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Moderate-intensity treadmill running promotes expansion of the satellite cell pool in young and old mice

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Abstract

Satellite cells, myogenic progenitors located at the myofiber surface, are essential for repair of adult skeletal muscle. There is ample evidence for age-linked decline in satellite cell numbers and performance in limb muscles. Hence, effective means to activate and expand the satellite cell pool may enhance muscle maintenance and reduce the impact of age-associated muscle deterioration (sarcopenia). Toward this aim, we explored the potential beneficial effect of endurance exercise on satellite cells in young and old mice. Animals were subjected to an 8-week moderate-intensity treadmill running approach that does not inflict apparent muscle damage (0° inclination, 11.5 meter/min, 30 min/day, 6 days/week). Myofibers of extensor digitorum longus muscles were then isolated from exercised and sedentary mice and used for monitoring satellite cell numbers and for harvesting individual satellite cells for clonal growth assays. We specifically focused on satellite cell pools of single myofibers, with the view that daily wear of muscles is likely inflicting individual myofibers rather than causing overall muscle damage. We found an expansion of the satellite cell pool in the exercised groups compared with the sedentary groups, with the same increase factor (~1.6) in both age groups. Current results accord with our findings with rat gastrocnemius, attesting for the consistent effect of exercise running on satellite cell expansion in limb muscles. The experimental paradigm established here is useful for studying satellite cell dynamic at the myofiber niche and for broader investigation of the impact of physiologically and pathologically relevant factors on adult myogenesis.

Keywords

Skeletal muscle; myofibers; satellite cells; aging; endurance exercise; Nestin-GFP

Introduction

Satellite cells are Pax7-expressing (Pax7⁺) myogenic progenitors, residing between the myofiber basal and plasma membranes and are essential for repair of adult skeletal muscles [1–4]. There is evidence that at least some of the satellite cells are bona fide stem cells, capable of self-renewing in addition to contributing progeny for myofiber repair, thereby ensuring maintenance of the satellite cell pool [5, 6]. Routine muscle activity, even if involves only subtle myofiber injuries, raises a continuous demand for functioning satellite

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cells throughout life. Rodent and human studies have shown, within the context of limb muscles, that there is an age-associated decline in satellite cell numbers and performance [7–17]. This diminution may contribute to age-related muscle deterioration, a condition known as sarcopenia. Sarcopenia is characterized by a decline in mass, strength, endurance and repair ability of skeletal muscles, leading to frailty, reduced life quality and even death [18–22]. Multiple factors, ranging from systemic to muscle-intrinsic, are thought to play a role in this process [22–26]. This muscle deterioration involves a reduction in myofiber number, atrophy of remaining myofibers and increased susceptibility to contraction-induced injuries [27]. Combating sarcopenia requires the identification of means to improve myofiber maintenance in aging muscle. Therefore, a better understanding of satellite cell dynamics at their natural niche, the myofiber, is essential.

The vast majority of satellite cells are typically quiescent in adult limb muscles. This inactivity combined with lack of evidence for satellite cell fusion with myofibers in uninjured muscles, has begun to cast doubts on the importance of satellite cells in “baseline” myofiber maintenance (homeostasis) through adult life. However, the sedentary lifestyle of rodents (most frequently used in satellite cell research, typically maintained in standard cage conditions) is not necessarily an optimal environment for promoting recruitment of satellite cell into performance.

Studies with isolated myofibers, using conditions that retain satellite cells at their niche, have shown that this positional intimacy with the myofiber is essential for satellite cell interplay between progeny expansion versus self-renewal [28–30]. Nevertheless, much of our current understanding of in-vivo myogenesis in adult life is based on models of induced muscle trauma [31, 32]. These studies have typically relied on using agents such as snake venom or barium chloride, or exhaustive eccentric exercise protocols that cause extensive muscle damage [33, 34]. While such muscle injury approaches have provided a wealth of knowledge about muscle regeneration, they do not retain muscle architecture and physiological milieu associated with daily muscle use. Furthermore, daily muscle use inflicts subtle myofiber damage that requires localized myofiber repair. It is reasonable to consider that this process relies on the performance of individual resident satellite cells in few myofibers at a time, rather than on “en masse” satellite activation that is characteristic to regeneration after massive muscle damage. Thus, in order to gain insights into the satellite cell pool through life, we have studied satellite cells at the single-cell level in isolated myofibers, demonstrating a drastic age-associated diminution of the satellite cell pool in mice and rats [9–11]. This decline may represent deterioration in the capacity of all or a subset of satellite cells to self-renew [10, 35].

We recently began using endurance exercise for in-vivo rodent satellite cell studies [11, 36], especially because this activity has been considered not to inflict muscle trauma and is even beneficial in certain myopathies [37–40]. While many human and rodent studies have shown enhanced satellite cell numbers following a single bout of eccentric activity [41–43] studies on satellite cells using in-vivo models that do not involve muscle damage are scarce. Endurance exercise is beneficial to many aspects of human health [44, 45]. Likewise, rodent studies have provided evidence for the beneficial effects of endurance exercise, including lifespan and health-span expansion [46–51]. The effect of endurance exercise on satellite cells has been analyzed only in a limited number of studies [11, 52–54]. One detailed study looked at satellite cell numbers in the rat plantaris muscle using voluntary wheel running [52] and we studied the effect of moderate-intensity, treadmill running (0° inclination) on gastrocnemius muscle of young and old rats [11]. Our preference of using treadmill running is based on the high reproducibility among mice subjected to exercise running, while mice subjected to voluntary wheel running vary widely in traveled distance outcomes. Treadmill-running endurance training engages muscle groups linked to toe and ankle functions [11, 55,

56]. These include extensor digitorum longus (EDL), tibialis anterior, flexor digitorum brevis, and gastrocnemius muscles. These fast twitch muscles are affected by aging [57]. Notably, exercise-induced effects on contralateral muscles that have not been engaged directly during physical activity have been reported [58]. Hence, exercise may also have systemic beneficial effects on satellite cells in distant muscles.

