THE EFFECT OF NONHYPERTROPHIC STIMULI ON SATELLITE CELLS

THE ROLE OF SATELLITE CELLS IN NONHYPERTROPHIC REMODELLING AND REGENERATION

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

McMaster University

McMaster University DOCTOR OF PHILOSOPHY (2015) Hamilton, Ontario (Kinesiology)

TITLE: The role of satellite cells in nonhypertrophic remodelling and regeneration

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NUMBER OF PAGES: xv, 154.

Lay Abstract

This thesis examined the role of muscle specific stem cells, commonly referred to as satellite cells, in skeletal muscle remodelling. The effect of resistance training on satellite cells has been extensively studied in humans; however, a paucity of information exists with respect to satellite cells and endurance exercise. We first demonstrate that interval training does not result in an increase in the satellite cell pool, but rather a greater number of active cells associated with remodelling fibres. We then describe that various modes of aerobic training lead to an increase in the number of active satellite cells without expansion of the satellite cell pool. Finally, we demonstrate that aerobic exercise training is able to improve skeletal muscle regeneration in old mice and this may be due to an increase in satellite cell content. Our findings highlight that satellite cells likely play a role in both mediating skeletal muscle remodelling and facilitating muscle regeneration to a greater capacity due to aerobic training.

Abstract

Skeletal muscle has the remarkable ability to remodel and repair when exposed to various stimuli such as exercise training and injury. Many factors contribute to the maintenance of healthy muscle mass throughout the lifespan including a functional population of resident muscle stem cells, commonly referred to as satellite cells (SC). When SC become active in response to a stimulus they proliferate and differentiate, eventually fusing to existing myofibres or to each other giving rise to new myotubes; while some SC revert to quiescence to maintain the SC pool. This process is termed 'the myogenic programme' and is governed by a complex network of transcription factors termed myogenic regulatory factors. SC are absolutely necessary for the repair of skeletal muscle, however, their role in mediating skeletal muscle remodelling following exercise training remains debatable. The effect of resistance exercise on SC content has been extensively studied in humans. However, a paucity of information exists in regards to the effect of 'non-resistance' type exercise training on SC content and function in healthy young adults. The purpose of this thesis was to determine the impact of nonhypertrophic exercise training on SC content. We examined the effect of high intensity interval training on the SC pool and determined that there was an increase in SC associated with remodelling hybrid fibres. We extended upon these findings by demonstrating that several high intensity interval training paradigms and traditional endurance training all resulted in an increase in SC pool activity without an overall expansion of the SC pool. Skeletal muscle regeneration is impaired in old rodents and is associated with a reduction in SC content. We therefore sought to determine whether nonhypertrophic exercise training in

old mice was able to improve the regenerative response following injury. Exercise training resulted in an increase in SC content and improved skeletal muscle regeneration in old mice. In addition to previous work implicating SC in mediating skeletal muscle hypertrophy induced by resistance exercise training, this thesis provides evidence that SC are able to respond to exercise stimuli that are nonhypertrophic in nature. In addition, we demonstrate that nonhypertrophic exercise training results in an increase in SC content in old mice and this likely contributed to the improvement in skeletal muscle regeneration observed in old mice pre-conditioned with regular exercise.

Acknowledgements

I'd first like to thank my advisor, Dr. Gianni Parise for his constant support and mentorship over the last several years. From the beginning you've supported my ideas and given me the opportunity and freedom to try new things. I appreciate the countless opportunities and the open environment you created which has allowed me to grow and learn so much during my time at McMaster. I really could not imagine having completed my PhD with another advisor.

To my advisory committee Dr. Phillips and Dr. Gibala thank you for the challenging questions and stimulating discussions during my committee meetings in addition to the numerous individual discussions. I am also grateful for the opportunity you have both given me to collaborate on different projects.

I would also like to thank Todd for all of his assistance and patience over the last four years, from this day forward I promise to properly use 'Chem labels'.

Thank you to all my lab mates: Josh, Tim, Jeff, Bryon, Dan, Carlo, Chris and Leeann you have definitely made coming to the lab everyday an experience on its own. Bryon, although our overlap in the lab was short I appreciate the time you put in getting me up to speed on everything. Josh, I've never met a harder working person; you've made the countless early mornings and late nights more enjoyable. Thank you to the rest of the EMRG lab group.

I'd also like to thank the rest of the Kin grad students for making these last 4 years a memorable life experience. Thank you to Thanh and Amy for your support and friendship. A special thanks to Jess and Alison for countless laughs and shenanigans and for keeping me sane these last few years.

To my family, Mom, Dad thank you for the support and encouragement through my 10+ years in University. I would not have been able to accomplish this without you and the work ethic you've instilled in me. Gabz I always looked forward to your visits and can't wait for you to come visit across the pond!

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Lists of figures and tables

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List of Abbreviations

ANOVA - Analysis of variance

BrdU - 5-bromo-2-deoxyuridine

BSA - Bovine serum albumin

CD56/NCAM - neural cell adhesion molecule

C/Fi - capillary to individual fibre ratio

CK - creatine kinase

CS - citrate synthase

CSA - cross sectional area

CTX - cardiotoxin

DAPI - 4',6-diamidino-2-phenylindole

DSHB - Developmental Studies Hybridoma Bank

EDL - extensor digitorum longus

eMHC - embryonic myosin heavy chain

FBS - Fetal bovine serum

FGF - firboblast growth factor

HGF - hepatocyte growth factor

GS - goat serum

HIT - high intensity interval training

HRmax - maximal heart rate

IGF-1 - insulin like growth factor 1

IL-6 - interleukin 6

LDH - lactate dehydrogenase

MHC - myosin heavy chain

MICT - moderate-intensity continuous exercise

MRF - myogenic regulatory factor

mRNA - messenger ribonucleic acid

nMHC - neonatal myosin heavy chain

OCT - optimum cutting temperature

PCNA - Proliferating cell nuclear antigen

PFA - paraformaldehyde

SC - satellite cell

SIT - high intensity sprint interval training

SDH - succinate dehydrogenase

TA - tibialis anterior

TUNNEL - terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling

VEGF - vascular endothelial growth factor

VO_2peak - peak oxygen consumption

Thesis format and organization

This thesis contains material from the Ph.D. work of Sophie Joanisse and has been prepared in the 'sandwich' format as outlined in the McMaster University School of Graduate Studies' Guide for the Preparation of Master's and Doctoral Theses, The thesis includes a general introduction (Chapter 1), followed by a description of 3 studies that have been prepared as separate manuscripts (Chapters 2-4) and is concluded with a general discussion (Chapter 5).

Declaration of Academic Achievement

Contribution to papers with multiple authorship

Chapter 2:
Sophie Joanisse, Jenna B. Gillen, Leeann M. Bellamy, Bryon R. McKay, Mark A. Tarnopolsky, Martin J. Gibala, and Gianni Parise. (2013). Evidence for the contribution of muscle stem cells to nonhypertrophic skeletal muscle remodeling in humans. *FASEB J.* 27, 4596–4605. DOI: 10.1096/fj.13-229799

Contributions:
G. Parise, M.J. Gibala, J.B. Gillen, and S. Joanisse, conceived and designed the experiments, M.A. Tarnopolsky, J.B. Gillen, and B.R. McKay, collected the data, S. Joanisse, L.M. Bellamy, B. R. McKay and G. Parise analyzed the data; S. Joanisse drafted the manuscript, with input from all other authors.

Chapter 3:
Sophie Joanisse, Bryon R. McKay, Joshua P. Nederveen, Trisha D. Scribbans, Brendon J. Gurd, Jenna B. Gillen, Martin J. Gibala, Mark A. Tarnopolsky, and Gianni Parise. (2015). Satellite cell activity, without expansion, after nonhypertrophic stimuli. *Am J Physiol Regul Integr Comp Physiol* 309: R1101–R1111. DOI: 10.1152/ajpregu.00249.2015

Contributions:
S. Joanisse, B.R. McKay, T. D. Scribbans, B. J. Gurd, J. B. Gillen, M. J. Gibala and G. Parise, conceived and designed the experiments; S. Joanisse, B.R. McKay, J. P. Nederveen, T. D. Scribbans, B. J. Gurd, J. B. Gillen and M.A. Tarnopolsky, performed experiments; S. Joanisse, B.R. McKay, and J. P. Nederveen analyzed data; S. Joanisse, B.R. McKay, J. P. Nederveen, and G. Parise, interpreted results of experiments; S. Joanisse and G. Parise drafted the manuscript; S. Joanisse, B.R. McKay, T. D. Scribbans, B. J. Gurd, J. B. Gillen, M. J. Gibala, M.A. Tarnopolsky, and G. Parise, approved final version of manuscript.

Chapter 4:
Sophie Joanisse, Joshua P. Nederveen, and Gianni Parise. Exercise conditioning in old mice improves skeletal muscle regeneration. Submitted to

Contributions:
S. Joanisse, and G. Parise conceived and designed the experiments; S. Joanisse, and J.P. Nederveen, performed the experiments; S. Joanisse analyzed the data; S. Joanisse, J.P. Nederveen, and G. Parise interpreted results of experiments; S. Joanisse and G. Parise drafted the manuscript; S. Joanisse, J.P. Nederveen, and G. Parise approved final version of manuscript

Chapter 1: General Introduction

1.1 Skeletal muscle satellite cells

Skeletal muscle is one of the largest organs in the human body and plays an indispensable role in whole body locomotion and posture. Muscle is also a highly metabolically active tissue and serves as a major site of glucose disposal and protein storage; and thus, plays an essential role in maintaining whole-body homeostasis. Skeletal muscle is composed of many individual multinucleated fibers that are able to work synergistically to produce force. Remarkably, muscle fibres are able to withstand significant mechanical stress induced through cycles of contraction and relaxation. This is in large part due to the highly organized cytoarchitecture of the muscle fibre. To maintain optimal structure and function, skeletal muscle must be able to respond and adapt to various physiological cues. Skeletal muscle nuclei, or myonuclei, are postmitotic. Therefore, it is believed that resident skeletal muscle stem cells commonly referred to as satellite cells (SC), are in part believed to play an important role in the maintenance of healthy muscle mass.

SC were first discovered by Mauro in 1961 and were aptly named based on their location, 'orbiting' on the periphery of muscle fibres (69). Specifically, SC reside between the sarcolemma and the basal lamina, referred to as the SC niche (59). The muscle fibre to which the SC is associated also composes part of the niche and thus SC respond to various signals originating from the muscle fibre (59). The establishment of skeletal muscle is an important part of embryogenesis in which SC play an important role.

All skeletal muscles arise from the somites. The somite is a division of the developing embryo's body and is composed of hypaxial and epaxial regions, eventually giving rise to the musculature of the limbs, abdomen and thorax, and the back, respectively (16, 37). During embryogenesis, cells up-regulate specific genes that render them committed to the myogenic lineage; the up-regulation of these genes is the result of signals from surrounding tissues (18). There is a subpopulation of myoblasts that remain undifferentiated and give rise to the SC population (119). SC content must be maintained through development and adulthood in order for repair and remodelling of skeletal muscle to occur in response to various external cues (15). Addition of 'de novo' nuclei via SC has been shown to be essential in maintaining muscle mass into adulthood and contributing to repair and regeneration (41, 70, 93). The progression of a SC from quiescence to terminal differentiation is governed by a network of transcription factors referred to as myogenic regulatory factors (MRF).

1.1.1 The myogenic programme and the activation of satellite cells

SC become active in response to various physiological stimuli such as exercise and injury (41). A number of growth factors (e.g., hepatocyte growth factor (HGF), fibroblast growth factor (FGF) interleukin 6 (IL-6), myostatin, insulin-like growth factor-1 (IGF-1)) have been proposed to regulate SC function (15). These factors serve to regulate transcriptional networks termed MRF. MRF are responsible for the progression of the SC from activation, proliferation through to differentiation; a process termed the 'myogenic programme' (15, 37, 44). Two primary MRF, Myf5 and MyoD are known to drive the proliferative phase, while two secondary MRF, MRF4 and myogenin are known

to drive differentiation. SC express Myf5 and MyoD during early regeneration in rodents (23) and MyoD expression is essential for the transition from proliferation to differentiation (118). A down-regulation of Myf5 and MyoD followed by an increase in MRF4 and myogenin results in SC differentiation (37).

Several studies in humans have explored the expression of MRF following acute exercise. Increased gene expression of Myf5 and MyoD occurs early following a bout of eccentric exercise, an increase in myogenin expression occurs early and is maintained up to 5 days following exercise, while the increase in MRF4 expression occurs at later time points (71). Additionally, there is an increase in SC co-localized with MyoD following resistance exercise (73). These results are reflective of what is observed in rodent models where Myf5 and MyoD are expressed early following SC activation and the expression of MRF4 and myogenin occurs later during SC differentiation (54).

