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

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Satellite cells, the myogenic progenitors located at the myofibre surface, are essential for the repair of adult skeletal muscle. There is ample evidence for an age-linked decline in the number of satellite cells and performance in limb muscles. Hence, an effective means of activating and expanding the satellite cell pool may enhance muscle maintenance and reduce the impact of age-associated muscle deterioration (sarcopaenia). Accordingly, in the present study, we explored the beneficial effects 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, $11.5\ \mathrm{m{\cdot}min^{-1}}$ for $30\ \mathrm{min{\cdot}day^{-1}}$, $6\ \mathrm{days{\cdot}week^{-1}}$). Myofibres of extensor digitorum longus muscles were then isolated from exercised and sedentary mice and used for monitoring the number of satellite cells, as well as for harvesting individual satellite cells for clonal growth assays. We specifically focused on satellite cell pools of single myofibres, with the view that daily wear of muscles probably affects individual myofibres rather than causing overall muscle damage. We found an expansion of the satellite cell pool in the exercised groups compared to the sedentary groups, with the same increase (~ 1.6-fold) in both ages. The results of the present study are in agreement with our findings obtained using rat gastrocnemius, indicating the consistent effect of exercise on satellite cell expansion in limb muscles. The experimental paradigm established in the present study is useful for investigating satellite cell dynamics at the myofibre niche, as well as for broader investigations of the impact of physiologically and pathologically relevant factors on adult myogenesis.

Introduction

Satellite cells are Pax7-expressing ($\mathrm{Pax7^+}$) myogenic progenitors residing between the myofibre basal and plasma membranes and are essential for the 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 myofibre repair, thereby ensuring the maintenance of the satellite cell pool [5,6]. Routine muscle activity, even if it involves only subtle myofibre injuries, raises a continuous demand for functioning satellite cells throughout life. Rodent and human studies have shown, within the context of limb muscles, that there is an age-associated decline in the number of satellite cells and performance [7–17]. This diminution may contribute to age-related muscle deterioration, a condition known as sarcopaenia. Sarcopaenia is

Abbreviations
ANOVA, analysis of variance; EDL, extensor digitorum longus; NES-GFP, nestin-green fluorescent protein.

characterized by a decline in the 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 assumed to play a role in this process [22–26]. Such muscle deterioration involves a reduction in myofibre number, atrophy of the remaining myofibres and an increased susceptibility to contraction-induced injuries [27]. Combating sarcopaenia requires the identification of a means to improve myofibre maintenance in ageing muscle. Therefore, a better understanding of satellite cell dynamics at their natural niche, the myofibre, is essential.

The vast majority of satellite cells are typically quiescent in adult limb muscles. This inactivity, combined with a lack of evidence for satellite cell fusion with myofibres in uninjured muscles, has cast doubt on the importance of satellite cells in 'baseline' myofibre maintenance (i.e. homeostasis) through adult life. However, the sedentary lifestyle of rodents (i.e. standard cage conditions, which is most frequently used in satellite cell research) is not necessarily an optimal environment for promoting the recruitment of satellite cells into performance.