In the present study, we have investigated satellite cell pools of individual EDL myofibers, with the view that daily wear of muscles is likely affecting individual myofibers rather than causing overall muscle damage as occurs in response to robust trauma. Specifically, we monitored satellite cell numbers as an overall measure for the satellite cell pool homeostasis and performance, which depends on the dynamic between quiescence, proliferation, renewal, and survival of satellite cells and their progeny [28, 35, 59]. We demonstrate that moderate-intensity treadmill running elevates the number of satellite cells in young and old mice. Current results accord with our findings with rat gastrocnemius, demonstrating the consistent effect of exercise running on satellite cell numbers in limb muscles. The experimental paradigm established here is beneficial for studying satellite cell dynamic at the myofiber niche and for broader investigations on the impact of physiological and pathological relevant factors on adult myogenesis.

Results and Discussion

Experimental approach

- a. In this study, we investigated the effect of moderate-intensity treadmill running on satellite cell number and performance in young (4-month old) and old (16-month old) male mice. Age of mice specified throughout the manuscript reflects their age at the beginning of the 8-weeks period of the experiment (i.e., exercise or sedentary schedule). Hindlimb (EDL) muscles were harvested at the end of this period for single myofiber isolation in order to quantify satellite cells and perform clonal growth analysis.
- b. The exercise groups were subjected to treadmill running (0° inclination) at a speed of 11.5 meters/minute, for 30 minutes/day, 6 days a week for 8 consecutive weeks. The moderate-intensity running conditions used in the present study are comparable to running conditions that were previously described by others for young and old mice [60, 61].
- c. Transgenic mice expressing GFP under the control of nestin gene regulatory elements (NES-GFP) were used throughout the study for detecting satellite cells in isolated myofibers according to GFP expression. As we previously published, NES-GFP⁺ satellite cells are easily distinguished from myonuclei that are GFP-negative, while both satellite cells and myonuclei are commonly stained with DAPI [62]. Regardless of mouse age, there is an over 95% agreement when satellite cells are detected by the endogenous marker Pax7 versus the transgenic marker NES-GFP [10, 62].

Running enhances the number of satellite cells in young and old mice

The number of satellite cells (NES-GFP⁺) per individual EDL myofibers was determined in sedentary and exercised mice of young and old age (Fig. 1). Fig 1A provides a summary, per each group, of total numbers of mice, myofibers and satellite cells analyzed, and average ($\pm$ SEM) number of satellite cells per myofiber. Further details of satellite cell distribution per individual myofibers are summarized in boxplots (Fig. 1B), depicting quartile analysis and average $\pm$ SEM satellite cell number per myofiber. Significant effects of age, exercise and a combination of age and exercise were observed (two-way ANOVA, $F_{1,569}=177.28$; 129.2

and 11.793, respectively, $p < 0.001$). The number of satellite cells declined with age, while running enhanced the number of satellite cells per myofiber of both young and old mice. Myofibers from young exercised mice contained the highest mean number of satellite cells compared to young sedentary, old exercised and old sedentary groups (average $\pm$ SEM, 14.0 ± 0.5 vs. 8.6 ± 0.3 , 7.9 ± 0.3 and 5.0 ± 0.2 , respectively).

Exercise induced a significant increase in average satellite cell numbers in both young and old mice ($p < 0.005$, for both ages). This is reflected in an overall shift toward higher number of satellite cells per myofiber with a noticeable increase in the lower end (from 1 to 6 and from 0 to 3 in young and old exercised mice, respectively). Furthermore, exercise brings the average number of satellite cells in myofibers of old exercised mice to the same level measured in myofibers of young sedentary mice. Remarkably, the increase factor (i.e. ratio between the average number of satellite cells per myofiber in exercised and sedentary mice) is conserved between the young and old group, amounting to 1.55 and 1.58, respectively. This observation suggests that satellite cells from old mice are equally capable of responding to the exercise as their younger counterpart.

Exercise effect on satellite cell numbers is further demonstrated in graphs depicting myofiber distribution according to satellite cell numbers (Fig. 1C). Myofibers are arranged in ascending order of the numbers of satellite cells per myofiber (X-axis) versus the number of myofibers containing a given number of satellite cells (Y-axis). The exercise-driven increase in the number of satellite cells per myofiber in both young and old mice is evident in the shift of the respective cumulative curves. Each data point in these cumulative curves depicts the percentage of myofibers (out of total myofibers analyzed) that contain less than, or equal to () the corresponding number of satellite cells per myofiber that is indicated on the X-axis. These cumulative plots (Fig. 1C) and the boxplots (Fig. 1B) demonstrate the persistent effect of exercise on increasing the relative abundance of myofibers containing high numbers of satellite cells. The median (50% value) for satellite cell number per myofiber rose from 8 (no-run) to 12 (run) for the young group (Fig. 1B & C). Median value for satellite cell numbers per myofiber in the old group rose from 5 (no-run) to 8 (run).

