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Age-related changes in satellite cell proliferation by compensatory activation in rat diaphragm muscles

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ABSTRACT

To investigate the age-related changes in satellite cell (SC) proliferation *in vivo*, we used a compensatory activation (CAC) model of the hemi-diaphragm muscle. Young (2-month), adult (14-month) and old (24-month) rats were randomly divided into control and CAC groups. In the CAC group, denervation surgery in the left hemi-diaphragm was performed to induce CAC of the right hemi-diaphragm. Six days after the surgery, the CAC diaphragm muscle was removed and separated into two blocks for immunohistochemical staining and real time RT-PCR procedures. The number of SCs in type I and IIa fibers were not affected significantly by the CAC in any age groups, but that in type IIx/b fibers was significantly increased in the young and adult groups. As compared to the age-matched control group, the Pax7 mRNA expression level was significantly higher in the young and adult CAC groups, but not in the old CAC group. These results may suggest that the mechanism of SC proliferation in type IIx/b fibers is impaired in aged diaphragm muscles.

Satellite cells (SCs) are known as muscle stem cells and are associated with growth, maintenance, repair and regeneration in postnatal muscle (4, 17, 20). In adult skeletal muscle, SCs are quiescent under normal physiological conditions. However, in response to activation signals resulting from exercise or injuries, SCs are activated, proliferate, and differentiate to provide new myonuclei to mature muscle fibers (4, 30). SCs constantly express Pax7, a closely related member of the paired box (Pax) family of transcription factors (15). Although the regulatory mechanisms of expression and target genes of Pax7 are still unclear, this gene is essential for the formation and function of SCs. Therefore, it is well recognized that Pax7 is the most reliable marker for SCs (15).

It is also well known that the SC population is maintained by self-renewal and appears to be self-sufficient as a source of new myonuclei (5, 6, 33). Some studies suggested that the impairment of this mechanism may lead to a reduction in SCs numbers with aging (8, 27), and that is linked to the age-related loss of muscle mass and regenerative ability. However, the age-related changes in SC capacity or content have not been elucidated. For example, a decline in the number of SCs in the skeletal muscle of aged rodents compared to that in adult animals has been reported (9, 29). On the other hand, some studies have found no decrease in the SC population of aged muscles (21, 26). The results for human skeletal muscle have also been equivocal. Previous studies have either shown a similar (11, 24) or lower (13, 23) SC content in older adults when compared to young adults.

In the present study, we determined the number of SCs in type identified muscle fibers and the Pax7 mRNA expression level in compensatory activated (CAC) hemi-diaphragm muscle. In previous studies (14, 19), significant increases in electromyogram

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(EMG) activity were reported in the CAC model, and it is speculated that the degree of these increased activities was much greater than those in overloading models in lower hindlimb muscles.

MATERIAL AND METHODS

Animal procedure and surgery. Forty-two young male Wistar rats (2-month old, mean body weight 250 ± 10 g), adult (14-month old, mean body weight 617 ± 36 g) and old (24-month old, mean body weight 630 ± 60 g) were used. They were randomly divided into sham-control and CAC groups (n = 7 for each group). All experimental and animal care procedures were approved by the Committee on Animal Care and Use in Yamaguchi University and followed the American Physiological Society Animal Care Guidelines.

The rats were anesthetized by intraperitoneal injections of sodium pentobarbital (50 mg/kg) and fixed on an anatomical table. Under a surgical microscope, the central line of the cervical ventral part was cut for about 4 cm, cutaneous muscles were opened and then the left phrenic nerve was transected. After ensuring that there was no bleeding in the operating field, the layers of muscles and skin were sutured separately. In the control group, the same surgery without phrenicotomy was performed.

At 6 days after the surgery, rats were anesthetized again, and the right hemi-diaphragm muscle was removed immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Fiber type identification. Serial cross sections of 10 μm thickness were obtained from all muscle samples on a cryostat (Leica CM510, Nussloch, Germany) at −20°C. The sections were allowed to warm to room temperature (RT) and were then preincubated in 1% normal goat serum in 0.1 M phosphate buffered saline (PBS; pH 7.6) at RT for 10 min. The mouse primary monoclonal antibodies were then applied: 1) Fast myosin (1 : 3,000; SIGMA, St. Louis, USA), which specifically reacts with the myosin heavy chain (MHC)-IIa and -IIx/b (Fig. 1 A); 2) SC-71 (1 : 1,000; Developmental Studies Hybridoma Bank; DSHB, Iowa city, USA), which specifically reacts with MHC-IIa (Fig. 1B). The sections were incubated overnight at RT, washed with PBS and reacted with a secondary antibody conjugated with horseradish peroxidase (1 : 1,000; Bio-Rad, Hercules, USA) at RT for 180 min, and then washed with PBS again. Diaminobenzidine tetrahydrochloride was used as a chromogen to localize peroxidase in the secondary antibody.