Studies with isolated myofibres, using conditions that retain satellite cells at their niche, have shown that this positional intimacy with the myofibre is essential for the satellite cell interplay between progeny expansion versus self-renewal [28–30]. Nevertheless, much of our present understanding of *in vivo* myogenesis in adult life is based on models of induced muscle trauma [31,32]. These studies have typically relied on the use of agents such as snake venom or barium chloride, or exhaustive eccentric exercise protocols that cause extensive muscle damage [33,34]. Although such muscle injury approaches have provided a wealth of knowledge about muscle regeneration, they do not retain the muscle architecture and physiological milieu associated with daily muscle use. Furthermore, daily muscle use inflicts subtle myofibre damage requiring localized myofibre repair. It is reasonable to assume that this process relies on the performance of individual resident satellite cells in a few myofibres at a time, rather than on the *en masse* satellite activation that is characteristic of regeneration after massive muscle damage. Thus, to gain insight into the satellite cell pool through life, we have studied satellite cells at the single-cell level in isolated myofibres, demonstrating a drastic age-associated diminution of the satellite cell pool in mice and rats [9–11]. This decline may represent a 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 was considered not to inflict muscle trauma and is even beneficial in certain myopathies [37–40]. Although many human and rodent studies have shown an enhanced number of satellite cells after a single bout of eccentric activity [41–43], studies of satellite cells using *in vivo* models that do not involve muscle damage are scarce. Endurance exercise is beneficial for many aspects of human health [44,45]. Similarly, rodent studies have demonstrated the beneficial effects of endurance exercise, including lifespan and healthspan 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 investigated the number of satellite cells in the rat plantaris muscle using voluntary wheel running [52] and we have studied the effect of moderate-intensity, treadmill running (0° inclination) on the 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, whereas mice subjected to voluntary wheel running vary widely with respect to travelled distance outcomes. Treadmill-running endurance training engages muscle groups that are linked to toe and ankle functions [11,55,56]. These include the extensor digitorum longus (EDL), tibialis anterior, flexor digitorum brevis and gastrocnemius muscles. These fast twitch muscles are affected by ageing [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 investigated satellite cell pools of individual EDL myofibres, with the view that the daily wear of muscles probably affects individual myofibres rather than causing overall muscle damage, as occurs in response to robust trauma. Specifically, we monitored the number of satellite cells as an overall measure of satellite cell pool homeostasis and performance, which depends on the dynamic between quiescence, proliferation, renewal and the survival of satellite cells, as well as their progeny [28,35,59]. We demonstrated that moderate-intensity treadmill running elevates the number of satellite cells in young and old mice. The results of the present study are in agreement with our findings obtained using rat gastrocnemius, demonstrating the consistent effect of exercise running on the number of satellite cells in limb muscles. The experimental paradigm established in the present study is beneficial for studying satellite cell dynamics at the myofibre niche, as well as for broader investigations of the impact of physiologically and pathologically relevant factors on adult myogenesis.

Results and Discussion

Experimental approach

In the present study, we investigated the effect of moderate-intensity treadmill running on the number of satellite cells and performance in young (4 months) and old (16 months) male mice. The age of mice reported throughout the present study reflects their age at the beginning of the 8-week period of the experiment (i.e. exercise or sedentary schedule). Hindlimb (EDL) muscles were harvested at the end of this period for single myofibre isolation to quantify the number of satellite cells and to perform clonal growth analysis.

The exercise groups were subjected to treadmill running (0° inclination) at a speed of $11.5\ \text{m}\cdot\text{min}^{-1}$ for $30\ \text{min}\cdot\text{day}^{-1}$ (6 days a week for 8 consecutive weeks). The moderate-intensity running conditions used in the present study are comparable to the running conditions that were previously described for young and old mice [60,61].

Transgenic mice expressing green fluorescent protein (GFP) under the control of nestin gene regulatory elements (NES-GFP) were used throughout the present study for detecting satellite cells in isolated myofibres according to GFP expression. As previously reported, NES-GFP^{+} satellite cells are easily distinguished from myonuclei that are GFP-negative, whereas both satellite cells and myonuclei are commonly stained with 4′,6-diamidino-2-phenylindole [62]. Regardless of mouse age, there is > 95% agreement when satellite cells are detected by the endogenous marker Pax7 versus the transgenic marker NES-GFP [10,62].

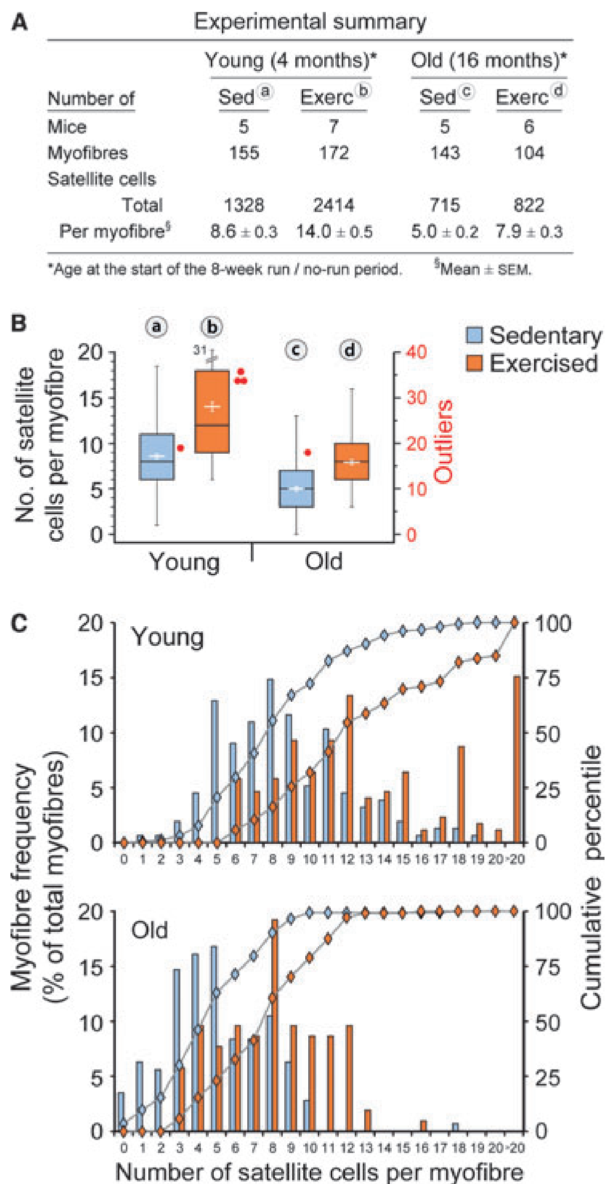
Running enhances the number of satellite cells in young and old mice