Collectively, the analysis of satellite cell content in individual myofibers has demonstrated that moderate-intensity treadmill running in both young and old mice leads to an increase in the frequency of myofibers with more satellite cells when compared to their non-exercised counterparts. Such an effect was also observed in our previous rat study where satellite cells were detected based on Pax7 immunostaining [11]. Myofibers lacking satellite cells, typically present in old mice and rats [9–11], are not detected following moderate-intensity treadmill running (Fig. 1C). The absence of such myofibers that lack satellite cells following long-term running could be due to inability of these myofibers to sustain the load/trauma and survive without their supporting satellite cells. We aim to explore this possibility by subjecting mice to the running protocol after inflicting age-specific ablation of satellite cells using Cre-loxP mice based on Pax7-Cre driven activation of diphtheria toxin in satellite cells. Using this paradigm, an absolute requirement for satellite cells in muscle regeneration following major trauma has been confirmed [32, 33]. Nevertheless, satellite cells do not appear to be required for muscle hypertrophy or recovery from unloading-induced atrophy in adult life [63, 64].

Alternatively, the absence of myofibers void of satellite cells in the exercised old mice could be due to the reconstitution of their satellite cell pool through contribution from neighboring progenitors during physical activity. Using a genetic model where ‘cell lineage trees’ can be re-constructed based on the similarity in somatic mutations (in non-coding sequences) between cells, we have previously shown that each myofiber has its own pool of satellite cells [65]. This suggests that during standard cage activity there is no cross over of satellite

cells between myofibers [65]. However, due to the fragility of these genetically modified mice, we are unable to age these animals and establish satellite cell numbers in combination with exercise running. Detection of clonal patches of revertant (dystrophin⁺) myofibers in mdx mice does, in fact, support the existence of pioneer myogenic progenitors that produce progeny, which populate adjacent myofibers during postnatal life of mdx mice [66].

Effects of age and exercise running on clonal performance of satellite cells

As an additional measure for the effect of exercise running on satellite cell performance, we analyzed the number of myogenic clones that developed from individual myofibers (Fig. 2). The same mice analyzed for satellite cell numbers (Fig. 1A) were used for the clonal studies, investigating 3–4 myofibers per mouse (Fig. 2A). Myofibers were individually triturated to strip the satellite cells. The resulting suspension of each myofiber was dispensed into 12 wells and then followed for the development of clones. Clones were identified following the 3rd culture day and defined as myogenic based on the presence of myotubes that are typically found by 7–10 days in culture [10]. Fig 2A provides per each group a summary of total myofibers and myogenic clones analyzed, and average ($\pm$ SEM) number of myogenic clones per myofiber. There was an overall age effect ($F_{1,88}=23.33$, $p<0.005$) showing a reduction in the number of myogenic clones in older mice, in agreement with the age-associated decline in satellite cells numbers detailed above (Fig. 1). The detection of a lower number of myogenic clones per myofiber compared to the number of satellite cell per myofiber (Fig. 1A vs. 2A) agrees with our early studies that have indicated about 50% satellite cell clonability in young and old mice [10]. The myogenic clone data analyzed in boxplots (Fig. 2B) depict quartile distribution and average $\pm$ SEM for the number of clones per myofiber. Myofiber distribution according to their number of myogenic clones and cumulative curves are also shown (Fig. 2C). These data demonstrate a trend toward more myofibers that are capable of generating more clones upon exercise for each age group. This exercise effect follows what we have observed previously with myogenic clones from exercised versus sedentary rats of young and old ages [11]. Noticeably, the 3rd quartile (75th percentile) value for the sedentary group is the median value for the exercised group (i.e., 5 for young and 3 for old mice). Furthermore, the increase factor between the average number of clones per myofiber in exercised and sedentary mice is conserved between the young and old group (1.31 and 1.27, respectively), as observed with the ratios of average number of satellite cells (Fig. 1).

At the morphological level, there was no apparent difference between the myogenic clones from the four groups analyzed. Noticeably, myofibers from both young and old exercised mice produced typical large myogenic clones depicting lower and higher cell densities (Fig. 2D–G) similar to the sedentary groups and in accordance with previous observations [10]. Hence, exercise-induced satellite cell expansion in vivo (Fig. 1) does not seem to be exhausting the post-exercise proliferative potential of satellite cells. Occasional non-myogenic clones were observed arising from a small number of the myofibers processed for satellite cell clonal analysis. Such non-myogenic clones (typically no more than 1 per myofiber) amounted to 2.3% and 2.9% of total clones for no-run and run groups, respectively in the young mice, and 14.8% and 8.8% for no-run and run groups, respectively in the old mice. These clones, which were void of myotubes, were comprised of distinctive large and flat cells negative for myogenic markers such as MyoD and sarcomeric myosin when analyzed by immunostaining. These non-myogenic clones have likely been derived from interstitial cells that are occasionally co-isolated with myofibers and their presence increases with age [10]. Future running studies with stable lineage marking of satellite cells such as MyoD^{Cre}-driven reporter expression [67, 68] can further assist in validating the non-satellite cell origin of the non-myogenic clones.

Concluding Remarks

In light of the importance of satellite cells in muscle maintenance and the possibility that reduced satellite cell performance constitutes an underlying factor of sarcopenia, we investigated the effects of aging on the number and function of satellite cells and their progeny in rodents [9–11]. Here, we focused on the individual and combined effects of exercise running and age on mouse satellite cells. The specific running approach applied in the present study (i.e. long-term moderate-intensity) has been considered not to inflict apparent muscle damage and even be beneficial in certain myopathies [37–40]. The positive effects of endurance exercise on satellite cell numbers parallel our previous work where we inspected the gastrocnemius muscle of young and old rats [11], attesting for the general effect of moderate-intensity treadmill running on satellite cell numbers, irrespective of the animal species or muscle type.