Microscopic images of the stained muscle fibers were obtained by microscopy and an image-processing system (Nikon DS-U1, Tokyo, Japan). On the basis of immunohistochemical staining images, the fibers were classified as one slow-twitch fiber (type I) and two fast-twitch fibers (IIa and IIx/b), and then the populations of each muscle fiber type were calculated from approximately 400 muscle fibers.

Satellite cell identification. Another serial section was fixed in 4% paraformaldehyde in PBS at RT for

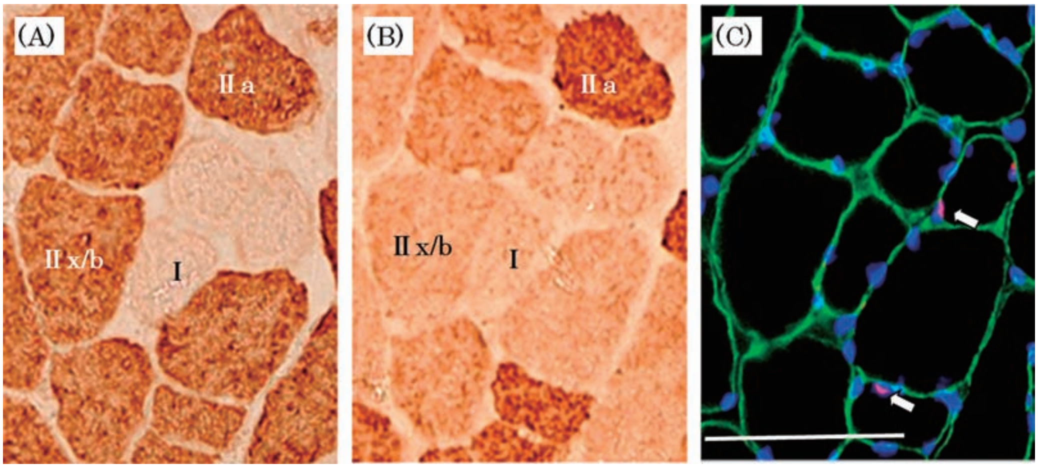

Fig. 1 Three serial transverse sections of diaphragm muscle from a young control rat. Two sections were stained with monoclonal antibodies against fast myosin (**A**) and myosin heavy chain-IIa (**B**). Muscle fibers were classified into type I (non-stained fiber in both **A** and **B**), IIa (stained fiber in both **A** and **B**) and IIx/b (fiber stained in **A** and non-stained in **B**). Triple-immunofluorescent stains for laminin (green), Pax7 (red) and nuclei (blue). The *white arrows* in merged image (**C**) indicate satellite cells. *Bar* 100 μm.

10 min and then washed with PBS. The section was pre-incubated in blocking solution containing 10% normal goat serum, 0.1% tritonX-100 (SIGMA) and 2% bovine serum albumin in PBS at RT for 30 min. After the pre-incubation, each section was washed with PBS and incubated for 60 min at RT with primary antibodies directed against a mouse Pax7 (1 : 1,000, DSHB), and a rabbit anti-laminin antibody (1 : 1,000, SIGMA) diluted in 2% bovine serum albumin/PBS. Then the section was washed with PBS, and appropriate secondary antibodies were applied: Cy™3-conjugated AffiniPure goat anti-mouse IgG (1 : 1,000; Jackson ImmunoResearch, West Grove, USA) and AlexaFluor488 goat anti-rabbit IgG (1 : 1,000; Molecular Probes, Breda, Netherlands), respectively. After incubating for 60 min with the secondary antibodies, the sections were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) diluted in PBS at RT for 2 min.