1.1.2 The identification of satellite cells in vivo

Identifying and quantifying SC content in cross sections of skeletal muscle using immunohistochemical techniques following a stimulus provides insight on their potential role and function in mediating muscle adaptation. SC have been identified using a variety of markers such as neural cell adhesion molecule (CD56/NCAM) (33, 49), C-Met (24, 108), M-cadherin (49), syndecan-3/-4 (24) and Pax7 (70, 93) in mice. SC have also been identified using various markers in humans such as CD56 (25, 32, 65, 68, 74), M-cadherin (92) and Pax7 (17, 68, 74, 113).

A limitation of using the markers described above is their inability to identify which stage of the myogenic programme a cell is in (quiescence, proliferation or differentiation). Several studies have identified SC in conjunction with markers of proliferation in humans like Ki67, proliferating cell nuclear antigen (PCNA) (68) or MyoD (73) in order to further describe the SC pool. Which marker of proliferation to use is still under considerable debate. Ki67 is expressed during the mid-G1 phase with increased expression throughout S and G2 phase and peaking in M phase of the cell cycle (35). PCNA is expressed during S phase and late G1 phase. The protein half-life of Ki67 is approximately 90 minutes (42) whereas the protein half-life of PCNA is approximately 20 hours (14). The discrepancy in the proportion of SC expressing Ki67 and PCNA 24 hours following eccentric exercise in muscle cross sections may be explained by the difference in protein half-life (17).

The expression of MyoD in SC has long been associated with cell proliferation. It is well established that MyoD is an important driver of myoblast proliferation (55, 75). Furthermore, MyoD remains up-regulated while Pax7 is down-regulated at the onset of differentiation (90). On this basis, it stands to reason that MyoD may be the best suited marker to identify active SC that are no longer in a quiescent state.

1.1.3 Satellite cells in muscle repair/regeneration

Skeletal muscle fibres undergo cycles of contraction and relaxation and must therefore undergo constant repair/remodelling in order to maintain healthy muscle function throughout life. Indeed, muscle injury can be induced in a variety of ways such

as mechanical insult like crush, puncture, cut or freeze injuries. Ischemia, injection of local anesthetics, myotoxins, whole muscle free grafts and exhaustive exercise have also been observed to result in skeletal muscle injury (10, 27). Inducing muscle injury has been used extensively in experimental rodent models in order to study the different phases of skeletal muscle regeneration.

A common method of inducing injury in mouse models is the injection of cardiotoxin (CTX), a snake venom from the Naja naja atra, or the Chinese cobra (63). Injury via injection of CTX occurs due to depolarization and degradation of the sarcolemma caused by Ca^{2+} overload and activation of Ca^{2+} dependent proteases (40, 85). Although injection of CTX causes extensive damage in skeletal muscle the basal lamina and blood vessels remain relatively intact and SC retain the ability to respond to this type of injury (40, 85). In order to overcome the damage induced by CTX in skeletal muscle, the regenerative process begins soon after injection.

Several overlapping healing phases are necessary for complete muscle fibre regeneration to occur following injury in rodent skeletal muscle (Figure 1). The first phase of muscle regeneration is the *destructive/inflammatory phase* (15). During this time, necrosis or degeneration of damaged muscle fibres, hematoma formation, and a large influx of inflammatory cells occur (15). Immune cells, namely neutrophils, followed by macrophages and leukocytes, infiltrate the muscle at the site of injury. These cells then secrete various factors that attract more inflammatory cells to the site of injury (38, 111) resulting in SC activation and proliferation (31). The second phase of muscle regeneration is the *repair/regenerative phase*. This phase is highlighted by the elimination of necrotic

debris by phagocytosis and the activation of SC (15). Activated SC migrate to the site of injury, proliferate and differentiate, either contributing their nuclei by fusing to damaged fibres or fuse together forming new multinucleated fibres (15). In the final *remodeling phase*, newly formed and repaired fibres grow and re-establish contractility (15). SC have repeatedly been observed to be an indispensable part of skeletal muscle regeneration. Several rodent studies have demonstrated severe impairment in muscle regeneration when skeletal muscle is devoid of SC (70, 93).

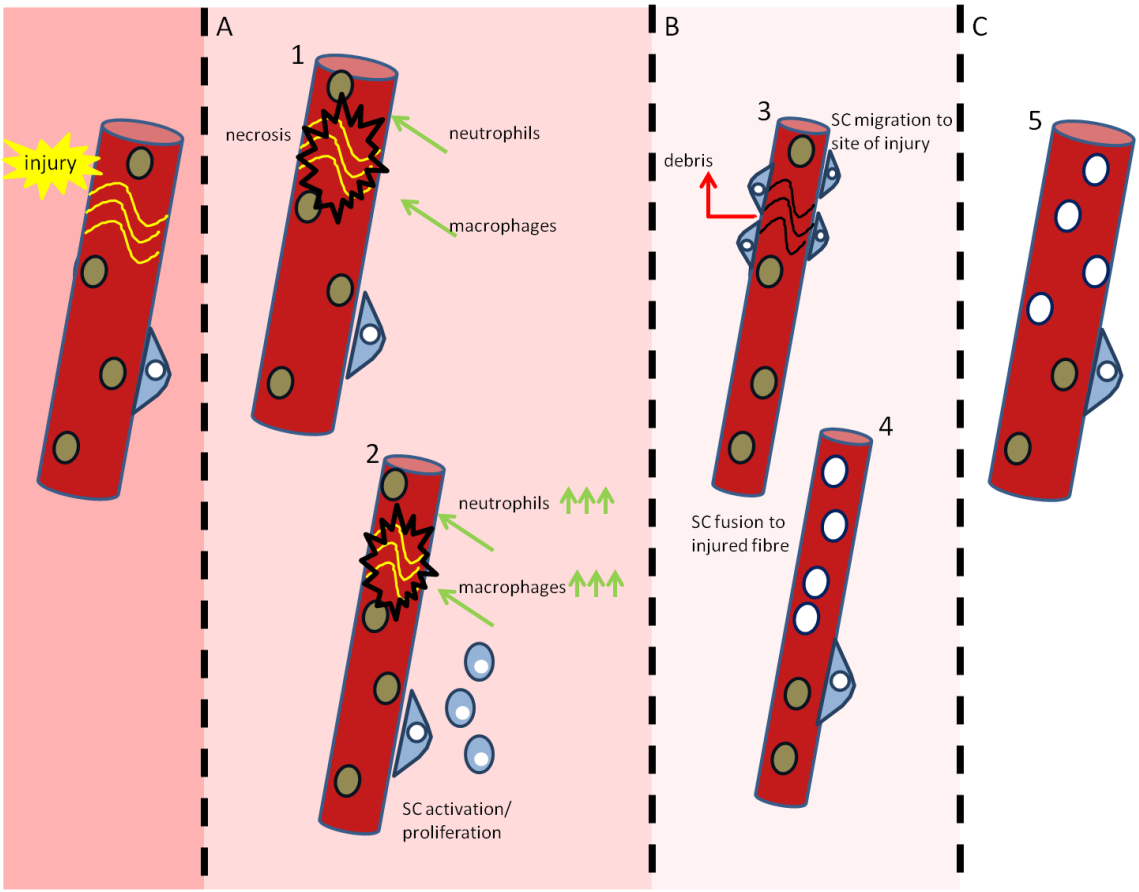

Figure 1. Stages of muscle regeneration. Upon muscle injury damaged muscle fibers undergo the first phase of regeneration; destructive/inflammatory phase (A). This phase in highlighted by necrosis of damaged tissue and an infiltration of inflammatory cells. Inflammatory cells release factors the lead to the activation and proliferation of SC. The

second, repair/regenerative, phase of muscle regeneration (B) is marked by the removal of debris from the injured muscle fibre and a migration of the SC to the site of injury. SC will then fuse to the muscle donating their nuclei. Not represented in this diagram but SC are also able to fuse to each other and form new myotubes (hyperplasia). The last phase of regeneration, the remodelling phase (C), muscle fibres grow and re-establish contractility.

The inflammatory response that occurs following injury also plays an important role in muscle regeneration as described above. Inflammatory cells infiltrate the injured skeletal muscle and factors that they release play an important role in activating SC and initiating their contribution to the regenerative process (41). The inflammatory response is observed as early as 2 to 6 hours following injury and continues for up to 4 days following the initial insult (84). The ability and efficiency of muscle regeneration is also, in part, dependent on inflammatory cell invasion into the muscle (84). An impaired inflammatory response could presumably impair the activation of SC in response to injury affecting the ability to re-establish muscle cross sectional area (CSA).

Regenerating muscles likely have a greater metabolic demand in comparison to healthy muscle and require adequate vascularization in order to supply the muscle with optimal nutrients and oxygen during repair/regeneration (81). Several studies have described the importance of angiogenesis following injury (39, 81) as there is an association between delayed angiogenesis and delayed muscle regeneration observed as early as 14 days following injury (9, 51, 81).

Fibrosis is also part of the regenerative process following skeletal muscle injury. Upon injury, inflammatory cells secrete factors that activate SC, as described above, but may also act on fibroblasts, located in skeletal muscle, which can result in scar tissue

formation (84). The activation of fibroblasts results in production of collagen (76). The early increase in collagen production acts as a scaffold in helping repair muscle fibres but if collagen production persists muscle regeneration can be impaired (76). An increase in collagen deposition results in the replacement of contractile tissue with non-contractile tissue thereby affecting muscle contractility (84). Regeneration and the processes discussed above are most relevant for studies using rodent models, however the study of SC activation in humans requires a less invasive method of inducing damage than toxin injections. Previous literature has shown that a bout of unaccustomed eccentric exercise (i.e., forced lengthening of the active muscle) is an effective approach to induce damage and initiate repair in humans (48).

1.2 The adaptive response of skeletal muscle to exercise training

1.2.1 The satellite cell response to acute exercise/damage

Skeletal muscle regeneration has been extensively used as a model to study SC biology. However, the ability of SC to respond to exercise has also been described and is therefore readily used to study SC function in humans. Following damage inducing eccentric contractions in humans there is an expansion of the SC pool (17, 25, 32, 74, 80, 113). An increase in SC content also occurs following a more traditional bout of resistance exercise in humans (8, 73). More recent work has also demonstrated that a bout of heavy resistance exercise is sufficient in not only increasing the SC pool in skeletal muscle but also increasing the number of proliferating SC (8, 73).

Acute exercise has also been used as a model to study SC biology in animals. When rodents were exposed to 1 bout of repeated eccentric contractions, in the form of

downhill running, an increase in SC content and in proliferating SC was observed 72 hours following exercise (28, 112). Eccentric contractions result in increased serum lactate dehydrogenase (LDH) and creatine kinase (CK) levels, an accumulation of macrophages and mononuclear cells, and a disruption of normal banding of some muscle fibres. Together these characteristics suggest that downhill running results in muscle damage (6). Although not a direct measure of SC content, as few as three stimulated maximal isometric contractions, not causing muscle injury, resulted in an increase in myogenin mRNA expression, which is up-regulated during SC differentiation (2). Taken together, data from both human and rodent models suggest that various modes and intensities of exercise can result in SC activation. To understand the role of SC in mediating exercise induced adaptations several studies have examined the SC response to exercise training.

1.2.2 The role of satellite cells in adaptation to resistance exercise training

Skeletal muscle fibres are a post mitotic tissue and therefore the addition of new nuclei must come from an exogenous source. The SC population has been postulated to be the primary source of new nuclei in skeletal muscle. The myonuclear domain theory suggests that each myonucleus governs a particular volume of cytoplasm. Once the volume of a cell exceeds the capacity of an individual nucleus (e.g. an increase in muscle fibre size) the addition of new nuclei is necessary to support a larger cell volume (4). This theory was originally supported by work in rodent models in which SC were ablated by gamma irradiation. Skeletal muscle that was void of SC did not respond to overload-induced hypertrophy whereas control, non-irradiated, rodents experienced significant

hypertrophy (1, 87). However, recent work has challenged common dogma that SC are necessary for inducing muscle fibre hypertrophy. Indeed, a novel mouse model was developed that achieved near complete ablation of SC in mature skeletal muscle. In this model, SC-ablated animals maintained the ability to respond to various hypertrophic stimuli (50, 70) suggesting that, at least in rodents, SC are not necessary for inducing skeletal muscle fibre hypertrophy.