The current study raises a number of questions worthy of future investigation. Do the satellite cells proliferate throughout the running period, or only during the initial days? Do the satellite cells or their progeny fuse with myofibers during running? Is it possible that the expansion of the satellite cell pool in exercised animals is due to Do some of the satellite cells observed at the end of the 8-week period represent the original satellite cells or do all cells go through at least one proliferative round giving rise to differentiated progeny and replenishing also the reserve pool of satellite cells? Is the proliferative potential of single satellite cells enhanced/reduced after the 8-week running? Our hypothesis is that there is no decline in satellite cell proliferative potential as a result of the running, a proposition supported by the clonal performance of the satellite cells and the large number of cells that developed in the myogenic clones from myofibers of exercised mice, regardless of age.

Our recent studies where we monitored the number of cells in day 10 myogenic clones, demonstrated an average of ~2100 (± 260 SEM, $n=54$) cells per clone when satellite cells were isolated from old mice, similar to what we measured for young mice (Stuelsatz P, Shearer A, Yablonka-Reuveni Z, unpublished). However, the production of satellite cell-like, reserve cells ($\text{Pax7}^+/\text{MyoD}^-/\text{NES-GFP}^+$) declined in clones from old age [10]. Hence, it would be of interest to investigate satellite cell dynamics in a paradigm of a long running period extending the duration of the exercise routine of the young group all the way to the age of the old group, and sampling myofibers and muscle sections for satellite cell numbers and expression of satellite cell proliferation/differentiation distinctive markers (i.e., Pax7, MyoD, myogenin, Ki67) at different time points throughout this extended running period. We hypothesize that such long-term running may support maintenance of satellite cell numbers in the old age group at a similar level to that of the young group, following the ‘use it or lose it’ concept [69, 70].

In particular, the use of the NES-GFP transgenic mouse to monitor satellite cells has introduced additional aspects that could be pursued in the future. Typically, when we have followed proliferation of NES-GFP⁺ satellite cells in isolated myofibers or primary cultures, the GFP signal was still evident, albeit at lower intensity, in proliferating (BrdU⁺) progeny by 3rd culture day, but diminished on day 4 [62]. While most proliferating cells ($\text{Pax7}^+/\text{MyoD}^+$) transited into the differentiating, myogenin⁺ state, some cells entered the renewal state ($\text{Pax7}^+/\text{MyoD}^-$), which also entailed cell cycle withdrawal and re-appearance of NES-GFP signal [10, 62]. At the conclusion of 8-week running essentially all satellite cells were positive for NES- GFP. Hence, satellite cells either underwent only a minimal number of proliferative rounds during the running period (and therefore, retained a detectable GFP level), or some of the GFP⁺ cells were renewal cells that re-expressed NES-GFP, or possibly GFP signal was not lost when satellite cells remained at the fiber niche, even if the cells might have proliferated multiple times.

Myofiber damage has been recognized as a trigger of satellite cell activation, leading to myonuclear fortification or formation of new myofibers. However, the current moderate-intensity treadmill running is considered not to induce muscle damage. Thus, it is important to determine in future studies whether the increased satellite cell pool detected herein does involve fortification of myofiber nuclei. It is attractive to consider the possibility that this satellite cell expansion may also play a “non-classical” role in muscle homeostasis, independent of myofiber repair. For example, the described cross talk between satellite cells and the microvasculature [71], could be in further demand during chronic treadmill exercise. It is also of interest to consider what might be the key factors involved in satellite cell pool expansion during moderate-intensity treadmill running, assuming that routine tissue removal and repair mechanism following damage are not occurring. The production of myokines (i.e., cytokines and growth factor produced by the muscle itself) appears to be enhanced following physical activity [72]. For example, IL-6 is produced by muscle cells during exercise, and considering its positively affects satellite cell proliferation, could represent a regulator of the exercise-induced satellite cell expansion observed in the current study [73, 74]. Other candidate myokines that could be involved in exercise-induced satellite cell expansion might include hepatocyte growth factor (HGF) and some of the fibroblast growth factors (FGFs) [75–77]. These factors are not only present in skeletal muscle, but promote satellite cell activation and expansion at the myofiber niche as shown in culture conditions where the satellite cells were retained by the parent myofiber [9, 28, 78–80].

In all, we established an experimental paradigm optimal for investigating the regulation of satellite cell performance in vivo in the context of muscle maintenance through life. This moderate-intensity endurance exercise approach can be used with Cre-loxP systems of knockout and overexpressing mice to study the impact of physiological relevance factors on satellite cell dynamics through life.

Materials and Methods

Animals

Transgenic male mice expressing GFP under nestin promoter (NES-GFP, heterozygous, C57Bl/6 strain background) [10, 62, 81] were used for experimental and control run/no-run groups. Animal procedures were conducted according to Tel Aviv University Institutional Animal Care and Use Committee (permit number M-06-095 and M-06-014).

Treadmill running procedure

Moderate-intensity treadmill running was performed using Horizon ID 100 treadmill custom adjusted for rodent experiments (running area= 41 × 114 cm) as previously described [11]. For the present mouse study, treadmill speed was adjusted to 0.69 kph (11.5 meters per minute) and mice ran for 30 minutes a day, 6 times a week, for 8 weeks. The age of young and old mice as specified throughout this study is the age at the beginning of the 8-week run/no-run period.

Treadmill acclimation conditions prior to the 8-week experimental period lasted about 2 weeks. At first, all mice were subjected to two 15-minute sessions (1 a day on consecutive days) in which the treadmill was turned on, while mice were kept in their cages, this was done in order to familiarize mice to the treadmill noise. For the next 3 days mice assigned to the exercise group were placed in the treadmill for 15–20 minutes without turning the treadmill on. Finally, mice assigned to the exercise group were allowed to run for 3 minutes for 2 days and then 1 or 2 minutes/day were added to the running period until all mice were able to run 30 minutes straight.