These three fluorescence images were merged (Fig. 1 C) and all fibers within each image were identified as Type I, IIa or IIx/b fibers based on matching of serial immunohistochemical images. Within each image, the mean fiber cross-sectional area (CSA), the number of myonuclei per fiber, and the mean myonuclear domain (MND) size (fiber CSA/number of myonuclei) were measured for the three fiber types separately. SCs were determined at the periphery of each fiber beneath the basal lamina stained with laminin and stained positive for both DAPI and Pax7. The numbers of SCs per fiber were calculated for the three fiber types separately, based on at least 100 muscle fibers.

Real time RT-PCR for mRNAs. Each muscle was analyzed for expression of Pax7 mRNA using a real-time RT-PCR system. Total RNA was extracted with TRIZOL reagent (Invitrogen, Tokyo, Japan). The purity and yield of total RNA were determined by measuring the absorbance of aliquots at 260 and 280 nm. Total RNA was then treated for 30 min at 37°C with TURBO™ DNase (Ambion, Austin, USA) to remove genomic DNA from samples. DNase-treated RNA (0.5 μg) was used to synthesize first-strand cDNA with an PrimeScript™ RT-PCR kit (Takara, Tokyo, Japan). Thereafter, the cDNA products were analyzed by real-time PCR using the SYBR Green PCR Master Mix protocol in StepOne™ Real Time PCR Systems (Applied Biosystems Japan, Tokyo, Japan).

The amplification program included an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, and annealing/extension at 58°C for 1 min. The amount of glyceraldehyde-3-phsphate dehydrogenase (GAPDH) mRNA was estimated as an internal control. Pax7 mRNA was normalized to GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from the Ct value of the gene target [ΔCt (target)]. The relative expression of the target gene was calculated as the relative quantification (RQ) value for the young control group. Following the relative expression, dissociation-curve analysis did not detect any nonspecific amplification in cDNA samples.

The sequences of the specific primers used in this experiment were as follows: Pax7 (forward 5’-AAA AGATTGAGGAGTATAAGAGGGAGAA-3’ and reverse 5’-GCCGGTCCCGGATTTC-3’) and GAPDH (forward 5’-GCTCTCTGCTCCTCCCTGTT-3’ and reverse 5’-GAGGCTGGCACTGCACAA-3’). Each PCR primer was designed using Primer Express® software, and the oligonucleotides were purchased from FASMAC (Kanagawa, Japan).

Statistics. The results obtained in this study were analyzed by two-way ANOVA with age (young, adult and old) and model (control and CAC) as grouping variables. Post-hoc analysis was performed using a *t*-test with the Bonferroni adjustment method. In all cases, statistical significance was set at $P < 0.05$. All values are reported as the mean ± standard deviation.

RESULTS

Fiber type population, CSA and MND size

In the young control group, the mean fiber type populations of type I, IIa and IIx/b were 36 ± 7%, 36 ± 6% and 28 ± 6%, respectively. There were no significant differences in the fiber type population among the three age groups (Fig. 2A). In the control group, the mean CSA values in the adult group (type I 1787 ± 348 μm^2; type IIa 1794 ± 257 μm^2; type IIx/b 4568 ± 617 μm^2) and old group (type I 2041 ± 286 μm^2; type IIa 2510 ± 573 μm^2; type IIx/b 4089 ± 485 μm^2) were significantly larger than those in the young group (type I 1201 ± 190 μm^2; type IIa 1390 ± 309 μm^2; type IIx/b 2515 ± 891 μm^2) in all fiber types (Fig. 2B).

The mean myonuclei numbers of the three fiber types in old control group (type I 1.9 ± 0.2; type IIa 2.2 ± 0.3; type IIx/b 2.5 ± 0.3) were significantly smaller than in the young control group (type I 2.6 ± 0.3; type IIa 2.9 ± 0.3; type IIx/b 3.6 ± 0.7). The mean myonuclei numbers of the three fiber types in