The number of satellite cells (NES-GFP^{+}) per individual EDL myofibre was determined in sedentary and exercised, young and old mice (Fig. 1). Per each group, the total number of mice, myofibres and satellite cells analyzed, and the mean (± SEM) number of satellite cells per myofibre, are provided in Fig. 1A. Further details of satellite cell distribution per individual myofibres are summarized in Fig. 1B, which includes quartile analysis and the mean ± SEM number of satellite cells per myofibre. Significant effects of age, exercise and a combination of age and exercise were observed [two-way analysis of variance (ANOVA), $F_{1,569} = 177.28$, 129.20 and 11.79, respectively; $P < 0.001$]. The number of satellite cells declined with age, whereas running enhanced the number of satellite cells per myofibre of both young and old mice. Myofibres from young exercised mice contained the highest mean number of satellite cells compared to young sedentary, old exercised and old sedentary groups (mean ± SEM: 14.0 ± 0.5 versus 8.6 ± 0.3, 7.9 ± 0.3 and 5.0 ± 0.2, respectively).

Exercise induced a significant increase in mean number of satellite cells in both young and old mice ($P < 0.005$ for both ages). This is reflected in an overall shift toward a higher number of satellite cells per myofibre 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 elevated the mean number of satellite cells in myofibres of old exercised mice to the same level as that measured in young sedentary mice. Remarkably, the ratio between the mean number of satellite cells per myofibre in exercised and sedentary mice is conserved across the young and old groups (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 counterparts.

The effect of exercise on satellite cells is further shown in Fig. 1C, which depicts the distribution of myofibres according to the number of satellite cells. Myofibres are arranged in ascending order of the number of satellite cells per myofibre (*x*-axis) versus the number of myofibres containing a given number of satellite cells (*y*-axis). The exercise-driven increase in the number of satellite cells per myofibre 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 myofibres (out of the total myofibres analyzed) that contain less than, or equal to ($\leq$) the corresponding number of satellite cells per myofibre 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 myofibres containing a high number of satellite cells. The median (50% value) number of satellite cells per myofibre rose from 8 (no-run) to 12 (run) for the young mice (Fig. 1B, C). The median number of satellite cells per myofibre in the old mice increased from 5 (no-run) to 8 (run).

Collectively, the analysis of satellite cell content in individual myofibres has demonstrated that moderate-intensity treadmill running in both young and old mice leads to an increase in the frequency of myofibres with a higher number of satellite cells compared to their non-exercised counterparts. Such an effect was also observed in our previous rat study where



A Experimental summary

Number of	Young (4 months)* Sed [a]	Young (4 months)* Exerc [b]	Old (16 months)* Sed [c]	Old (16 months)* Exerc [d]
Mice	5	7	5	6
Myofibres	155	172	143	104
Satellite cells				
Total	1328	2414	715	822
Per myofibre[§]	8.6 ± 0.3	14.0 ± 0.5	5.0 ± 0.2	7.9 ± 0.3

*Age at the start of the 8-week run / no-run period. [§]Mean ± SEM.

