in muscle regeneration (21).

The finding that voluntary running-induced cell proliferation in plantaris muscle reversed beyond day 14 despite continued exercise suggests that cell proliferation is associated with a process as a response to running activity in untrained muscle, but not in trained muscle, and/or associated with the adaptation process. It is likely that both muscle injury and muscle adaptation contribute to the increased cell proliferation in this voluntary running model. If it were due only to muscle injury, it would suggest that chronic exercise results in remodeling of the muscle-rendering resistance to the same injury stimulus.

Regardless of whether satellite cell proliferation is required for fast-to-slow fiber-type switching has been an intriguing question in the field of exercise physiology. Previous findings in animal models of compensatory hypertrophy (surgical removal of synergistic muscles) show that adaptations in MHC gene expression are not significantly affected by satellite cell sterilization (1, 27, 28), which argues against the hypothesis that satellite cell proliferation plays an essential role in contractile activity-induced fiber-type transformation. However, before our present study, it remained questionable whether the results were applicable to endurance exercise. Our findings have extended these previous findings and provide clear evidence, in a physiological model of endurance exercise, that

Fig. 3. Resident stem cell proliferation is required for voluntary running-induced muscle growth, but not for fiber-type switching and angiogenesis. A: quantitative data for indirect immunofluorescence for pulse-labeled BrdU-positive nuclei in nonirradiated (NI) or irradiated (IR) plantaris muscles in sedentary control or 2-wk exercise trained mice (n = 5). ***P < 0.001. B: indirect immunofluorescence staining of type Ila myofibers. An increased percentage of type Ila myofibers was present in trained plantaris muscles, regardless of X-ray irradiation. C: quantitative data for type Ila myofiber surface area in plantaris muscles (n = 5). *P < 0.05. ***P < 0.001. D: immunoblot analysis for MHC type Ila protein. Representative images of MHC type Ila and loading control γ-tubulin are shown.
fiber-type switching does not require active proliferation of resident myogenic stem cells.

An unexpected finding in this study is that irradiated muscles showed significant amount of residual activity of cell proliferation during voluntary running. There are several possibilities that could explain the residual cell proliferation in the irradiated skeletal muscles. First, it is possible that the radiation dose used was insufficient to ablate all of the resident stem cells, although we previously used this radiation dose in the same strain of mice to block cardiotoxin-induced skeletal muscle regeneration (34). The fact that irradiation resulted in a complete block of voluntary running-induced muscle growth provides evidence that the X-ray radiation dosage was sufficient to block myogenic cell function. Second, some of the resident stem cells may have survived irradiation to promote delayed cell proliferation. Recent reports have indicated that there are stem cell populations within skeletal muscle that appear to be resistant to radiation-induced damage (11). Third, stem cells from extramuscular tissues may migrate via the circulation to the skeletal muscle to compensate for the loss of resident stem cells. This latter scenario is particularly intriguing and could be of important functional significance. Although increasing evidence supports the hypothesis that resident satellite cells are self-sufficient as a source of muscle regeneration (9), we cannot exclude the possibility that irradiated muscle could undergo hypertrophy with the participation of stem cells other than resident myogenic stem cells if the mice were allowed to run for a longer period.

Irradiation had no significant impact in this study on angiogenesis induced by exercise. This finding raised several inter-

Fig. 3—Continued. E: indirect immunofluorescence staining of CD31-positive capillary endothelial cells in plantaris muscles that were not irradiated (NI) and in those that were X-ray-irradiated (IR) from sedentary control or 4-wk exercise trained mice. An increase in CD31-positive capillary endothelial cells in trained plantaris muscles occurred regardless of X-ray irradiation. F: quantitative data for capillary density in plantaris muscles (n = 5). ***P < 0.001. G: comparison of muscle mass (normalized to body wt) in soleus and plantaris muscles that either were or were not irradiated from sedentary control or exercise-trained mice. *P < 0.05, **P < 0.01, ***P < 0.001.
testing questions regarding the cellular processes for activity-induced angiogenesis in skeletal muscle. Angiogenesis in skeletal muscle can occur by intussusception or sprouting (for review, see Ref. 24), which requires increases in endothelial cells, presumably through cell proliferation. Our findings suggest that either 1) endothelial cells are resistant to X-ray irradiation, or 2) other progenitor cells from extramuscular sources participate in exercise-induced angiogenesis. It should be pointed out that irradiation at the dose used in this study had previously been shown to block the proliferation of endothelial cells in vitro (10). It is unlikely that endothelial cells in skeletal muscle are uniquely resistant to irradiation. Thus endothelial progenitor cells may be recruited from the circulation. The possibility that circulating endothelial progenitor cells plays an essential role in endurance exercise-induced angiogenesis is worthy of future investigation.

In summary, in the present study, we have defined for the first time some temporal and functional features of cell proliferation in skeletal muscle in a physiological model of endurance exercise. The results indicate that cell proliferation induced by voluntary running is required for activity-dependent muscle growth but not for fiber-type switching and angiogenesis.

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Present address of T. Akimoto: Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Waseda-Tsurumaki 513, Shinjuku, Tokyo 162-0041, Japan.

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