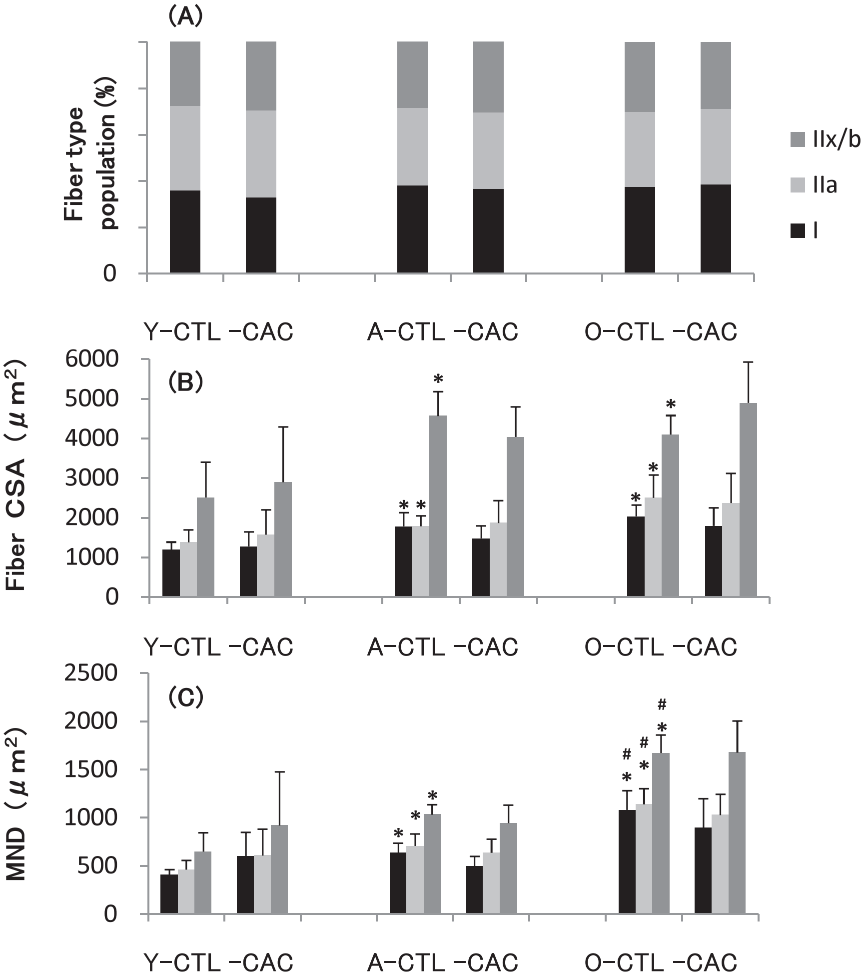

Fig. 2 Fiber type population (**A**), cross sectional area (CSA) (**B**), and myonuclear domain size (MND) (**C**) of diaphragm muscle from young (Y: 2-month), adult (A: 14-month), and old (O: 24-month) rats in the control (CTL) and compensatory activation (CAC) groups. Values are means + standard deviations. (*) Significantly different from the same fiber type of the young CTL ($P < 0.05$); (#) significantly different from the same fiber type of the adult CTL ($P < 0.05$)

the adult control group (type I 2.8 ± 0.6; type IIa 2.6 ± 0.3; type IIx/b 4.4 ± 0.5) were not significantly different to those in the young control group. Consequently, as shown in Fig. 2C, there were significant differences in mean MND sizes among the young control (type I 411 ± 54 μm^2; type IIa 463 ± 95 μm^2; type IIx/b 650 ± 197 μm^2), adult control (type I 637 ± 100 μm^2; type IIa 707 ± 125 μm^2; type IIx/b 1038 ± 102 μm^2) and old control groups (type I 1083 ± 198 μm^2; type IIa 1142 ± 160 μm^2; type IIx/b 1673 ± 187 μm^2).

No significant effects of CAC on the fiber type population, CSA or MND sizes were found in any age group.

Number of satellite cells in each fiber type

In the control groups, the numbers of SCs per type I fiber were 0.18 ± 0.07, 0.19 ± 0.05 and 0.22 ± 0.06 in young, adult and old groups, respectively. The numbers of SCs per type IIa fiber were 0.13 ± 0.06, 0.14 ± 0.08 and 0.17 ± 0.04 in young, adult and old groups, respectively. The numbers of SCs per type IIx/b fiber were 0.08 ± 0.04, 0.11 ± 0.04 and 0.11 ± 0.05 in young, adult and old groups, respectively. In all fiber types, there were no significant differences in the SCs number among three age groups.

In type I and IIa fibers in the three age groups, there were no significant differences in the number of SCs between the CAC and age-matched control groups. In type IIx/b fibers, however, the number of SCs significantly increased with CAC in the young (0.23 ± 0.16) and adult groups (0.23 ± 0.07), but not in the old group (0.21 ± 0.12) (Fig. 3).