A hallmark of resistance exercise training is skeletal muscle fibre hypertrophy. Several studies in humans have reported a concomitant increase in fibre CSA and SC content following resistance training. An increase in fibre CSA of the trapezius was observed following resistance training focused on the neck and shoulder muscles in women which was also associated with an increase in SC content (53, 64). Resistance training focusing on the lower limbs that resulted in hypertrophy was also associated with an increase in SC content (8, 83). Interestingly, subjects who had the greatest increase in fibre CSA were the only ones to increase SC content following prolonged training (83). In addition to the increase in SC content following periods of hypertrophy induced by resistance training, a simultaneous increase in the number of myonuclei per muscle fibre to support muscle fibre growth was reported (8, 53, 83). A relationship between muscle fibre CSA and the number of myonuclei per fibre has been described, where larger fibers have a greater number of myonuclei compared to smaller fibres (34, 53, 99). Taken together, these data further support the notion that in humans nuclear addition appears to be an important part of muscle hypertrophy therefore reinforcing the role of SC in contributing to muscle hypertrophy.

Although an expansion of the SC pool is commonly associated with an increase in fibre CSA, one study has reported an increase in SC content following light- and high-load resistance exercise training without a significant increase in muscle fibre size (66). Collectively, the data would suggest that SC play a role in mediating adaptations to hypertrophic stimuli, in various muscle groups, by contributing nuclei to maintain the mynocluear domain. However, SC may also play a role in mediating nonhypertrophic adaptations as an increase in SC content was reported without an increase in fibre CSA (64).

1.2.3 The role of satellite cells in adaptation to aerobic exercise training

Skeletal muscle adaptation to aerobic exercise has been extensively studied. Aerobic exercise results in skeletal muscle remodelling with a hallmark adaptation being a shift towards a more oxidative phenotype in the absence of hypertrophy. Aerobic exercise training in rodents consistently results in an increase in SC content. Several studies have reported an expansion of the SC pool following various treadmill training protocols (61, 97, 98, 114). An increase in SC content was reported when rats were exposed to voluntary wheel running for 8 weeks (60). A more recent study suggests that exercise intensity may be important in expanding the SC pool (61). Rats underwent 10 weeks of either high or low intensity exercise of short or long duration. Only rats that completed high intensity exercise whether it be of short or long duration experienced an expansion in the SC pool (61). Importantly, none of the aerobic training protocols led to increases in fibre CSA, thus reinforcing the possibility that SC play a role in muscle adaptation other than hypertrophy induced via resistance training. Additionally, the

intensity at which aerobic exercise is performed may dictate, to some extent, the increase in SC content.

The SC response to aerobic exercise in humans has not been as extensively studied and is much less consistent than that observed in rodent models. In addition, all studies describing the SC response to aerobic exercise have been performed in older adults. Some studies report an increase in SC content following 14 weeks of interval training consisting of 4 minutes of cycling at 65-75% HR_{max} followed by 1 minute of cycling at 85-95% HR_{max} for a total of 45 minutes (19, 117). While a more traditional endurance training program consisting of 40 minutes of aerobic exercise at 75% of $VO_{2\ max,}$ did not result in an increase in SC content (103). These results are in accordance with rodent studies suggesting that intensity may play a role in mediating SC pool expansion with respect to aerobic exercise.

It is however, important to note that the interval training described above resulted in an increase in CSA of type IIa fibres (19, 117) whereas the traditional endurance training protocol did not lead to an increase in CSA of any fibre type (103). The intensity of the aerobic interval training program may have been a potent enough stimulus in older participants to induce increases in fibre CSA. Therefore the increase in SC content may have occurred in order to mediate fibre hypertrophy and not adaptations normally associated with aerobic exercise. Although the evidence is inconclusive in humans, it suggests that SC may be involved in mediating skeletal muscle adaptations associated with aerobic exercise.

1.3 Skeletal muscle and aging

In 2011, average life expectancy in Canada for males was 79 years and 83 years for females. This is expected to reach 84 and 87 years respectively by 2036 (105). The proportion of the Canadian population over the age of 65 was 14.6% in 2011 and is expected to increase to over 25% by 2061 (105). Aging, in part, is characterized by a progressive loss of muscle mass and strength, referred to as sarcopenia (101). The progressive loss of muscle mass is believed to begin as early as the fifth decade of life (45) and results in a decline in functional capacity and independence, with an increase in the incidence of injury (89, 101). The age-associated loss of muscle mass and strength severely impacts older individuals ultimately leading to a loss of independence and reduced quality of life. Sarcopenia not only negatively affects the aging individual but also has a tremendous impact on caregivers and places a heavy economic burden on the Canadian health care system. Therefore, there is an emphasis on understanding the physiological responses that occur with aging so that appropriate therapeutic strategies can be developed.

1.3.1 Satellite cells and aging

Studies in rodents have described the age-related changes that occur in skeletal muscle. Enumeration of the SC pool in old animals has led to the theory that an age-related reduction in the SC pool may contribute to sarcopenia. Table 1 summarizes several rodent studies in which SC content of various muscles were compared to that of young animals.

Table 1. The effect of age on SC content in rodents.

Reference	Species	Muscle	Age	SC pool
(104)	Mouse	Soleus	30 mts	decreased
(36)	Rat	Soleus, EDL	24 mts	decreased in both muscles
(79)	Rat	Levator ani	32 mts	no change
(29)	Rat	TA	24 mts	decreased
(94)	Mouse	Soleus	200 to 720 days	no change
(11)	Mouse	TA	24 mts	decrease
(96)	Mouse	Soleus, EDL	7-10 mts 18-27 mts 28-33 mts	decrease in each age group for soleus; decrease in 18-27 and 28-33 mts in EDL
(20)	Mouse	EDL	22-30 mts	decreased
(98)	Rat	Gastrocnemius	15-17 mts	decreased
(97)	Mouse	EDL	16 mts	decreased

These studies examined the age-related response of SC in both mice and rats and in various muscles, from primarily oxidative soleus muscle, to primarily glycolytic extensor digitorum longus (EDL) muscle (7). Interestingly, a decrease in SC content is often reported with advanced age. Age-related decreases in SC content are reported as early as 10 months in the mouse soleus (96). Not only does aging lead to a decrease in SC content but function has also been reported to be dramatically affected. Old rodents have reduced capacity to produce myogenic progeny from myofibre associated SC (20, 97, 98). Additionally, SC from old rodents tend to produce a greater proportion of non-myogenic progeny (97). These results suggest that the SC pool from old animals may have an impaired ability to progress through the myogenic programme.

Of late, much emphasis has been placed on determining the effect of age on SC content in order to better understand the age-related loss of muscle mass observed in humans. Data regarding SC content in older adults is equivocal with some studies reporting no change while others report a decrease (summarized in Table 2). The differences in SC content between studies are likely due to the varying age and health status of subjects. When the average age of subjects is over 70 only one study reports no change in SC content. The reduction in SC associated with type II and not type I fibres may be explained by the type II specific atrophy associated with aging (78). Although some information is gained by describing the basal SC pool in old adults, valuable insight on SC function can be gained by determining the ability of the SC pool to become activated in response to various stimuli. Old adults fail to increase the number of SC associated with type II fibres compared to young adults following exercise (72, 73, 102). The activation of SC following exercise is also impaired in older adults (73, 102). These data suggest not only that the SC pool is reduced with age but also that its functionality is impaired which may exacerbate the ability of skeletal muscle to regenerate and recover from injury.

Table 2. The effect of age in SC content in humans.

Reference	**Age**	**Muscle**	**Change in SC pool**
(43)	65	Vastus lateralis	no change
(86)	74	Masseter, biceps	decreased in both muscles
(88)	Men: 69 Women: 67	Vastus lateralis	no change
(52)	70-83	Tibialis anterior	decreased
(92)	~71	Vastus lateralis	decreased
(32)	60-75	Vastus lateralis	no change
(82)	~64 yrs old	Vastus lateralis	no change

(116)	76	Vastus lateralis	decreased in Type II no change Type I
(73)	70	Vastus lateralis	decreased in Type II no change Type I
(115)	75	Vastus lateralis	decreased in Type II no change Type I
(67)	66	Vastus lateralis	no change

1.3.2 Aging, adaptation and exercise

Although age-related changes in skeletal muscle are numerous, muscle retains the ability to positively respond to stimuli such as exercise. Studies examining the benefits of resistance exercise in rodents are few, however, aerobic exercise has been shown to result in an increase in SC content in old mice (97, 98). Resistance training in old rodents has also been shown to slow the age-related progressive loss of muscle mass (58). In addition, resistance training reversed the age-associated increase in type I fibres of the soleus, and the age-associated increase and decrease in type IIa and type IIb fibres, respectively of the superficial plantaris (56). Both endurance and strength training resulted in improvements of various enzyme activities in skeletal muscle of old rats (56). In addition to improved enzyme activities, resistance training improved strength and contractile properties of skeletal muscle in aged rats (57). Adaptation of skeletal muscle following endurance exercise is not limited to improved muscle qualities as improved spontaneous locomotion (98) and even increased lifespan in old animals have been observed (77). These studies highlight the vast benefits of exercise and reinforce the notion that skeletal muscle retains the ability to positively respond to exercise stimuli even with advancing age.

The response of old adults to resistance training has been extensively studied as an interventional strategy to mitigate the effects of sarcopenia. Generally, resistance training results in increased muscle mass and strength in the elderly (101). Resistance training in old adults is often associated with an expansion of the SC pool, which is believed to be necessary in order to counterbalance the increase in fibre CSA and maintain the myonuclear domain (101). However, fewer studies have explored skeletal muscle adaptation to aerobic exercise in the elderly. Interval type aerobic training in old adults led to increases in $VO_{2\ peak}$ and citrate synthase (CS) activity, which are adaptations consistent with aerobic training. However, an increase in SC content and increased CSA of type IIA fibres have also been reported (19, 117). Following a traditional aerobic training protocol in older adults with type 2 diabetes, no increase in CSA or SC content was observed, however, consistent with other studies improvements in $VO_{2\ peak}$ and maximal workload capacity were observed (103). High intensity interval training (HIT) has also been reported to improve various performance measures, such as VO_{2peak}, anaerobic threshold and peak power output in older adults diagnosed with various pathologies (26, 110). Although these data are not direct measures of skeletal muscle adaptation they would suggest that at least some metabolic adaptations may have occurred. There are certainly fundamental differences in the nature of the response to resistance and aerobic exercise training but most importantly older individuals retain the ability to respond to either mode of exercise.

1.3.3 Skeletal muscle regeneration during aging

SC are indispensable for muscle regeneration (70, 93). As SC content is decreased with aging (refer to Table 1), it stands to reason that impaired skeletal muscle repair may also be associated with aging. In the initial report of age-related impaired muscle regeneration, signs of impaired regeneration were evident even 2 months following injury (91). More recent work demonstrated that the early stages of the regenerative process are also impaired in old mice (12, 22). Indeed this impairment was highlighted by fewer regenerating fibres, greater fibrosis and fewer active SC in old compared to young animals 5 days after injury (13, 22). However, these results are markedly inconsistent as others have reported that regeneration occurs to the same extent in young and old rodents when examined at later time points following injury (62, 95, 100).

Interestingly, models of parabiosis have demonstrated that heterochronic pairings between younger and older animals are able to improve muscle regeneration in older animals (12, 21). These observations suggest that there are systemic, factors that control SC function and that these micro-environmental factors are of key importance in the impaired SC response during aging. Furthermore, these findings suggest that the extrinsic systemic environment can significantly impact skeletal muscle function. Additional work has demonstrated that myogenic cells are able to adopt alternate cell lineages such as fibroblasts (12) or adipocytes (109) based on external cues. This ability of SC from aged animals to adopt an alternate lineage fate may further exacerbate the impaired regeneration observed with aging.

Although studies inducing muscle regeneration are readily used in rodent models to study SC biology and their role in the repair process, these types of interventions cannot be carried out in humans. Therefore, alternative models must be employed in order to determine the role of SC in humans. Following periods of atrophy, induced by immobilization or reduced activity, skeletal muscle must undergo a period of remodelling to re-establish baseline fibre CSA; a process that also occurs during muscle repair. Although muscle repair is not directly measured in old adults, insight on the muscle's ability to repair may be gained from studies examining immobilization with subsequent re-loading.

The reduction in muscle mass and strength are similar in both young and older adults following two weeks of immobilization. Additionally, following 14 days of re-training these parameters are re-established to baseline measures in both groups (46, 107). More recent work suggests a reduction in strength and fibre CSA following only four days of immobilization in old and young men (47). After seven days of re-training young adults re-established fibre CSA and strength whereas strength and type I fibre CSA remained lower in old adults (47). Two weeks of complete immobilization in young and older adults lead to decreases in type I and II fibre CSA. This reduction was still observed following four weeks of re-training in older adults but was re-established in young adults (106). An increase in SC content was reported in young adults during the re-training period, which was not observed in old adults and likely contributed to the impaired re-establishment of fibre CSA (106). Impaired recovery of skeletal muscle fibre CSA after a period of disuse followed by subsequent re-loading was similar to that observed during

muscle repair in old animals. Therefore the impaired recovery observed in old adults may in part be due to a reduction and function of SC as they are necessary for complete muscle repair.