Satellite cell quantification in isolated myofibers

Satellite cells were quantified based on the number of NES-GFP⁺ cells in individual myofibers isolated from the hindlimb EDL muscles. As we previously demonstrated, there is 95–100% agreement when monitoring satellite cells by NES-GFP in live or fixed myofibers versus Pax7 immunostaining using fixed myofibers [10, 62]. Each mouse was processed separately for myofiber isolation and satellite cell quantification using our standard protocols for EDL myofiber isolation from young and old mice. Myofibers were released by repetitive gentle trituration of the EDLs after digestion with 0.2% collagenase type I (Sigma-Aldrich, St. Louis, Missouri) for 90–120 minutes [9, 82] and the released myofibers were rinsed extensively as detailed in our recent protocol updates to eliminate residual interstitial cells released during the procedure [10, 83]. GFP⁺ satellite cells were counted on live myofibers using fluorescent microscope [10]. Per each group, data from individual mice were pooled after establishing a common satellite cell distribution pattern across mice using ANOVA.

Satellite cell clonal analysis

Cloning of satellite cells from individual EDL myofibers of NES-GFP mice was performed following our published procedure [9]. The growth medium consisted of DMEM (high glucose, with l-glutamine, 110 mg/L sodium pyruvate, and pyridoxine hydrochloride supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen) supplemented with 10% horse serum, 20% fetal bovine serum and 1% chicken embryo extract (Biological Industries, Beit Haemek, Israel). Individual myofibers were transferred into 1.2 ml of warm growth medium in individual tube and then triturated to strip satellite cells from the parent myofiber. Each triturated suspension was dispensed in 100-µl aliquots into 12 Matrigel-coated wells in 24-well tissue culture trays. Trays were left undisturbed for 4 days to allow cell adherence and initiation of clonal growth. Each well was then fortified with 250 µl of fresh growth medium followed by replacement of the culture medium with fresh 500 µl growth medium every other day, starting on culture day 5. Clonal growth was first analyzed on culture days 4–5 and all wells were monitored for clonal growth for a total of 14 days. Myogenic clones were identified morphologically by the presence of myotubes, typically found by 7–10 days in culture. Some clones were defined as non-myogenic by the absence of myotubes and the presence of distinctive large and flat morphology of the cells. These non-myogenic clones were negative for myogenic markers such as MyoD and sarcomeric myosin when analyzed by immunostaining.

Microscopy

Observations were done with an inverted fluorescent microscope (Zeiss, Axiovert 200M), controlled by Axiovision 4.4 Imaging System. Images were acquired with an AxioCam MRm monochrome CCD camera and composites of digitized images were assembled using Adobe Photoshop software.

Statistics

Statistical analyses of satellite cell numbers per myofiber were performed using Statistica 9 software. Analysis of variance was tested with the parametric MANOVA (multiple analyses of variance) test. When significant differences were found they were followed by post-hoc Fisher LSD test for comparisons. For all tests, *P* values less than 0.05 were considered significant. Descriptive statistics of the data are depicted in boxplots. Data are organized in quartiles with outlier (defined in the legend to Fig. 1) depicted in circles

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Abbreviations

NES-GFP	Nestin-GFP
EDL	extensor digitorum longus

References

1. Mauro A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol.* 1961; 9:493–5. [PubMed: 13768451]
2. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 Is Required for the Specification of Myogenic Satellite Cells. *Cell.* 2000; 102:777–786. [PubMed: 11030621]
3. Yablonka-Reuveni Z. The skeletal muscle satellite cell: still young and fascinating at 50. *J Histochem Cytochem.* 2011; 59:1041–59. [PubMed: 22147605]
4. Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development.* 2012; 139:2845–56. [PubMed: 22833472]
5. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell.* 2005; 122:289–301. [PubMed: 16051152]
6. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature.* 2008; 456:502–6. [PubMed: 18806774]
7. Gibson MC, Schultz E. Age-related differences in absolute numbers of skeletal muscle satellite cells. *Muscle Nerve.* 1983; 6:574–80. [PubMed: 6646160]
8. Brack AS, Bildsoe H, Hughes SM. Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy. *J Cell Sci.* 2005; 118:4813–4821. [PubMed: 16219688]
9. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z. Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. *Dev Biol.* 2006; 294:50–66. [PubMed: 16554047]
10. Day K, Shefer G, Shearer A, Yablonka-Reuveni Z. The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. *Dev Biol.* 2010; 340:330–43. [PubMed: 20079729]
11. Shefer G, Rauner G, Yablonka-Reuveni Z, Benayahu D. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS ONE.* 2010; 5:e13307. [PubMed: 20967266]
12. Collins CA, Zammit PS, Ruiz AP, Morgan JE, Partridge TA. A population of myogenic stem cells that survives skeletal muscle aging. *Stem Cells.* 2007; 25:885–894. [PubMed: 17218401]
13. Neal A, Boldrin L, Morgan JE. The satellite cell in male and female, developing and adult mouse muscle: distinct stem cells for growth and regeneration. *PLoS ONE.* 2012; 7:e37950. [PubMed: 22662253]
14. Renault V, Thorne LE, Eriksson PO, Butler-Browne G, VM. Regenerative potential of human skeletal muscle during aging. *Aging Cell.* 2002; 1:132–139. [PubMed: 12882343]
15. Verdijk LB, Dirks ML, Snijders T, Prompers JJ, Beelen M, Jonkers RA, Thijssen DH, Hopman MT, LJ VANL. Reduced satellite cell numbers with spinal cord injury and aging in humans. *Med Sci Sports Exerc.* 2012; 44:2322–30. [PubMed: 22776875]
16. Carlson BM, Faulkner JA. Muscle transplantation between young and old rats: age of host determines recovery. *Am J Physiol.* 1989; 256:C1262–6. [PubMed: 2735398]

17. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*. 2005; 433:760–4. [PubMed: 15716955]
18. Rosenberg IH. Epidemiologic and Methodologic Problems in Determining Nutritional-Status of Older Persons - Proceedings of a Conference Held in Albuquerque, New Mexico, October 19–21, 1988 - Summary Comments. *Am J Clin Nutr*. 1989; 50:1231–1233.
19. Rosenberg IH. Sarcopenia: origins and clinical relevance. *Clin Geriatr Med*. 2011; 27:337–9. [PubMed: 21824550]
20. Carmeli E, Coleman R, Reznick AZ. The biochemistry of aging muscle. *Exp Gerontol*. 2002; 37:477–89. [PubMed: 11830351]
21. Thompson LV. Age-related muscle dysfunction. *Exp Gerontol*. 2009; 44:106–11. [PubMed: 18657920]
22. Arthur ST, Cooley ID. The effect of physiological stimuli on sarcopenia; impact of Notch and Wnt signaling on impaired aged skeletal muscle repair. *Int J Biol Sci*. 2012; 8:731–60. [PubMed: 22701343]
23. Brack AS, Rando TA. Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. *Stem Cell Rev*. 2007; 3:226–37. [PubMed: 17917136]
24. Carlson ME, Conboy MJ, Hsu M, Barchas L, Jeong J, Agrawal A, Mikels AJ, Agrawal S, Schaffer DV, Conboy IM. Relative roles of TGF-beta1 and Wnt in the systemic regulation and aging of satellite cell responses. *Aging Cell*. 2009; 8:676–89. [PubMed: 19732043]
25. Brooks SV, Faulkner JA. Contractile properties of skeletal muscles from young, adult and aged mice. *J Physiol*. 1988; 404:71–82. [PubMed: 3253447]
26. Grounds MD. Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. *Ann N Y Acad Sci*. 1998; 854:78–91. [PubMed: 9928422]
27. Faulkner JA, Larkin LM, Claflin DR, Brooks SV. Age-related changes in the structure and function of skeletal muscles. *Clin Exp Pharmacol Physiol*. 2007; 34:1091–6. [PubMed: 17880359]
28. Yablonka-Reuveni Z, Rivera AJ. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol*. 1994; 164:588–603. [PubMed: 7913900]
29. Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol*. 2004; 166:347–57. [PubMed: 15277541]
30. Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell*. 2007; 129:999–1010. [PubMed: 17540178]
31. Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*. 2001; 91:534–51. [PubMed: 11457764]
32. Lepper C, Partridge TA, Fan CM. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*. 2011; 138:3639–46. [PubMed: 21828092]
33. Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development*. 2011; 138:3625–37. [PubMed: 21828091]
34. Sambasivan R, Yao R, Kissenpfennig A, Van Wittenberghe L, Paldi A, Gayraud-Morel B, Guenou H, Malissen B, Tajbakhsh S, Galy A. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development*. 2011; 138:3647–56. [PubMed: 21828093]
35. Chakkalakal JV, Jones KM, Basson MA, Brack AS. The aged niche disrupts muscle stem cell quiescence. *Nature*. 2012; 490:355–60. [PubMed: 23023126]
36. Shefer G, Benayahu D. SVEP1 is a novel marker of activated pre-determined skeletal muscle satellite cells. *Stem Cell Rev*. 2010; 6:42–9. [PubMed: 20052625]
37. Joya JE, Kee AJ, Nair-Shalliker V, Ghoddusi M, Nguyen MAT, Luther P, Hardeman EC. Muscle weakness in a mouse model of nemaline myopathy can be reversed with exercise and reveals a novel myofiber repair mechanism. *Hum Mol Genet*. 2004; 13:2633–2645. [PubMed: 15367485]