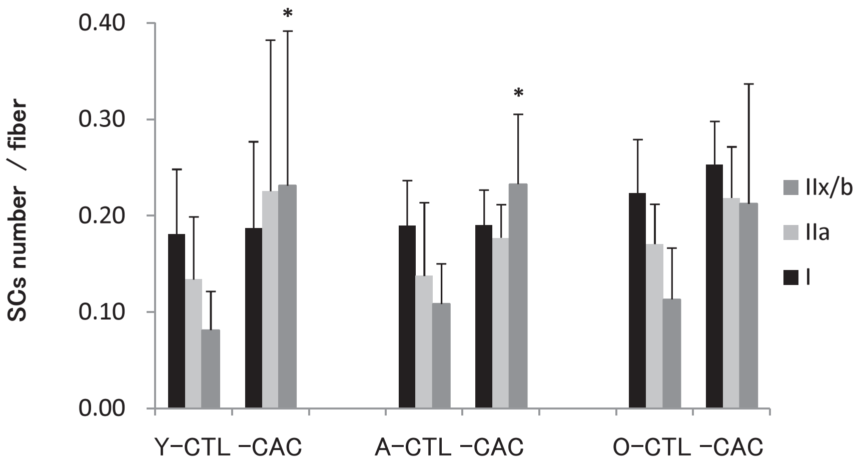

Fig. 3 Number of satellite cells (SCs) in each fiber type of diaphragm muscle from young, adult and old rats in the CTL and CAC groups. Values are means + standard deviations. (*) Significantly different from the same fiber type of the age-matched CTL ($P < 0.05$)

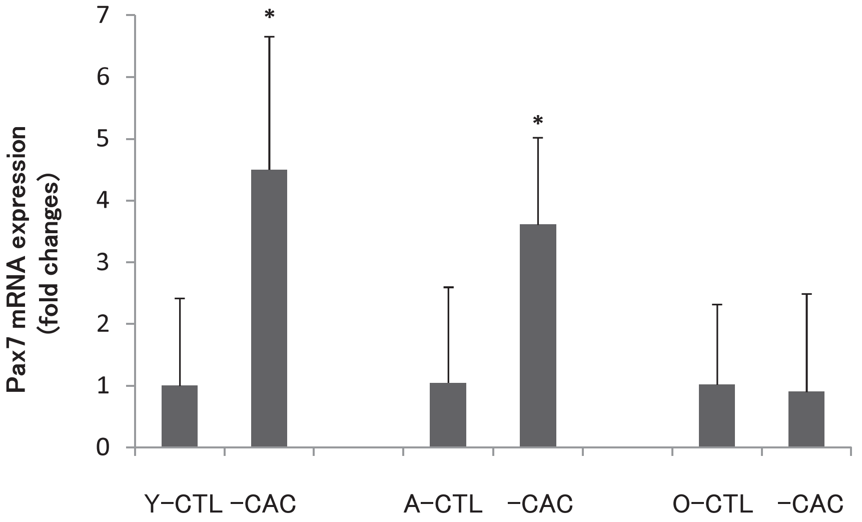

Fig. 4 Relative value of Pax7 mRNA expression as fold change from the young CTL group. Values are means + standard deviations. (*) Significantly different from the age-matched CTL ($P < 0.05$)

Expression of Pax7 mRNA

In the control groups, the relative values of the Pax7 mRNA level were identical among the three age groups. Although CAC increased the Pax7 mRNA expression to 4.5- and 3.6-fold for each control level in the young and adult groups, respectively, there was no difference between control and CAC in the old group (Fig. 4).

DISCUSSION

In this study, we demonstrated that the SCs number in type IIx/b fibers and Pax7 mRNA expression level of young and adult CAC muscles were significantly higher than those in age-matched control muscles, while significant effects were not found in old CAC muscles.

Aging effect on fiber type composition, CSA and MND size

A previous study showed that rat diaphragm muscle includes three subtypes of type II fiber; MHC-IIa, -IIx and -IIb, and that type IIx and IIb fibers have very similar contractile and metabolic properties (32). Therefore, we classified fiber types into type I, IIa and IIx/b for convenience.