No differences were observed in myogenic cells isolated from young and old adults with respect to proliferation and differentiation in an *in vitro* setting (5). However, albeit to a lesser extent than muscle derived fibroblasts, when myogenic cells were exposed to fatty acids or conditions promoting adipocyte growth an increased expression of genes involved in adipogenic regulation was observed in old cells (3). Advanced age results in an increase in intramuscular fat (30). Therefore, determining the propensity of SC from old compared to young adults to adopt an adipogenic lineage may be of interest. Although speculative, the increase in intramuscular fat infiltration may further impact impaired SC activation and ultimately muscle repair in older adults.

The effect of age on muscle repair has been extensively studied in rodent models; however, due to the inability to induce regeneration in humans, there is a marked paucity of information on the repair process of injured muscle in older adults. It may be argued that immobilization with subsequent re-loading is not a model of injury in human skeletal muscle. Nonetheless, the muscle undergoes a period of atrophy followed by a period of hypertrophy suggesting significant remodelling of the fibre architecture. During regeneration skeletal muscle also undergoes significant remodelling where fibre CSA is reduced followed by a period of hypertrophy to re-establish fibre size. Similar to what is observed in young adults following a period of immobilization, fibre CSA is reduced in older adults. However, older adults fail to re-establish baseline fibre size after a period of

re-loading. Therefore, the studies described above suggest that muscle repair may be impaired in older adults.

1.4 Purpose of the thesis

The research experiments that comprise this thesis were designed to investigate the role of SC in response to nonhypertrophic exercise training. The effect of hypertrophy-inducing resistance training on SC content had been studied but a gap in knowledge exists on the role, if any, that SC play in promoting adaptation to nonhypertrophic aerobic training. In the first study the response of SC following six weeks of HIT was examined. The effect of HIT on the SC pool had never been previously described. Moreover, the effect of aerobic type training on the SC pool had only been examined in an older population. We hypothesized that HIT would not result in an expansion of the SC pool in the basal state but rather would result in an increase in the number of active SC associated with remodelling fibres. Immunoflurescent experiments of muscle cross sections were used to determine fibre type distribution and SC content pre and post training.

The aim of the second study was to describe the SC pool following traditional endurance exercise and various HIT models. Based on results from the first study it was hypothesized that SC content would remain unchanged with training but an increase in SC pool activity would be observed. Various immunofluorescent experiments were used to determine the number of active and differentiating SC pre and post training

The final study extended the results from the previous two studies. The general aim of the third study was to determine the impact of nonhypertrophic exercise training on skeletal muscle regeneration in old animals. As described in previous sections some reports suggest that older animals have an impaired regenerative response following muscle injury and this may be due to an age related decline in SC content and function. Previous work in rodent models demonstrated that treadmill running was able to increase SC content in older animals, the first two studies of this thesis demonstrated an increase in SC activity without pool expansion following aerobic training. Therefore, we hypothesized that old mice that had completed an aerobic exercise conditioning program would experience improvements in skeletal muscle regeneration and that this improvement would be mediated by an increase in SC pool content. Various immunohitochemical techniques were used to determine SC content and the extent of regeneration in old sedentary and exercised animals and young animals following injury.

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Chapter 2:

Evidence for the contribution of muscle stem cells to non-hypertrophic skeletal muscle remodeling in humans (2013). *FASEB J.* 27, 4596–4605.

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Short title: Satellite cells and muscle fibre remodeling

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Nonstandard abbreviations

CSA, cross sectional area

DSHB, Developmental Studies Hybridoma Bank

HR_{max}, maximal heart rate

MHCI, myosin heavy chain type I

MHCII, myosin heavy chain type II

eMHC, embryonic myosin heavy chain

nMHC, neonatal myosin heavy chain

MRF, myogenic regulatory factors

OCT, optimum cutting temperature

PFA, paraformaldehyde

SC, muscle stem cell (satellite cell)

VO_2 peak, maximal oxygen uptake

Abstract

The purpose of this study was to explore the possible role of muscle stem cells, also referred to as satellite cells (SC), in adaptation and remodeling following a non-hypertrophic stimulus in humans. Muscle biopsies were obtained from the vastus lateralis of previously untrained women (n=15, age: 27±8 y, BMI: 29±6 kg/m^2) before and after 6 wk of aerobic interval training. The fibre type specific SC response to training was analyzed using immunofluorescent microscopy of muscle cross sections. Following training, the number of SC associated with fibres expressing myosin heavy chain type I and II isoforms (hybrid fibres) increased (pre: 0.062±0.035 SC/hybrid fibre; post: 0.38±0.063 SC/hybrid fibre; $p<0.01$). Additionally, there was a greater number of MyoD+/Pax7- SC, indicative of differentiating SC, associated with hybrid fibres (0.18±0.096 MyoD+/Pax7- SC/hybrid fibre) compared to type I (0.015±0.00615 MyoD+/Pax7- SC/type I fibre) or II (0.012±0.00454 MyoD+/Pax7- SC/type II fibre) fibres ($p<0.05$). There was also a training-induced increase in the number of hybrid fibres containing centrally located nuclei (15.1%) compared to either type I (3.4%) or II fibres (3.6%) ($p<0.01$). These data are consistent with the hypothesis that SC contribute to the remodeling of muscle fibres even in the absence of hypertrophy.

Keywords: satellite cells, Pax7, fibre remodeling, aerobic interval training

Introduction

Many factors are essential for maintaining healthy skeletal muscle function, including a functional population of resident stem cells which are commonly referred to as satellite cells (SC). SC were first discovered in the early 1960's and play an active role in skeletal muscle regeneration and maintenance of muscle mass (1). Following mechanical stress such as weight-bearing physical activity (2) or a trauma-inducing injury (3;4), SC are activated, proliferate and differentiate contributing to the repair and formation of new functional muscle fibres. This process is referred to as myogenesis and is controlled by a specific group of transcription networks termed myogenic regulatory factors (MRF) (5-8).

Although the role of SC in skeletal muscle regeneration following injury is well established, the necessity of SC in mediating exercise induced adaptations in skeletal muscle remains debatable. The potential role of SC in mediating gains in muscle mass following hypertrophic stimuli has been highlighted in rodent irradiation studies (9-11), while other groups using SC specific ablation transgenic rodent models (12-14), have proposed that SC are unnecessary for muscle hypertrophy. Importantly, several studies have shown that the increase in muscle mass after resistance training in humans is associated with a concomitant increase in nuclei per fibre, suggesting the donation of SC nuclei in response to resistance exercise training (15;16), and also an expansion of the SC pool (15;17-19), which represent normal physiological functions of SC. Much emphasis has been placed on defining the role of SC when the skeletal muscle faces a hypertrophic stimulus (i.e. resistance training) but less focus has been placed on investigating the role for SC in muscle adaptation facing non-hypertrophic stimuli (traditional endurance or aerobic interval training, which lead to enhanced oxidative capacity without muscle growth).

The response of the SC to endurance training is limited and inconclusive as some groups report an increase in the SC pool using a more intense form of endurance training (40 to 45 min of cycling consisting of 4 min of sustained low intensity cycling followed

by 1 min of high intensity cycling based on either maximal heart rate (HR_{max}) or maximal oxygen uptake (VO_2 peak)) (20;21) while others report no change using a more traditional endurance training protocol (40 min of exercise at 75% VO_2 peak) (22). Both studies reporting an increase in the number of SC also showed an increase in type IIa fibre cross sectional area (CSA) with intermittent cycle ergometer training (20;21). Additionally, the number of SC per myofibre is increased in rat plantaris muscle after high intensity endurance training of both short and long duration, but not after low intensity endurance training regardless of duration with no increase in muscle mass or fibre area (23). These data suggest that intensity, rather than duration of exercise plays an important role in SC pool expansion. Following endurance training, skeletal muscle is remodeled to adapt metabolic and structural characteristics of aerobic exercise stress without evidence of muscle fibre hypertrophy (24;25). A question that remains unknown is whether SC play a role in mediating these non-hypertrophic adaptations.

Aerobic interval training is characterized by a series of high intensity workloads interspersed with low intensity periods of recovery. Recently studies have established that this method of training is a time-efficient strategy to induce physiological adaptations that resemble traditional endurance training, such as mitochondrial biogenesis and skeletal muscle remodeling towards a more oxidative phenotype (26). While there are many different forms of aerobic interval training, it is generally not associated with skeletal muscle hypertrophy (27;28). Aerobic interval training is therefore an attractive model to investigate whether SC play an active role in skeletal muscle remodeling and the potential role of exercise intensity. Different stimuli (i.e. injury, strength or endurance training) elicit varying SC responses. The largely debated question of whether SC are necessary for inducing hypertrophy may not be the most important question. Rather, identifying the role of SC in human muscle adaptation as it relates to exercise and whether it differs based upon the exercise stimulus may be more appropriate. Therefore the objective of this study was to further define the role of SC in human skeletal muscle in response to exercise. We specifically investigated whether a non hypertrophic stimulus (i.e. aerobic interval training) would expand the SC pool. More specifically, we wished to investigate the

possible role of SC in muscle remodeling following training. We hypothesized that following training there would be an increase in active SC associated with remodeling muscle fibres.

Materials and Methods

Ethical approval

The study was approved by the Hamilton Health Sciences/McMaster University Faculty of Health Sciences Research Ethics Board. Informed written consent was obtained from all participants prior to the start of the study.

Subjects

Fifteen previously untrained women who were overweight but otherwise healthy (age: 27±8 y, BMI: 29±6 kg/m^2) underwent a 6 week aerobic interval training program. Participants were deemed sedentary based on physical activity self-reports of less than 2 sessions of structured exercise per wk lasting less than 30 minutes.

Baseline Measures and Exercise Training

Each subject completed a VO_2 peak test on an electronically braked cycle ergometer (Lode Excalibur Sport V 2.0, Groningen, Netherlands) where their HR_{max} was determined. The power output required to elicit a training intensity of approximately 90% of a HR_{max} was determined on two separate occasions.

The training protocol consisted of 18 supervised sessions over 6 wk (3 sessions/wk). Each training session involved 10 x 60 sec bouts of cycling interspersed with 60 sec of recovery. Individual workloads were selected to elicit approximately 90% of a subject's HR_{max}. During the 60 sec recovery periods subjects rested or pedalled slowly against a resistance of 50 W.

Sample Collection

Two muscle biopsies were obtained from the *vastus lateralis*, under local anesthetic (1% Lidocaine) using a Bergstrom needle adapted using manual suction, before and 96 hours following the last training session (25). Upon excision muscle biopsies were dissected into pieces that were either immediately frozen in liquid nitrogen (RNA isolation) or embedded in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek, USA) and frozen in isopentane cooled with liquid nitrogen (immunohistochemical analysis).

Immunohistochemistry

Muscle cross-sections (7 µm) were prepared from OCT embedded sections and allowed to air dry and subsequently stored at -80˚C. Immunofluorescent analysis for Pax7, A4.951 [myosin heavy chain type I (MHCI; slow isoform) and MyoD were similar to our previously published methods (26;27). Briefly tissue sections were fixed in 4% PFA for 10 min, washed 3x5min in PBST, blocked for 60 min at room temperature in PBS containing 2% bovine serum albumin, 5% fetal bovine serum, 0.2% Triton x-100, 0.1% NaAzide, and 2% goat serum. Slides were then stained with antibodies against Pax7 (neat; DSHB); MyoD (anti-MyoD1; clone 5.8A; 1:50; Dako, Burlington, ON, Canada); A4.951 [myosin heavy chain type I (MHCI; slow isoform); neat; DSHB]; myosin heavy chain type II (MHCII; fast isoform; 1:1000; ab91506, Abcam, Cambridge, MA, USA); neonatal myosin heavy chain (nMHC 1:10; VP-M666, Vector Laboratories, Burlingame, CA, USA); laminin (1:1000; ab11575, Abcam, Cambridge, MA, USA). Secondary antibodies used were Pax7 (Alexa Fluor 488 or 594, 1:500; Invitrogen, Molecular Probes, Carlsbad, CA, USA); MyoD (biotinylated secondary antibody, 1:200; Vector Canada, Burlington, ON, Canada, and streptavidin-594 fluorochrome, 1:500; Invitrogen, Molecular Probes); A4.951 (Alexa Fluor 488, 1:500); myosin heavy chain type II (Alexa Fluor 647, 1:500); neonatal myosin heavy chain (Alexa Fluor 488, 1:500); and laminin (Alexa Fluor 647, 1:500). Staining specificity was confirmed using appropriate negative controls. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI, 1:20000; Sigma-Aldrich, Oakville, ON, Canada). Samples were viewed with the Nikon Eclipse Ti microscope at 20x magnification and captured with a Photometrics CoolSNAP HQ2

fluorescent camera (Nikon Instruments, Melville, NY, USA), and images were captured and analyzed using the Nikon NIS Elements AR software (Nikon instruments).