Fig. 1. Quantification of GFP+ satellite cells in EDL myofibres isolated from sedentary and exercised NES-GFP male mice. Satellite cells were recorded using individual myofibres isolated from young (4 months) and old (16 months) mice. The reported age of the mice is the age at the beginning of the 8-week run/no-run period. (A) Summary of experimental details. 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) The number of satellite cells per individual myofibres summarized as boxplots. The box represents the interquartile range (IQR), which spans from the first to the third quartile (Q1 and Q3), the dividing line within the box is the median, and the white marks show the mean ± SEM. Outliers (red circles, right *y*-axis) are defined as being either > Q3 + 1.5 × IQR or < Q1 – 1.5 × IQR. The whiskers on each side of the box extend to the minimum and maximum values or, when outliers are present, to 1.5 × IQR from Q1 and Q3 (http://www.physics.csbsju.edu/stats/box2.html). Outliers identified in the young/exercised group emanated from two mice. (C) Myofibre distribution according to the number of satellite cells. Myofibres are arranged in ascending order according to the number of satellite cells per myofibre (*x*-axis) versus the number of myofibres containing a given number of satellite cells (*y*-axis). The exercise-driven increase in the number of satellite cells per myofibre is also shown in the cumulative curves. Each data point (diamond-shaped, right *y*-axis) in these cumulative curves shows the percentage of myofibres out of total myofibres analyzed that contain less than, or equal to (≤) the corresponding number of satellite cells per myofibre indicated on the *x*-axis.

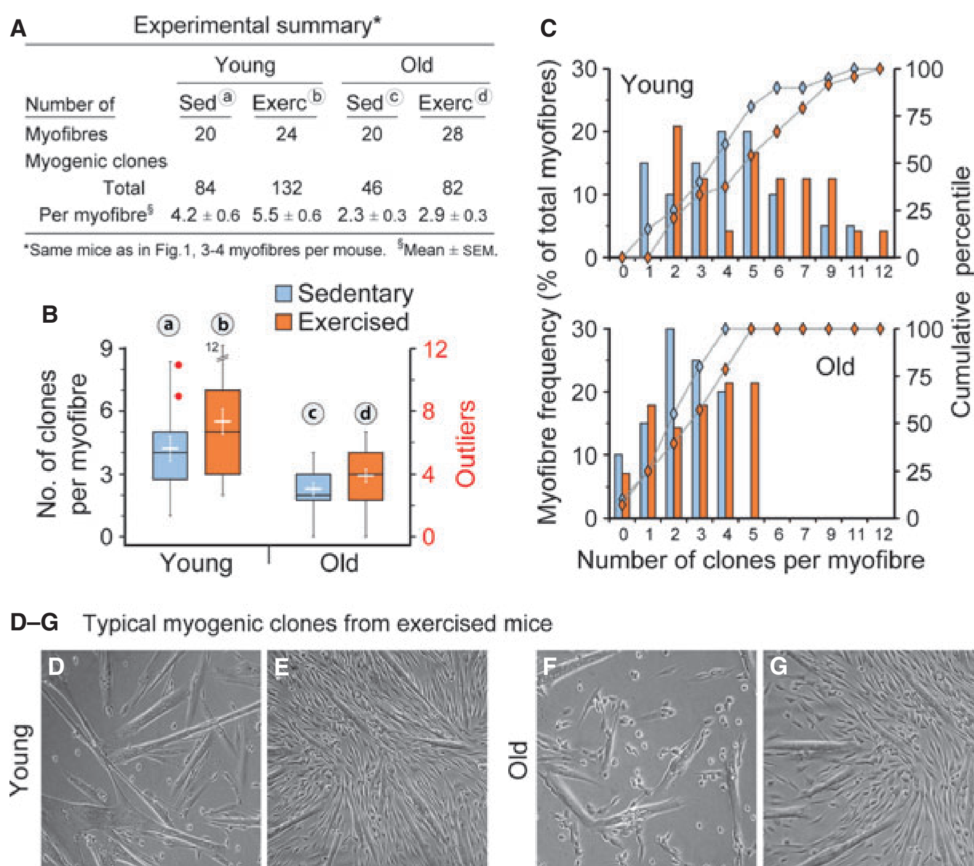
satellite cells were detected based on Pax7 immunostaning [11].