Our data showed that there was no difference in the fiber type population among the three age control groups. It is well documented that the many factors contribute to skeletal muscle atrophy with aging, such as type II fiber specific hypoplasia, degeneration of the neuromuscular junction (31) and fast-to-slow motor unit remodeling (25). However, Brooks *et al.* (2) reported a significant fast-to-slow fiber transition in the aged rat plantaris, but not in the rat soleus muscle. Similar results have been shown extensively in both human and murine studies (22). These differences may be associated with not only muscle contractile properties, but also daily activity related to functional specificity in the each muscle. Hodgson *et al.* (12) demonstrated that intramuscular EMG activity in rat soleus muscle was 2- to 8-fold higher than other fast-twitch muscles (tibialis anterior, medial gastrocnemius and vastus lateralis). Regardless of age, the diaphragm muscle is always active for respiration and thus its fiber type composition in the old control group was similar to that in the young and adult control groups.

On the other hand, we found that CSA and MND sizes of all fiber types in the aged diaphragm were significantly larger compared to young animals (Fig. 2B, C). During growth, increased CSA is often accompanied by an increased number of myonuclei (18). However, it is generally considered that muscle fiber does not maintain a constant MND size during development or hypertrophy. For example, Mantilla *et al.* (16) showed that MND sizes were significantly increased in all fiber types from postnatal 14- to 24-days in the rat diaphragm. In a previous study at their laboratory, MND size in adult rats (body weight was ~ 300 g) was also larger than in animals at postnatal day 24 (1). There is evidence that the existing myonuclei are able to increase protein synthesis and support the initial increase in cytoplasm prior to an increase in myonuclear number (13). The changes in MND size related to atrophy are unclear. In atrophied muscle exposed to microgravity, the MND size is maintained constantly because the myonuclear number decreases simultaneously with a decrease in CSA (10), whereas the MND size was reduced in the hindlimb muscle of aged mice (3). It is unknown whether the reduction in myonuclear number precedes muscle fiber atrophy, or vice versa. Our result may suggest that the myonuclear number decreases prior to the loss of fiber volume, and subsequently muscle atrophy occurs with further aging.

Survival and proliferation of aged satellite cells

In the control group, the numbers of SCs per fiber in old animals were similar to those in young and adult animals (Fig. 3). Several studies have shown a decrease in SCs number with aging (9, 13, 23, 29). In contrast, there are many studies also reported no change in SCs number in aged muscle (11, 24). These conflicting results may be due to the numerous methods used for measuring SCs. Moreover, we raised the point that these studies investigated human leg or animal hindlimb muscles. As mentioned above, the daily activities of these muscles are much lower than that of the diaphragm, especially in older or aged animals. Combining our findings in the rat diaphragm with the EMG data (12, 19), we suggest that the SCs number may be not affected by aging, at least in a muscle functioning constantly.

In the young and adult groups, the CAC in the diaphragm for 6 days significantly increased the SCs number of type IIx/b fibers, but not in type I or IIa fibers (Fig. 3). As compared to type I and IIa fibers, type IIx/b fibers are relatively inactive in eupnea (28). Therefore, we speculate that the impact of CAC on type IIx/b fibers was much greater than those on type I and IIa fibers. In the old CAC group, although the number of SCs per fibers tended to increase only in type IIx/b fibers compared with that in the old control ($P = 0.08$), no significant changes were found in the SCs number in any fiber types (Fig. 3). In addition, the Pax7 mRNA expression level of the old CAC group did not change from the baseline level at all, whereas the relative value was increased approximately 4-fold in the young and adult CAC groups (Fig. 4). Some studies have demonstrated that the proliferative potential of SCs decreases with aging, and it is thought that this may in part account for the lack of the hypertrophic response in aged skeletal muscle (27). Considering the fact that Pax7 is essential for SC survival, especially self-renewal (33), our results support the notion of a reduction in proliferative potential of SC in aged skeletal muscle.

In our experiment, we obtained muscle samples only at 6 days after CAC surgery. A pilot study in our laboratory showed that the SCs number in young rats was most increased at 6 days after CAC surgery (data not shown). In old rats, however, it remains unclear whether prolonged CAC induces activation and proliferation of SCs in the diaphragm within 6 days after surgery. Furthermore, Collins *et al.* (7) emphasized that a subpopulation of SCs in aged muscle fibers, which are Pax7-negative and located in a niche of SCs, retains equivalent capacity to generate large clusters of progeny that contain both differentiating cells and other cells. Further studies are needed to clarify these points.

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