For fibre type specific MyoD detection serial sections were used to visualize fibre type (MHCI and MHCII) and MyoD and Pax7 localization (MyoD, Pax7, Laminin, MHCI and DAPI). For detection of nMHC expression all sections were treated with the nMHC Ab and only a subset of sections possessed fibres expressing nMHC. To determine nMHC expression within hybrid fibres in subjects who had fibres expressing nMHC, serial sections were used to identify hybrid fibres (MHCI and MHCII) and fibres expressing nMHC (MHCn, laminin, Pax7 and DAPI). The association of SC with fibres expressing nMHC was also determined.

SC and CSA quantification was conducted on an average of 400 fibres/subject/timepoint. Quantification of MyoD and of centrally located nuclei was conducted on an average of 200 fibres/subject. All quantification was conducted in a blinded fashion.

RNA Isolation

RNA was isolated from 15 to 25 mg of muscle tissue using the Trizol/RNeasy method (Qiagen Sciences, Valencia, CA, USA). All samples were homogenized with 1mL of Trizol® Reagent (Life Technologies, Burlington, ON, Canada), in Lysing Maxtrix D tubes (MP Biomedicals, Solon, OH, USA), with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA) for a duration of 40 sec at a setting of 6m/sec, samples were then stored at -80˚C until further processing. Samples were thawed and 200µl of cholorform reagent (Sigma-Aldrich, Oakville, ON, Canada) was added to each sample, mixed for 15 sec, incubated at room temperature for 5 min and centrifuged for 10 min at 12000g at 4˚C. The RNA phase was then transferred to a RNeasy mini-spin column and RNA was purified following the commercially available E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) manufacturer's instructions. The RNA concentration and purity was quantified using the Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA).

Quantitative real time RT-PCR

Individual samples were reverse transcribed in 20 µl using the commercially available highcapacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, using an using an Eppendorf Mastercycler epgradient thermal cycler (Eppendorf, Mississauga, ON, Canada). Reactions were run using RT^2 real-time SYBR Green qPCR master mix (SABioscience; Qiagen Sciences), prepared using the epMotion 5075 Eppendorf automated pipetting system (Eppendorf, Mississauga, ON, Canada) and carried out in duplicate an Eppendorf $realplex^2$ Master Cycler epgradientS (Eppendorf, Mississauga, ON, Canada). Primer sequences are shown in Supplemental Table 1.

Statistical Analysis

Statistical analysis was performed using SigmaStat 3.1.0 analysis software (Systat Software Inc., Chicago, IL, USA). A 2-way repeated-measures ANOVA with one factor for time (pre, post) and one factor for fibre type (slow, fast, hybrid) was conducted for the fibre type specific SC analysis, significant interactions and main effects were analyzed using the Tukey's HSD post hoc test. A one way ANOVA was conducted for the fibre type specific centrally located nuclei and for the MyoD and Pax7 populations, significant group differences were analyzed with the Holm-Sidak method for pairwise multiple comparisons. A paired two-tailed t-test was conducted for pre-post comparison myonuclear domain, nuclei per fibre, the SC domain and mRNA expression. Statistical significance was accepted at $p \leq .05$, all results are reported as means ± standard error of the mean (SEM).

Results

Fibre Cross Sectional Area

Fibre cross sectional area (CSA) was assessed with immunofluorescence staining for type I (MHCI) and type II (MHCII) fibres and fibres expressing both MHCI and

MHCII were identified as hybrid fibres. Six wks of aerobic interval training resulted in no significant increase in CSA for either type I (pre: 5962±365 μm^2; post: 5899±358 μm^2), II (pre: 5469±390 μm^2; post: 5291±373 μm^2) or hybrid fibres (pre: 5499±426 μm^2; post: 4881±424 μm^2) (Fig. 1A). In accordance with fibre CSA the number of nuclei per fibre (pre: 4.5±0.34; post: 4.6±0.24 nuclei/fibre) as well as the myonuclear domain, defined as the fibre CSA per nuclei (pre: 1159±46; post: 1223±66 μm^2/nuclei) remained unchanged with training (Fig. 1B/C). Additionally, the SC domain, defined as the fibre CSA per Pax7 positive cell, was also maintained following 6 wks of training (pre: 35912±3147; post: 34829±2679 μm^2/SC) (Fig. 1D). Together these data indicate that 6 wks of aerobic interval training did not lead to skeletal muscle hypertrophy.

Fibre type distribution

Fibre type distribution was determined via immunofluorescent staining of muscle cross sections for type I and type II fibres, fibres expressing both MHCI and MHCII were identified as hybrid fibres. Although the proportion of hybrid fibres pre training was low, 10 of the 15 subjects did have hybrid fibres. Following training, only one subject had no visible hybrid fibres but the proportion of hybrid fibres varied considerably between subjects (from 0% to 20%). Training resulted in a significant decrease in type II fibres (pre: 61±12%; post: 54±13%; $p<.05$), a strong trend for an increase in hybrid fibres (pre: 0.7±0.7%; post: 5±6%; $p=0.074$), whereas the proportion of type I fibres was unchanged (pre: 38±12%; post: 40±13%) (Table 1).

Fibre Type Specific SC Response

The fibre type specific SC response was quantified using immunofluorescent staining of Pax7, laminin, MHCI, MHCII and DAPI (Fig. 2Aa-d). Training did not expand the SC pool associated with either type I (pre: 0.20±0.022; post: 0.21±0.023 SC/type I fibre) or type II fibres (pre: 0.16±0.020; post: 0.17±0.016 SC/ type II fibre) (Fig. 2B). Rather, a specific expansion of the SC pool was observed in association with hybrid fibres (pre: 0.062±0.035; post: 0.38±0.063 SC/ hybrid fibre; $p<.01$) (Fig. 2B). Prior to training a greater number of SC were associated with type I fibres (pre:

0.20±0.022 SC/type I fibre) compared to hybrid fibres (pre: 0.062±0.035 SC/ hybrid fibre; $p<.05$) (Fig. 2B). Additionally following training a greater number of SC were associated with hybrid fibres (0.38±0.063 SC/ hybrid fibre) compared to either type I (0.21±0.023 SC/type I fibre; $p<.01$) or type II fibres (0.17±0.016 SC/type II fibre; $p<.01$) (Fig 3.B). In accordance with the higher number of SC associated with hybrid fibres compared to either type I or II fibres following training, the SC domain of hybrid fibres (11816±1718 μm^2/hybrid associated SC) was smaller than the SC domain of either type I (39506±5158 μm^2/per type I associated SC; $p<.01$) or II fibres (41996±3847 μm^2/type II associated SC; $p<.01$) following training (Fig. 3C). Collectively, training resulted in SC expansion but only in association with hybrid fibres.

Remodeling Hybrid Fibres Following Training

A hallmark observation of repair and remodeling in skeletal muscle is the presence of central nuclei. The percentage of fibres with centrally located nuclei was quantified using immunofluorescent staining of MHCI, MHCII and DAPI (Fig. 3Aa-b). Hybrid fibres had significantly more centrally located nuclei (15.1±4.33%) then either type I (3.4±0.65%; $p<.01$) or type II fibres (3.6±0.98%; $p<.01$) following training suggesting that hybrid fibres were undergoing remodeling (Fig. 3B).

Immunofluorescent staining of all muscle cross sections did not reveal any fibres expressing eMHC. Before training, 1 of 15 subjects possessed fibres expressing nMHC, while 5 of 15 subjects had fibres expressing nMHC following training. This indicates that not all hybrid fibres were positive for nMHC. To determine the proportion of hybrid fibres expressing nMHC serial cross sections were prepared with sections stained for MHCI and MHCII and corresponding sections stained with laminin, nMHC and Pax7 (Fig. 4). This analysis was restricted to 4 of the 5 subjects that had fibres expressing nMHC post training due to lack of muscle tissue. Fibres expressing nMHC represented 9.3% of total fibres while hybrid fibres represented 9.4% of total fibres. Of all hybrid fibres analyzed 58.0% of them also expressed nMHC, while 40.2% of fibres expressing nMHC were not hybrid fibres but rather either type I or type II muscle fibres (Table 2).

The number of SC associated with fibres expressing nMHC was also determined. The average number of SC associated with hybrid fibres not expressing nMHC was 0.45 (SC/ hybrid not expressing nMHC) and 0.40 to hybrid fibres expressing nMHC (SC/hybrid expressing nMHC). The number of SC associated with all fibres expressing nMHC (both hybrid and non-hybrid fibres) was 0.31 (SC/fibre expressing nMHC) and the number of SC associated with hybrid fibres was 0.39 (SC/hybrid fibre) (Table 3).

MyoD and Pax7 Populations

To further describe the SC response following training we assessed Pax7+ and MyoD+ cells in a fibre type specific manner through immunofluorescent staining (Fig. 5Aa-d). Hybrid fibres were identified using serial sections stained with MHCI and MHCII. These fibres were then identified on complementary sections stained for Pax7, MyoD, laminin and MHCII. MyoD is a key MRF expressed by SC during proliferation and during the transition between proliferation and differentiation. Hybrid fibres were associated with significantly more quiescent SC (Pax7+/MyoD-) per myofibre (0.28±0.088 Pax7+/MyoD- SC/hybrid fibre) then type I or II fibres (0.099±0.0080, Pax7+/MyoD- per type I fibre p=.01; 0.088±0.01 Pax7+/MyoD- SC/type II fibre, p<.01), differentiating SC (Pax7-/MyoD+) per myofibre (0.18±0.096 Pax7-/MyoD+ SC/hybrid fibre) then type I or II fibres (0.015±0.0062 Pax7-/MyoD+ SC/type I fibre, p<.05; 0.012±0.0045, Pax7+/MyoD- SC/type II fibre p<.05) and there was a strong trend towards a greater number of activated SC (Pax7+/MyoD+) per myofibre (0.048±0.023 Pax7+/MyoD+ SC/hybrid fibre) as compared to type I and II fibres (0.015±0.0052 Pax7+/MyoD+ SC/type I fibre; 0.018±0.0055 Pax7+/MyoD+ SC/type II fibre, p=.133) following training. Collectively, this data suggests that there are not only more quiescent SC associated with hybrid fibres following training but also significantly more active SC.

Discussion

The primary novel finding from the present study was a fibre type specific expansion of the SC pool following 6 wk of aerobic interval training. More specifically we describe an increase in active SC associated with hybrid fibres following training.

These results suggest that SC are not only activated following resistance training leading to skeletal muscle hypertrophy but are also activated and may contribute to non-hypertrophic muscle fibre remodeling. Establishing the underlying mechanisms that govern skeletal muscle adaptation is essential in understanding how skeletal muscle remodels in response to different stimuli and ultimately may have significant implications for many pathological conditions including aging.

There was no increase in CSA for any fibre type after training, consistent with other reports (23;24), showing the stimulus was not hypertrophic in nature. The myonuclear domain theory suggests that each myonuclei in a fibre is responsible for governing a defined volume of cytoplasm. If this volume exceeds the capacity of a given nucleus (i.e. under hypertrophic conditions), the addition of new nuclei are necessary to support growth (32). Similar to the myonuclear domain the SC domain is the volume of cytoplasm that each SC may be responsible for governing. The lack of hypertrophy measured by muscle CSA is further supported by the observation that neither myonuclear domain nor SC domain changed as a function of training. The shift in expression of MHC isoforms is not dependent on the addition of additional myonuclei as demonstrated with gamma-irradiation in rodent models (9-11) again supporting the maintenance of the myonuclear domain with training.

Many studies have examined the SC pool in response to exercise training in humans. It is well documented that following resistance training, where an increase in muscle mass is observed, there is a concomitant expansion of the SC pool (15-18;33). In contrast, there are limited and equivocal data regarding the SC pool response to endurance training in humans with two studies reporting an increase in SC content (20;21) and one other reporting no change (22). Interestingly, an increase in CSA of type IIa fibres following endurance training and a concomitant increase in type II associated SC has been described (21). Both studies reporting an increase in SC content following training had training protocols consistent with interval type training where subjects completed 4 min of low intensity cycling followed by 1 min of high intensity cycling for a total of 40 to 45 min 3 to 4 d/wk for 14 wk (20;21). The study that reported no change

in SC content had a more modest endurance training protocol in which subjects completed 40 min of low intensity exercise 3d/wk for 6 months (22). Given the induction of muscle hypertrophy using the training protocols with high intensity bouts of exercise it is plausible that high intensity type activity for prolonged periods of time may induce hypertrophy and hence SC activity. In addition, each study reporting on the expansion of the SC pool following endurance type training in humans reported no change in fibre type distribution (20-22). This further highlights the fact that the expansion of the SC pool associated with training likely resulted from muscle hypertrophy and was not due to a fibre type transition induced by training. This notion is supported by a study in rats that demonstrated an expansion of the type II associated SC pool following high intensity endurance training as compared to no change in the SC pool of rats undergoing low intensity endurance training (23). Taken together these results suggest that SC may play a role in endurance type training but their contribution to adaptation is likely intensity dependent.