38. Sveen ML, Jeppesen TD, Hauerslev S, Køber L, Krag TO, Vissing J. Endurance training improves fitness and strength in patients with Becker muscular dystrophy. *Brain*. 2008; 131:2824–2831. [PubMed: 18776212]
39. Wenz T, Diaz F, Hernandez D, Moraes CT. Endurance exercise is protective for mice with mitochondrial myopathy. *J Appl Physiol*. 2009; 106:1712–9. [PubMed: 19286571]
40. Sveen ML, Jeppesen TD, Hauerslev S, Krag TO, Vissing J. Endurance training: an effective and safe treatment for patients with LGMD2I. *Neurology*. 2007; 68:59–61. [PubMed: 17200494]
41. Cermak NM, Snijders T, McKay BR, Parise G, Verdijk LB, Tarnopolsky MA, Gibala MJ, Loon LJ. Eccentric exercise increases satellite cell content in type II muscle fibers. *Med Sci Sports Exerc*. 2012 Oct 2. [Epub ahead of print].
42. Snijders T, Verdijk LB, Beelen M, McKay BR, Parise G, Kadi F, van Loon LJ. A single bout of exercise activates skeletal muscle satellite cells during subsequent overnight recovery. *Exp Physiol*. 2012; 97:762–73. [PubMed: 22327327]
43. Walker DK, Fry CS, Drummond MJ, Dickinson JM, Timmerman KL, Gundersmann DM, Jennings K, Volpi E, Rasmussen BB. PAX7+ satellite cells in young and older adults following resistance exercise. *Muscle Nerve*. 2012; 46:51–9. [PubMed: 22644638]
44. Freiburger E, Sieber C, Pfeifer K. Physical activity, exercise, and sarcopenia -future challenges. *Wien Med Wochenschr*. 2011; 161:416–25. [PubMed: 21792532]
45. Trappe S, Hayes E, Galpin AJ, Kaminsky LA, Jemiolo B, Fink WJ, Trappe TA, Jansson A, Gustafsson T, Tesch PA. New Records In Aerobic Power Among Octogenarian Lifelong Endurance Athletes. *J Appl Physiol*. 2012
46. Radak Z, Naito H, Kaneko T, Tahara S, Nakamoto H, Takahashi R, Cardozo-Pelaez F, Goto S. Exercise training decreases DNA damage and increases DNA repair and resistance against oxidative stress of proteins in aged rat skeletal muscle. *Pflugers Arch*. 2002; 445:273–8. [PubMed: 12457248]
47. Baltgalvis KA, Call JA, Cochrane GD, Laker RC, Yan Z, Lowe DA. Exercise training improves plantarflexor muscle function in mdx mice. *Med Sci Sports Exerc*. 2012 Mar 28. [Epub ahead of print].
48. Boveris A, Navarro A. Systemic and mitochondrial adaptive responses to moderate exercise in rodents. *Free Radic Biol Med*. 2008; 44:224–9. [PubMed: 18191758]
49. Navarro A, Gomez C, Lopez-Cepero JM, Boveris A. Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer. *Am J Physiol Regul Integr Comp Physiol*. 2004; 286:R505–11. [PubMed: 14615275]
50. Pasini E, Le Douaron Lahaye S, Flati V, Assanelli D, Corsetti G, Specia S, Bernabei R, Calvani R, Marzetti E. Effects of treadmill exercise and training frequency on anabolic signaling pathways in the skeletal muscle of aged rats. *Exp Gerontol*. 2012; 47:23–8. [PubMed: 22015326]
51. Kohman RA, DeYoung EK, Bhattacharya TK, Peterson LN, Rhodes JS. Wheel running attenuates microglia proliferation and increases expression of a proneurogenic phenotype in the hippocampus of aged mice. *Brain Behav Immun*. 2012; 26:803–10. [PubMed: 22056294]
52. Kurosaka M, Naito H, Ogura Y, Kolima A, Goto K, Katamoto S. Effects of voluntary wheel running on satellite cells in the rat plantaris muscle. *J Sports Sci Med*. 2009; 8:51–57.
53. Kurosaka M, Naito H, Ogura Y, Machida S, Katamoto S. Satellite cell pool enhancement in rat plantaris muscle by endurance training depends on intensity rather than duration. *Acta Physiol*. 2012; 205:159–66.
54. Li P, Akimoto T, Zhang M, Williams RS, Yan Z. 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*. 2006; 290:C1461–8. [PubMed: 16407413]
55. Leblond H, L'Esperance M, Orsal D, Rossignol S. Treadmill locomotion in the intact and spinal mouse. *J Neurosci*. 2003; 23:11411–9. [PubMed: 14673005]
56. Jeneson JAL, De Snoo MW, Verlinden NAT, Joosten BJLJ, Doornenbal A, Schot A, Everts ME. Treadmill but not wheel running improves fatigue resistance of isolated extensor digitorum longus muscle in mice. *Acta Physiol*. 2007; 190:151–161.

57. Lang T, Streeper T, Cawthon P, Baldwin K, Taaffe DR, Harris TB. Sarcopenia: etiology, clinical consequences, intervention, and assessment. *Osteoporos Int.* 2010; 21:543–59. [PubMed: 19779761]
58. Catoire M, Mensink M, Boekschoten MV, Hangelbroek R, Muller M, Schrauwen P, Kersten S. Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle. *PLoS ONE.* 2012; 7:e51066. [PubMed: 23226462]
59. Tajbakhsh S. Losing stem cells in the aged skeletal muscle niche. *Cell Res.* 2013
60. LeBrasseur NK, Schelhorn TM, Bernardo BL, Cosgrove PG, Loria PM, Brown TA. Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. *J Gerontol A Biol Sci Med Sci.* 2009; 64:940–8. [PubMed: 19483181]
61. Savage KJ, McPherron AC. Endurance exercise training in myostatin null mice. *Muscle Nerve.* 2010; 42:355–62. [PubMed: 20544938]
62. Day K, Shefer G, Richardson JB, Enikolopov G, Yablonka-Reuveni Z. Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. *Dev Biol.* 2007; 304:246–59. [PubMed: 17239845]
63. Jackson JR, Mula J, Kirby TJ, Fry CS, Lee JD, Ubele MF, Campbell KS, McCarthy JJ, Peterson CA, Dupont-Versteegden EE. Satellite cell depletion does not inhibit adult skeletal muscle regrowth following unloading-induced atrophy. *Am J Physiol Cell Physiol.* 2012; 303:C854–61. [PubMed: 22895262]
64. McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C, Van Zant G, Campbell KS, Esser KA, Dupont-Versteegden EE, Peterson CA. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development.* 2011; 138:3657–66. [PubMed: 21828094]
65. Wasserstrom A, Adar R, Shefer G, Frumkin D, Itzkovitz S, Stern T, Shur I, Zangi L, Kaplan S, Harmelin A, Reisner Y, Benayahu D, Tzahor E, Segal E, Shapiro E. Reconstruction of cell lineage trees in mice. *PLoS ONE.* 2008; 3:e1939. [PubMed: 18398465]
66. Lu QL, Morris GE, Wilton SD, Ly T, Artem'yeva OV, Strong P, Partridge TA. Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J Cell Biol.* 2000; 148:985–96. [PubMed: 10704448]
67. Kanisicak O, Mendez JJ, Yamamoto S, Yamamoto M, Goldhamer DJ. Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD. *Dev Biol.* 2009; 332:131–41. [PubMed: 19464281]
68. Starkey JD, Yamamoto M, Yamamoto S, Goldhamer DJ. Skeletal muscle satellite cells are committed to myogenesis and do not spontaneously adopt nonmyogenic fates. *J Histochem Cytochem.* 2011; 59:33–46. [PubMed: 21339173]
69. Adamo ML, Farrar RP. Resistance training, and IGF involvement in the maintenance of muscle mass during the aging process. *Ageing Res Rev.* 2006; 5:310–31. [PubMed: 16949353]
70. Power GA, Dalton BH, Behm DG, Vandervoort AA, Doherty TJ, Rice CL. Motor unit number estimates in masters runners: use it or lose it? *Med Sci Sports Exerc.* 2010; 42:1644–50. [PubMed: 20142771]
71. Christov C, Chretien F, Abou-Khalil R, Bassez G, Vallet G, Authier FJ, Bassaglia Y, Shinin V, Tajbakhsh S, Chazaud B, Gherardi RK. Muscle satellite cells and endothelial cells: close neighbors and privileged partners. *Mol Biol Cell.* 2007; 18:1397–409. [PubMed: 17287398]
72. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat Rev Endocrinol.* 2012; 8:457–65. [PubMed: 22473333]
73. Hiscock N, Chan MH, Bisucci T, Darby IA, Febbraio MA. Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB J.* 2004; 18:992–4. [PubMed: 15059966]
74. Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab.* 2008; 7:33–44. [PubMed: 18177723]

75. Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol.* 1998; 194:114–28. [PubMed: 9473336]
76. Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, Allen RE. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. *Mol Biol Cell.* 2002; 13:2909–18. [PubMed: 12181355]
77. Shefer, G.; Yablonka-Reuveni, Z. Ins and outs of satellite cell myogenesis: the role of the ruling growth factors. In: Partridge, SSaT, editor. *Skeletal Muscle Repair and Regeneration, Advances in Muscle Research.* Springer; Netherlands: 2008. p. 107-144.
78. Kastner S, Elias MC, Rivera AJ, Yablonka-Reuveni Z. Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. *J Histochem Cytochem.* 2000; 48:1079–96. [PubMed: 10898801]
79. Wozniak AC, Anderson JE. Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. *Dev Dyn.* 2007; 236:240–50. [PubMed: 17117435]
80. Yablonka-Reuveni Z, Seger R, Rivera AJ. Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. *J Histochem Cytochem.* 1999; 47:23–42. [PubMed: 9857210]
81. Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J Comp Neurol.* 2004; 469:311–24. [PubMed: 14730584]
82. Shefer G, Yablonka-Reuveni Z. Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. *Methods Mol Biol.* 2005; 290:281–304. [PubMed: 15361669]
83. Keire P, Shearer A, Shefer G, Yablonka-Reuveni Z. Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. *Methods Mol Biol.* 2013; 946:431–68. [PubMed: 23179849]

Fig. 1.

Quantification of GFP⁺ satellite cells in EDL myofibers isolated from sedentary and exercised NES-GFP male mice. Satellite cells were recorded per individual myofibers isolated from young (4 months) and old (16-months) mice. Age of mice specified is the age at the beginning of the 8-week run/no-run period. **(A)** Summary of experimental details of the current figure. Each mouse was processed separately for satellite cell quantification and data within each group were pooled after establishing (by ANOVA) a common satellite cell distribution pattern across mice. **(B)** Satellite cell numbers per individual myofibers summarized as boxplots. The box represents the interquartile range (IQR) which spans from the 1st to the 3rd quartile (Q1 and Q3), the dividing line within the box is the median, and the white marks show the average ± SEM. Outliers (red circles, right Y-axis) are defined as being either greater than $Q3 + 1.5 \times IQR$ or less than $Q1 - 1.5 \times IQR$. The whiskers on each side of the box are taken to the minimum and maximum values or when outliers are present to $1.5 \times IQR$ from Q1 and Q3 (<http://www.physics.csbsju.edu/stats/box2.html>). Outliers identified in the young/exercised group were contributed by two mice. **(C)** Myofiber distribution according to satellite cell numbers. Myofibers are arranged in ascending order according to the number of satellite cells per myofiber (X-axis) versus the number of myofibers containing a given number of satellite cells (Y-axis). The exercise-driven increase in the number of satellite cells per myofiber is also illustrated in the cumulative curves. Each data point (diamond-shaped, right Y-axis) in these cumulative curves shows the percentage of myofibers out of total myofibers analyzed that contain less than, or equal to () the corresponding number of satellite cells per myofiber that is indicated on the X-axis.

Fig. 2. Quantification of myogenic clones derived from individual EDL myofibers of young and old mice. **(A)** Summary of the number of myofibers and clones analyzed for the current figure. Clones were prepared from myofibers isolated from mice detailed in Fig. 1, investigating 3–4 myofibers per mouse and data were then pulled within each group. **(B)** Myogenic clone data are illustrated in boxplots, depicting quartile distribution, average ± SEM (white marks) and outliers (red circles) for the number of clones per myofiber, as detailed in Fig. 1B. Right Y-axis provides the scale of outlier data points. Outliers identified in the young/sedentary group were contributed by two mice. **(C)** Myofiber distribution according to the number of myogenic clones per each myofiber and cumulative curves. Myofibers are arranged in ascending order according to the number of myogenic clones per myofiber (X-axis) versus the number of myofibers containing a given number of myogenic clones (Y-axis). In the cumulative curves, each data point (diamond-shaped, right Y-axis) shows the percentage of myofibers out of total myofibers analyzed that contain less than, or equal to () the corresponding number of myogenic clones per myofiber that is indicated on the X-axis. **(D–G)** Phase images of myogenic clones developed from individual myofibers isolated from exercised young (D and E) and old (F and G) mice. Images were taken at 2-weeks and depict for each age group representative lower (D and F) and higher (E and G) density clones (10x objective).