Myofibres lacking satellite cells, which are typically present in old mice and rats [9–11], are not detected after moderate-intensity treadmill running (Fig. 1C). The absence of such myofibres that lack satellite cells after long-term running could be a result of the inability of these myofibres 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 after major trauma has been confirmed [32,33]. Nevertheless, satellite cells do not appear to be essential for muscle hypertrophy or recovery from unloading-induced atrophy in adult life [63,64].

Alternatively, the absence of myofibres void of satellite cells in the exercised old mice could be a result of the reconstitution of their satellite cell pool through contributions from neighbouring progenitors during physical activity. Using a genetic model where ‘cell lineage trees’ can be reconstructed based on a similarity in somatic mutations (in non-coding sequences) between cells, we have previously shown that each myofibre has its own pool of satellite cells [65]. This may suggest that, during standard cage activity, there is no cross-over of satellite cells between myofibres. However, because of the fragility of these genetically modified mice, we are unable to age such animals and establish the number of satellite cells in combination with exercise running. The detection of clonal patches of revertant (dystrophin^{+}) myofibres in mdx mice does support the existence of pioneer myogenic progenitors producing progeny that populate adjacent myofibres during the 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 myofibres (Fig. 2). The same mice that were analyzed for the number of satellite cells (Fig. 1A) were used for the clonal studies (Fig. 2A). Three or four myofibres per mouse were individually triturated to strip the satellite cells. The resulting suspension of each myofibre was dispensed into 12 wells and then followed for the development of clones. Clones were first identified following the third day of culture and subsequently defined as myogenic based on the presence of myotubes that are typically found by 7–10 days in culture [10]. Figure 2A provides a summary for each group of the total number of myofibres and myogenic clones analyzed, and the mean ± SEM number of myogenic clones per myofibre. There was an overall effect of age ($F_{1,88}$ = 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 described above (Fig. 1). The detection of a lower number of myogenic clones per myofibre compared to the number of satellite cell per myofibre (Figs 1A and 2A) is in agreement with previous studies demonstrating a satellite cell clonability of


A Experimental summary*

Number of	Young Sed (a)	Young Exerc (b)	Old Sed (c)	Old Exerc (d)
Myofibres	20	24	20	28
Myogenic clones				
Total	84	132	46	82
Per myofibre§	4.2 ± 0.6	5.5 ± 0.6	2.3 ± 0.3	2.9 ± 0.3

*Same mice as in Fig.1, 3-4 myofibres per mouse. §Mean ± SEM.

Fig. 2. Quantification of myogenic clones derived from individual EDL myofibres of young and old mice. (A) Summary of the number of myofibres and clones analyzed. Clones were prepared from myofibres isolated from mice shown in Fig. 1, investigating three or four myofibres per mouse, and data were then pulled within each group. (B) Myogenic clone data are illustrated as boxplots, depicting the quartile distribution, mean ± SEM (white marks) and outliers (red circles) for the number of clones per myofibre (as detailed in Fig. 1B legend). The right *y*-axis provides a scale for the outlier data points. Outliers identified in the young/sedentary group emanated from two mice. (C) Myofibre distribution according to the number of myogenic clones per myofibre and cumulative curves. Myofibres are arranged in ascending order according to the number of myogenic clones per myofibre (*x*-axis) versus the number of myofibres 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 myofibres out of total myofibres analyzed that contain less than, or equal to (≤) the corresponding number of myogenic clones per myofibre indicated on the *x*-axis. (D–G) Phase images of myogenic clones developed from individual myofibres isolated from exercised young (D, E) and old (F, G) mice. Images were taken at 2 weeks and depict, for each age group, representative lower (D, F) and higher (E, G) density clones (× 10 objective).

~ 50% in young and old mice [10]. The quartile distribution and mean ± SEM number of clones per myofibre are shown for myogenic clones in Fig. 2B. The distribution of myofibres according to the number of myogenic clones and corresponding cumulative curves are shown in Fig. 2C. These data demonstrate a trend toward more myofibres that are capable of generating more clones upon exercise for each age group. This exercise effect is in agreement with that observed previously with myogenic clones from exercised versus sedentary rats of young and old age [11]. Noticeably, the third quartile (75th percentile) value for the sedentary group is equal to the median value for the exercised group (i.e. 5 for young; 3 for old). Furthermore, the increase in the mean number of clones per myofibre in exercised and sedentary mice is conserved between young and old mice (1.31- and 1.27-fold, respectively), as observed for the ratio of the mean number of satellite cells (Fig. 1).