Aerobic interval training has been shown to elicit skeletal muscle remodeling similar to traditional endurance training, characterized most prominently by increased mitochondrial content (34-36). Contrary to previous studies (20;21) there was no expansion of the SC pool associated with either type I or II fibres after training in the present study. Rather, training expanded the SC pool only in association with hybrid fibres, whose proportion tended to increase with training. In the small number of subjects who had hybrid fibers expressing nMHC the number of SC associated with these fibers (0.40 SC/hybrid fibre) was similar to the number of SC associated with hybrid fibres not expressing nMHC (0.45 SC/hybrid fibre). These results demonstrate that the number of SC associated with hybrid fibres is similar whether the fibres also express nMHC or not. We hypothesize that the increase in SC associated with hybrid fibres following training was necessary in order to remodel fibres expressing only either MHCI or MHCII to hybrid fibres expressing both MHC I and II. In pre muscle biopsies hybrid fibres were identified in 8 of the 15 subjects and in 14 out of the 15 subjects post training biopsies. Although identifiable prior to training the frequency at which hybrid fibres were

identified following training was greater. This may suggest that under normal physiological conditions in some individuals hybrid fibres are present but do not require a greater association of SC compared to type I or II fibres. Prior to the onset of training fewer SC were associated with hybrid fibres compared to type I fibres. It is possible that fewer SC are associated with hybrid fibres before training as these fibres are not necessarily undergoing active remodeling (or to the same degree as with exercise) to express both MHCI and II isoforms but rather express both these isoforms in normal physiological conditions. Following training hyrbid fibres had more centrally located nuclei compared to both type I and II fibres, which is indicative of regenerating or remodeling fibres (37). This result would also suggest that SC play a role in muscle remodeling.

In addition to the presence of centrally located nuclei as a marker of fibre regeneration or remodeling we also performed immunofluorescent staining for embryonic MHC (F1.652 [embryonic myosin heavy chain (eMHC; embryonic isoform);DSHB]) (data not shown) and neonatal MHC on muscle cross sections. In newly formed fibres or regenerating fibres eMHC is expressed followed by nMHC (38). These stains revealed no fibres staining positive for eMHC. However we determined that in subjects who had fibers expressing nMHC post training 42.0% of these fibres were not hybrid fibres, some fibres expressing solely MHCI or MHCII also expressed nMHC indicating that a small number of type I and II fibres were undergoing regeneration. We are however unable to definitively state whether hybrid fibres are regenerating fibres no longer expressing eMHC/nMHC or whether they are mature muscle fibres undergoing remodeling.

Fast-to-slow and slow-to-fast fibre type transitions are well documented in skeletal muscle following different stimuli. When a fast-to-slow fibre type transition is induced in rabbit skeletal muscle via chronic low frequency stimulation an increase in hybrid fibres is observed (39). Our results suggest that the training intervention was able to induce a fast-to-slow fibre type transition as there tended to be a greater proportion of hybrid fibres post training and a reduction in type II fibres. It may be possible that hybrid fibres undergo bouts of degeneration and regeneration in order to remodel their

phenotypic profile and thus also express nMHC at early time points during this process which may explain why not all hybrid fibres express nMHC. We hypothesize that the decrease in type II fibres represents a small population of fibres that are undergoing active remodeling and begin to express both MHC isoforms and may ultimately contribute to the fast-to-slow fibre type transition. This further supports the evidence that interval training induces similar skeletal muscle adaptations observed with endurance training (26) as endurance training increases the proportion of type I fibers (40).

In support of our findings, a fast-to-slow fibre type transition induced via chronic low frequency stimulation, resulted in an increase in the proportion of hybrid fibres and SC content (41). The increase in the number of hybrid fibres was accompanied by an increase in fibres expressing developmental MHC, indicative of fibres undergoing a transition in their MHC isoform expression, although there was no indication of fibre regeneration (41). These findings suggest that the fibre type transition occurred in adult fibres and was not the result of newly formed myotubes. While gamma-irradiation studies in rodents have demonstrated that even in the absence of SC skeletal muscle is able to undergo fibre type transition from type II to I (9-11) our results suggest that when present SC play an active role in the remodeling of fibres. Perhaps what is most important to consider is not whether SC are necessary for a transition in MHC isoform expression but rather their role in remodeling of the fibres when they are present. Together these results highlight the importance of SC in the remodeling of muscle tissue.

In addition to the higher number of SC associated with hybrid fibres, we also observed a higher number of active SC associated with hybrid fibres following training. MyoD is a member of the MRF family and its expression is up-regulated in proliferating and differentiating SC to promote progression through the myogenic program (8;42). We further highlight the role that SC play in remodeling fibres as an increase in differentiating (Pax7-/MyoD+), and a strong trend to an increase in activated (Pax7+/MyoD+) SC was associated with hybrid fibres following training. The increase in MyoD+ SC suggests an increase in the number of active SC associated with hybrid fibres likely contributing to the remodeling of the muscle fibre. In accordance with the larger

pool of active SC associated with hybrid fibres an increase in mRNA expression of MRFs expressed during SC proliferation, myf5 and MyoD, and differentiation, MyoD, MRF4 and myogenin, (43;44), was observed at the whole muscle level following training (Sup.Fig1).

In summary, evidence presented herein suggests that satellite cells are active in muscle fibres that appear to be remodeling in response to non-hypertrophic adaptation to exercise-training. The precise role of the satellite cell in myofiber remodeling remains unclear and further investigation is necessary. If exposed to a hypertrophic stimuli (i.e. resistance training) the SC may play a role in muscle hypertrophy but when exposed to a non-hypertrophic stimuli (i.e. aerobic interval training) the SC may play a role in the remodeling of muscle fibres. We also show that following training a greater number of hybrid fibres compared to other fibre types were undergoing remodeling based on the presence of central nuclei. Finally, we report an expansion of the SC pool in response to training specific to hybrid fibres, which also showed a larger pool of activated SC compared to either type I or II fibres following training. These data are the first to describe the ability of aerobic interval training to induce a fibre type specific SC response implicating an active role for SC in skeletal muscle remodeling.

Acknowledgments

The Pax7 hybridoma cells developed by Dr. A. Kawakami; and the A4.951 hybridoma cells, developed by Dr. H. Blau, were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA52242 USA.
Contributions: G.P., M.J.G, J.B.G., S.J conceived and designed the experiments, M.A.T., J.B.G, B.R.M collected the data, S.J., L.M.B., B.R.M, G.P analyzed the data; S.J., G.P prepared the manuscript. S.J., G.P., J.B.G., M.J.G., L.M.B., B.R.M., M.A.T edited the manuscript. All authors approved the final version of the manuscript for publication. The current study was completed at McMaster University, Hamilton, Ontario, Canada.

Tables

Table 1. Fibre type distribution PRE and POST training

	Type I	**Type II**	**Hybrid**
PRE	38.1 **	61.1 **	0.7 **
POST	39.9 ***	54.5*, ***	5.5#

Fibre type distribution of type I, II and hybrid fibres PRE and POST training reported as percentages. (*) indicates a significant change from PRE; (**) indicates a significant difference from the two other fibre types; (***) indicates a significant difference from hybrid fibres ($p<.05$); (#) indicates a trend ($p=.074$) for a difference from PRE.

Table 2. Proportion of hybrid fibres expressing nMHC

Hybrid (% of total fibres)	**nMHC positive (% of total fibres)**	**% hybrid fibres expressing nMHC**	**% hybrid fibres not expressing nMHC**	**% nMHC non hybrid fibres**
9.4	9.3	58.0	42.0	40.2

Proportion of hybrid fibres, fibres expressing nMHC reported as a percentage of total muscle fibres. Proportion of hybrid fibers expressing and not expressing nMHC reported as a percentage of total hybrid fibres. Proportion of non hybrid fibres expressing nMHC reported as a percentage of total nMHC fibres. This analysis was completed on 4 subjects POST training who had fibres expressing nMHC.

Table 3. Association of SC with fibres expressing nMHC

Pax7 positive cells associated with hybrid fibres (SC/fibre)	Pax7 positive cells associated with fibres expressing nMHC (SC/fibre)	Pax7 positive cells associated with hybrid fibres expressing nMHC (SC/fibre)	Pax7 positive cells associated with hybrid fibres not expressing nMHC (SC/fibre)
0.39	0.31	0.40	0.45

Number of SC associated with hybrid fibres, fibres expressing nMHC, hybrid fibres expressing nMHC and hybrid fibres not expressing nMHC. This analysis was completed on 4 subjects POST training who had fibres expressing nMHC.

Figures and Legends

Figure 1. Absence of hypertrophy following training

Fibre-type specific CSA, myonuclei and SC were quantifed by visualization of myofibre borders (laminin and MHCI/II) nuclei (DAPI) and SC (Pax7) **(A)** Mean CSA of type I, II and hybrid fibres PRE and POST training. Calculations of **(B)** nuclei per fibre, **(C)** myonuclear domain (fibre area (μm^2) per nucleus) and **(D)** SC domain (fibre area (μm^2) per Pax7+ cell) PRE and POST training.

Figure 2. Fibre type specific association of satellite cells pre and post training

(A) representative image of a Pax7/Laminin/MHCI/MCHII/nuclei immunofluorescent stain on a muscle cross section. Yellow arrows denote Pax7+ cells associated with a hybrid fibre, white arrow denotes a Pax7+ cell associated to a type I fibre. Single channel views of **(a)** MHCI (green), **(b)** MHCII and laminin (red), **(c)** Pax7 (pink) and, **(d)** nuclei (blue-DAPI) are provided, scale bar measures 10μm. **(B)** the SC response of type I, type II hybrid fibres associated SC PRE and POST training expressed per myofibre. * indicates a significant difference from type I fibres, ($p<0.05$); ** indicates a significant difference from PRE and from type I and II fibres, ($p<0.01$); mean ± SEM.

Figure 3. Fibre type specific satellite cell domain and centrally located nuclei following training

(A) representative image of a MHCI/MHCII/nuclei immunofluorescent stain on a muscle cross section. Yellow arrows denote hybrid fibres, white arrows denote centrally located nuclei within a hybrid fibre. Single channel views of **(a)** MHCI (green) and **(b)** MHCII (red) are provided, scale bar measures 10μm. **(B)** The percentage of type I, II and hybrid fibres with centrally located nuclei following training. **(C)** The fibre type specific SC domain following training expressed as fibre area (μm^2) per Pax7+ cell. ** indicates a significant difference from type I and II fibres ($p < 0.01$); mean $\pm$ SEM.

Figure 4. Association of satellite cells to fibres expressing MHCn

(A) representative image of a Pax7/nMHC/Laminin/nuclei immunofluorescent stain on a muscle cross section, scale bar measures 100 µm. Boxed area is magnified in **(B)**, the white arrows denote Pax7+ cells associated with a fibre expressing nMHC, scale bar measures 10 µm. **(a)** single channel views of nMHC (green), **(b)** Pax7 (red)/laminin (orange) note the Pax7 positive cells, denoted by white arrows lie beneath the basal lamina (orange) and are associated with a fibre expressing nMHC, **(c)** Pax7 (red) and **(d)** nuclei (blue-DAPI).

Figure 5. Fibre type specific MyoD response of satellite cells following training

(A) representative image of a Pax7/MyoD/Laminin/MCHII/nuclei immunofluorescent stain on a muscle cross section. The yellow arrow denotes a Pax7-/MyoD+ cell associated with a hybrid fibre, the white arrow denotes a Pax7+/MyoD- cell associated with a hybrid fibre and the green arrow denotes a Pax7+/MyoD- cell associated with a type II fibre. Single channel views of **(a)** Pax7 (green), **(b)** MHCII and laminin (red), **(c)** MyoD (pink) and **(d)** nuclei (blue-DAPI) are provided, scale bar measures 10 µm. **(B)** the fibre type specific association of quiescent (Pax7+/MyoD-), **(C)** activated (Pax7+/MyoD+) and **(D)** differentiating (Pax7-/MyoD+) SC following training expressed per myofibre. ** indicates a significant difference from type I and II fibres ($p<0.05$); mean $\pm$ SEM.

Supplemental material

Supplemental Figure 1. Gene Expression Analysis

Whole muscle mRNA expression of (A) Pax7, (B) Myf5, (C) MyoD, (D) MRF4 and (E) myogenin PRE and POST training. Data are expressed as fold change from PRE. *indicates a significant difference from PRE ($p<0.05$).

Supplemental Table 1. Quantitative RT-PCR primer sequences

Gene	Forward sequence	Reverse sequence
Pax7	5’-GCTCCGGGGCAGAACTACC-3’	5’-GCACGCGGCTAATCGAACTC-3’
Myf5	5’-ATGGACGTGATGGATGGCTG-3’	5’-GCGGCACAAACTCGTCCCCAAATT-

		3’
MyoD	5’-GGTCCCTCGCGCCCAAAAGATT-3’	5’-CAGTTCTCCCGCCTCTCCTACCTCAA-3’
Myogenin	5’-CAGTGCACTGGAGTTCAGCGCCAA-3’	5’-TTCATCTGGGAAGGCCACAGACACAT-3’
MRF4	5’-CCCCTTCAGCTACAGACCCAAACAAGAA-3’	5’-CCCCCTGGAATGATCGGAAACAC-3’

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Figure 1.

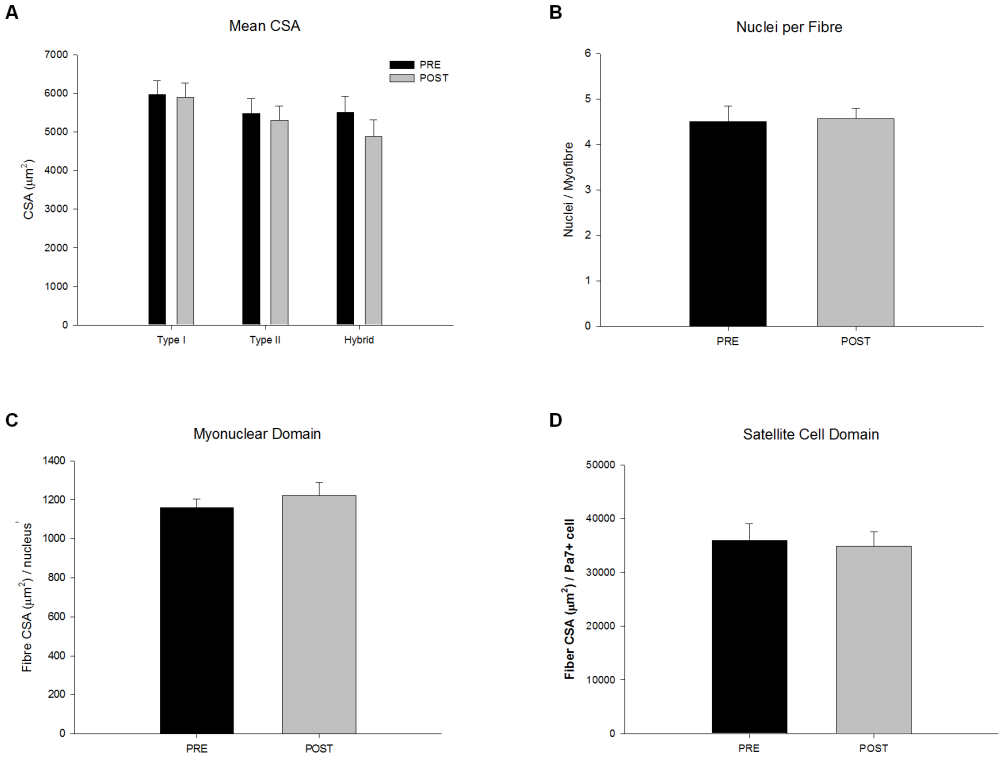
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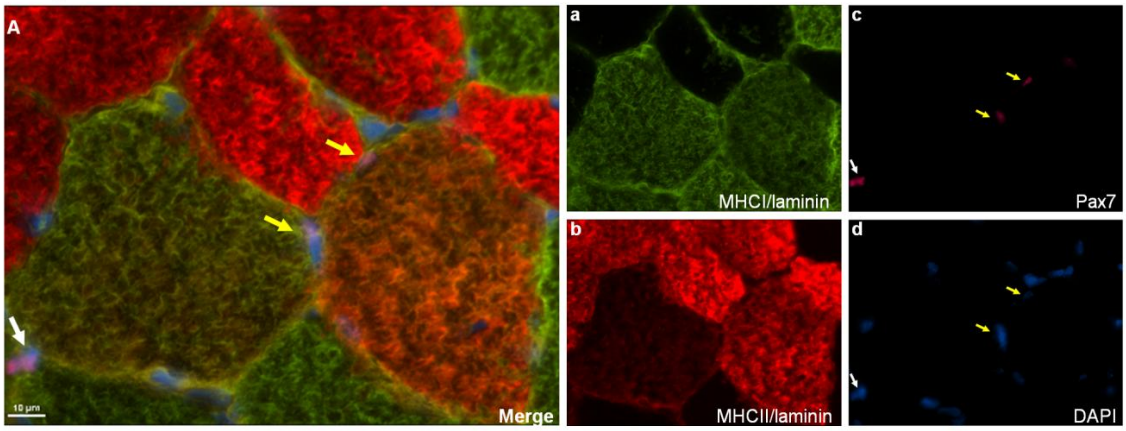
Figure 2.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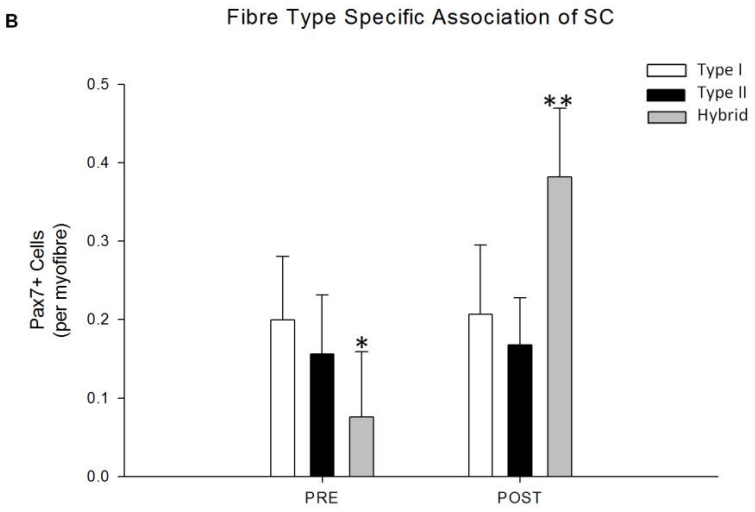
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Figure 3.

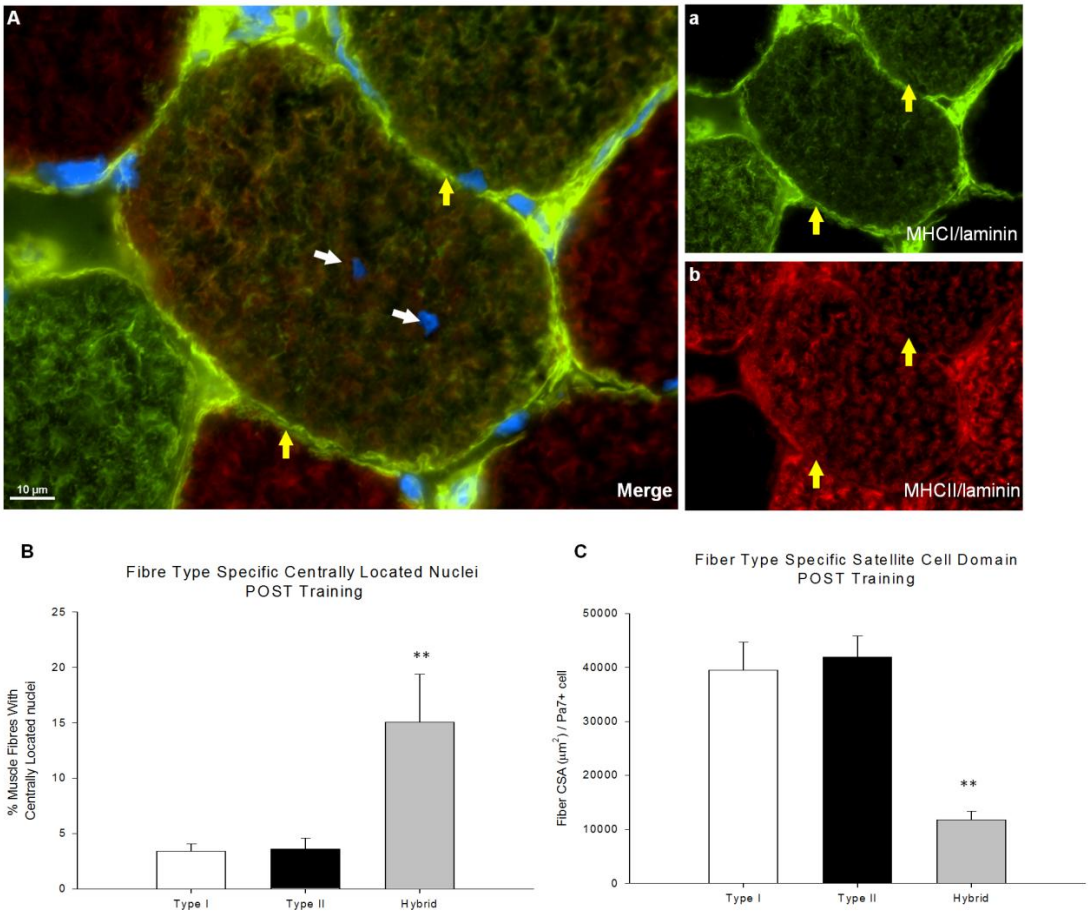
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Figure 4.

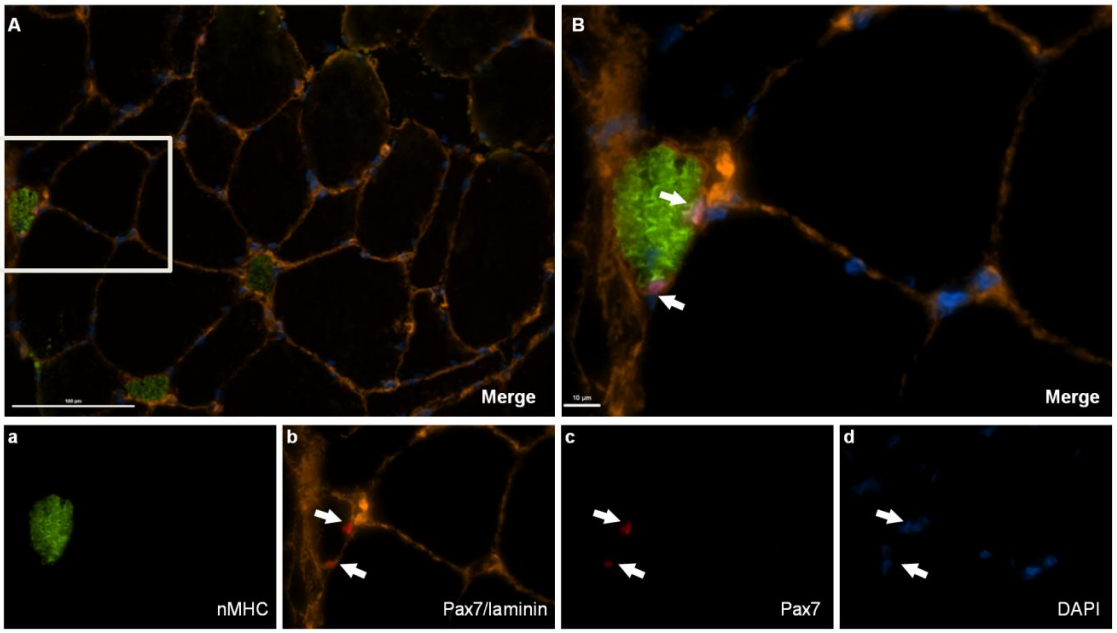
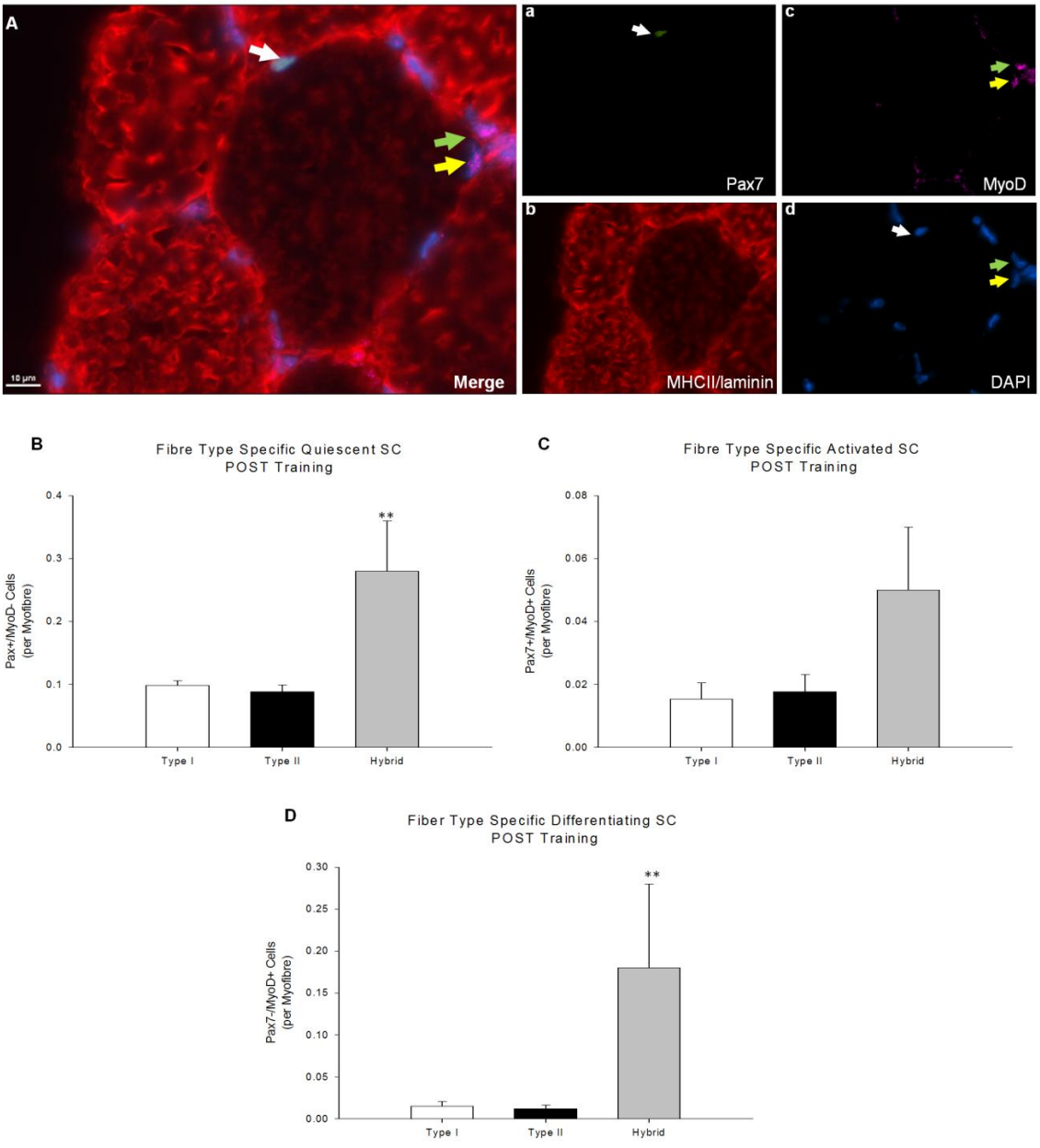