At the morphological level, there was no apparent difference between the myogenic clones from the four groups analyzed. Notably, myofibres from both young and old exercised mice produced typical large myogenic clones, with 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 appear to exhaust the post-exercise proliferative potential of satellite cells. Occasional non-myogenic clones were observed arising from a small number of the myofibres that were processed for satellite cell clonal analysis. Such non-myogenic clones (typically no more than one per myofibre) 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, comprised distinctive large and flat cells that were negative for myogenic markers such as MyoD and sarcomeric myosin when analyzed by immunostaining. These non-myogenic clones probably derive from interstitial cells that are occasionally co-isolated with myofibres and their presence increases with age [10]. Future studies using stable lineage markers of satellite cells, such as $MyoD^{Cre}$-driven reporter expression [67,68], should 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 sarcopaenia, we have investigated the effect of ageing on the number and function of satellite cells and their progeny in rodents [9–11]. In the present study, we focused on the individual and combined effects of exercise running and age on mouse satellite cells. The specific running approach applied in the current study (i.e. long-term moderate-intensity) was previously considered not to inflict apparent muscle damage and even to be beneficial in certain myopathies [37–40]. The positive effects of endurance exercise on the number of satellite cells parallel the findings of our previous study inspecting the gastrocnemius muscle of young and old rats [11]. This attests to the general effect of moderate-intensity treadmill running on the number of satellite cells, irrespective of animal species or muscle type.

The present 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 myofibres during running? 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 the reserve pool of satellite cells? Is the proliferative potential of single satellite cells enhanced or reduced after the 8-week running period? Our hypothesis is that there is no decline in satellite cell proliferative potential as a result of the running. This is supported by the clonal performance of the satellite cells and the large number of cells that developed in the myogenic clones from myofibres of exercised mice, regardless of their age.

Our recent studies where we monitored the number of cells in day 10 myogenic clones, demonstrated a mean ± SEM of 2100 ± 260 (n = 54) cells per clone when satellite cells were isolated from old mice, similar to that measured for young mice (P. Stuelsatz, A. Shearer, Z. Yablonka-Reuveni Z, unpublished data). However, the production of satellite cell-like, reserve cells ($Pax7^{+}/MyoD^{-}/NES\text{-}GFP^{+}$) declined in clones from old age [10]. Hence, it would be interesting 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 myofibres and muscle sections for the number of satellite cells and the expression of satellite cell proliferation/differentiation distinctive markers (i.e. Pax7, MyoD, myogenin, Ki-67) at different time points throughout this extended running period. We hypothesize that such long-term running may support the maintenance of the number of satellite cells in the old age group at a

level similar to that of the young group, in accordance with 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 worthy of further investigation. Typically, when we have followed the proliferation of NES-GFP$^+$ satellite cells in isolated myofibres or primary cultures, the GFP signal was still evident, albeit at lower intensity, in proliferating (BrdU$^+$) progeny by culture day 3 but diminished on day 4 [62]. Although most proliferating cells (Pax7$^+$/MyoD$^+$) transited into the differentiating, myogenin$^+$ state, some cells entered the renewal state (Pax7$^+$/MyoD$^-$), which also entailed cell cycle withdrawal and re-appearance of the NES-GFP signal [10,62]. At the conclusion of running period at 8 weeks, 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 the GFP signal was not lost when satellite cells remained at the fibre niche, even if the cells might have proliferated multiple times.

Myofibre damage has been recognized as a trigger of satellite cell activation, leading to myonuclear fortification or the formation of new myofibres. However, the moderate-intensity treadmill running in the present study is considered not to induce muscle damage. Thus, in future studies, it is important to determine whether the exercise-induced satellite cell expansion involves the fortification of myofibre nuclei. It is possible that this satellite cell expansion also plays a 'non-classical' role in muscle homeostasis, independent of myofibre 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 the key factors involved in satellite cell pool expansion during moderate-intensity treadmill running, assuming that routine tissue removal and repair does not occur. The production of myokines (i.e. cytokines and growth factor produced by the muscle itself) appears to be enhanced after physical activity [72]. For example, interleukin–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 present study [73,74]. Other candidate myokines that could be involved in exercise-induced satellite cell expansion include hepatocyte growth factor and some of the fibroblast growth factors [75–77]. These factors are not only present in skeletal muscle, but also promote satellite cell activation and expansion at the myofibre niche, as shown in culture conditions where the satellite cells were retained by the parent myofibre [9,28,78–81].

In summary, we have established an experimental paradigm that is optimal for investigating the regulation of satellite cell performance *in vivo* in the context of muscle maintenance. This moderate-intensity endurance exercise approach can be used with Cre-loxP systems of knockout and overexpressing mice to study the impact of physiologically relevant factors on satellite cell dynamics throughout life.

Materials and methods

Animals

Transgenic male mice expressing GFP under nestin promoter (NES-GFP, heterozygous, C57Bl/6 strain background) [10,62,82] were used for the experimental and control run/no-run groups. Animal procedures were conducted in accordance with the Tel-Aviv University Institutional Animal Care and Use Committee (permit numbers M-06-095 and M-06-014).

Treadmill running procedure

Moderate-intensity treadmill running was performed using a Horizon ID 100 treadmill (Horizon Fitness, Cottage Grove, WI, USA) adjusted for rodent experiments (running area = 41 × 114 cm), as described previously [11]. For the present study, the treadmill speed was adjusted to 0.69 km·h^{-1} (11.5 m·min^{-1}) and mice ran for 30 min·day^{-1}, six times a week, for 8 weeks. The age of young and old mice as reported throughout the present 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 ~ 2 weeks. Initially, all mice were subjected to two 15-min sessions (one per day on consecutive days) in which the treadmill was turned on when mice were kept in their cages. This was carried out to familiarize mice to the treadmill noise. For the next 3 days, mice assigned to the exercise groups were placed in the treadmill enclosure for 15–20 min without turning the treadmill on. Finally, mice assigned to the exercise groups were allowed to run for 3 min for 2 days, and then 1 or 2 min·day^{-1} were added to the running period until all mice were able to run continuously for 30 min.

Satellite cell quantification in isolated myofibres

Satellite cells were quantified based on the number of NES-GFP$^+$ cells in individual myofibres isolated from the hindlimb EDL muscles. As previously demonstrated, there is 95–100% agreement when monitoring satellite cells by

NES-GFP in live or fixed myofibres versus Pax7 immunostaining using fixed myofibres [10,62]. Each mouse was processed separately for myofibre isolation and satellite cell quantification using our standard protocols for EDL myofibre isolation from young and old mice. Myofibres were released by repetitive gentle trituration of the EDLs after digestion with 0.2% collagenase type I (Sigma-Aldrich, St Louis, MO, USA) for 90–120 min [9,83] and the released myofibres were rinsed extensively to eliminate residual interstitial cells released during the procedure, as described in our recent protocol updates [10,84]. GFP^{+} satellite cells were counted on live myofibres [10]. For 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 myofibres of NES-GFP mice was performed in accordance with a previously described method [9]. The growth medium consisted of DMEM (high glucose, with L-glutamine, 110 $mg{\cdot}L^{-1}$ sodium pyruvate, and pyridoxine hydrochloride supplemented with 50 $U{\cdot}mL^{-1}$ penicillin and 50 $\mu g{\cdot}mL^{-1}$ streptomycin) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% horse serum, 20% fetal bovine serum and 1% chicken embryo extract (Biological Industries, Beit Haemek, Israel). Individual myofibres were transferred into 1.2 mL of warm growth medium in individual tube and then triturated to strip satellite cells from the parent myofibre. 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 500 μL of fresh 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, as typically found after 7–10 days in culture. Some clones were defined as non-myogenic by the absence of myotubes and the presence of a 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 performed using an inverted fluorescent microscope (Axiovert 200M) controlled by AXIOVISION, version 4.4 (Carl Zeiss Inc., Thornwood, NY, USA). Images were acquired with an Axiocam MRm monochrome charge-coupled device camera (Carl Zeiss Inc.) and composites of digitized images were assembled using ADOBE PHOTOSHOP (Adobe Systems, San Jose, CA, USA).

Statistical analysis

Statistical analysis of the number of satellite cells per myofibre was performed using STATISTICA, version 9 (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance was tested with the parametric multiple ANOVA test. When significant differences were found, they were followed by a post-hoc Fisher's least significant difference test for comparisons. $P < 0.05$ was considered statistically significant. Descriptive statistics of the data are depicted as boxplots. Data are organized into quartiles with outliers depicted as circles.

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