Figure 5.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POST Training
Pax+/MyoD- Cells
(per Myofibre)
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Type I
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Fibre Type Specific Activated SC
POST Training
Pax7+/MyoD+ Cells
(per Myofibre)
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Type I
Type II
Hybrid
D
Fiber Type Specific Differentiating SC
POST Training
Pax7-/MyoD+ Cells
(per Myofibre)
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0.25
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Type I
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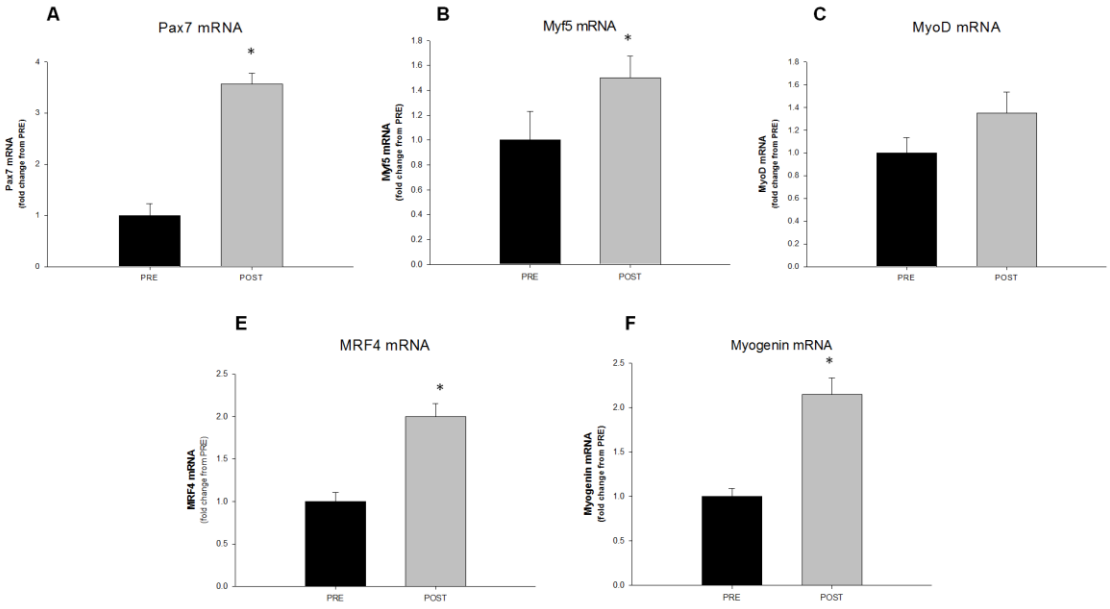
Hybrid

Figure S1.


A
Pax7 mRNA
Pax7 mRNA (fold change from PRE)
PRE
POST
B
Myf5 mRNA
Myf5 mRNA (fold change from PRE)
C
MyoD mRNA
MyoD mRNA (fold change from PRE)
E
MRF4 mRNA
MRF4 mRNA (fold change from PRE)
F
Myogenin mRNA
Myogenin mRNA (fold change from PRE)

Chapter 3:

Satellite cell activity, without expansion, following non-hypertrophic stimuli (2015). *Am J Physiol Regul Integr Comp Physiol* 309: R1101–R1111

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Abbreviated title

Contribution of satellite cells to non-hypertrophic stimuli

Abstract

The purpose of this study was to determine the effect of various non-hypertrophic exercise stimuli on satellite cell (SC) pool activity in human skeletal muscle. Previously untrained males and females (males: 29±9yr; females: 29±2yr, n=7 each) completed six weeks of very low volume high intensity sprint interval training (SIT-1). In a separate study, recreationally active males (n=16) and females (n=3) completed 6 weeks of either traditional moderate-intensity continuous exercise (MICT) (n=9: 21±4yr) or low volume sprint interval training (SIT-2) (n=10: 21±2yr). Muscle biopsies were obtained from the *vastus lateralis* pre- and post-training. The fibre type specific SC response to training was determined as was the activity of the SC pool using immunofluorescent microscopy of muscle cross sections. Training did not induce hypertrophy as assessed by muscle cross sectional area (CSA) nor did the SC pool expand in any group. However, there was an increase in the number of active SC following each intervention. Specifically, the number of activated ($Pax7^+/MyoD^+$, $p \leq .05$) and differentiating ($Pax7^-/MyoD^+$, $p \leq .05$) SC increased following each training intervention. Here we report evidence of activated and cycling SC that may or may not be contributing to exercise-induced adaptation while the SC pool remains constant following three non-hypertrophic exercise training protocols.

Key Words

muscle stem cells, Pax7, MyoD, endurance training, sprint interval training

Introduction

Skeletal muscle repair is largely possible due to a pool of resident muscle stem cells, referred to as satellite cells (SC) (31). Although essential in mediating skeletal muscle regeneration (24, 30, 32, 41, 49), SC become active following various stresses such as eccentrically loaded muscle contractions (8, 10, 33, 38, 42) and bouts of resistance exercise (34, 37), the acute response of SC to various exercise stimuli have been well characterized (26). Upon activation, SC proliferate and differentiate driving muscle repair, while a proportion of SC revert to quiescence following proliferation to maintain the muscle SC pool. This process is referred to as the myogenic program and is regulated by a transcriptional network collectively referred to as the myogenic regulatory factors (MRF) and includes Myf5, MyoD, Mrf4 and Myogenin (2, 15, 47, 56). Shortly after activation, Myf5 is expressed by SC which is followed by the up-regulation of MyoD through proliferation and early differentiation. Following proliferation SC undergo differentiation, which is achieved by the up-regulation of MRF4 and myogenin (5, 9).

The contribution of SC to skeletal muscle hypertrophy following resistance training in humans has been extensively studied (20, 27, 43, 44, 53, 59). Although gains in muscle mass are often associated with an expansion of the SC pool, more recent work demonstrated that an increase in fibre cross sectional area (CSA) is not always associated with expansion (11). Albeit to a lesser extent, the SC response to endurance training has also been studied. An expansion of the SC pool was observed following 14 weeks of interval training consisting of one minute of high intensity cycling interspersed by 4 minutes of low intensity cycling for a total of 40 to 45 minutes (6, 61). In contrast, others have suggested that there is no change in SC content following a more traditional lower intensity endurance training program (54). These data suggest that exercise intensity may play an important role in mediating SC pool expansion with respect to aerobic exercise. It is important to note that studies reporting an expansion in the SC pool following high intensity cycle training also reported an increase in CSA (6, 61). We recently reported an expansion of SC specifically associated with hybrid fibres in the absence of both an expansion of the total SC pool and muscle hypertrophy (18). Endurance exercise in rodent

models has been shown to result in an increase in SC content (23, 51, 52). However, there is a paucity of information, regarding the influence of SC on exercise-induced skeletal muscle adaptations/remodeling. Our recent work suggests that SC may support muscle adaptation and remodeling in the absence of muscle hypertrophy. We have demonstrated that a non-hypertrophic stimuli like aerobic interval training leads to an expansion of the SC pool associated with remodeling fibres (18).

Studies examining the response of SC to various modes of exercise commonly use immunofluorescent techniques to label SC with various markers such as Pax7 (4, 29, 37, 38, 58) and neural cell adhesion molecule (NCAM, CD56) (8, 10, 28, 29, 38, 42). Previous literature has identified SC concomitantly with various markers of proliferation such as Ki67, PCNA (29) or MyoD (18, 36) to further describe the SC pool and provide more in depth information on its activity status. In addition to the increase in the number of SC associated with hybrid fibers following aerobic interval training described above, we also observed an increase in the number of active SC associated with these fibres (18). These results suggest that enumerating the number of SC per fibre following exercise training may not be sufficient in describing the contribution of SC to muscle adaptation or remodeling. The current study aimed to investigate whether the number of proliferating or differentiating SC increased following 3 non-hypertrophic stimuli. We hypothesized that there would be no expansion of the SC pool; however, there would be evidence of an increase in the number of active SC following training.

Materials and Methods

Subjects:

Study 1

Nineteen healthy, recreationally active males (n= 16) and females (n= 3) volunteered to participate in this training study. Subjects were matched by VO_2-peak and assigned to either the low volume high intensity sprint interval training (SIT-2; n =10, 21 ± 2 yr; 175 ± 10 cm; 71 ± 17 kg) or moderate-intensity continuous exercise (MICT; n= 9, 21 ± 4 yr; 180 ± 7 cm; 74 ± 9 kg) groups. Complete subject characteristics have been

previously reported (50). The subjects were considered recreationally active and were not involved in more than 3 hours of aerobic exercise (recreational sports, jogging, etc.) per week or involved in any structured training program within the past six months.

Study 2

Fourteen overweight/obese men (n =7; 29 ±9 yr; 176 ± 5 cm; 97 ± 8 kg) and women (n = 7; 29±20yr; 162 ± 8 cm; 75 ± 12 kg) performed low-volume high intensity sprint interval training (SIT-1). Complete subject characteristics are reported elsewhere. The subjects were considered sedentary based on their self-reported habitual physical activity, which consisted of less than 2 sessions per week of structured exercise lasting less than 30 min.

Physiological Testing:

Study 1

Pre-training VO_2-peak was assessed by a VO_2-peak incremental ramp test to exhaustion as described previously (50). During baseline testing subjects reported to the lab in the morning following an overnight fast (≥8 h). Subjects were fed a standardized breakfast [plain bagel (190 kcal; 1 g fat, 36 g CHO, 7 g protein) with 15 g of peanut butter (90 kcal; 8 g fat, 4 g CHO, 3 g protein) and 200 mL of apple juice (90 kcal; 0 g fat, 22 g CHO, 0 g protein)] and rested for 1 hour before a muscle biopsy was taken from the *vastus lateralis* muscle under superficial local anaesthesia (2% lidocaine, with epinephrine) using the Bergstrom needle biopsy technique (3) adapted with suction. Forty-eight hours following the muscle biopsy participants returned to the lab to complete a VO_2-peak incremental ramp test to exhaustion as described previously (50). A VO_2-peak incremental ramp test to exhaustion was performed half way through training on the first day of week 4 in order to adjust training loads. As a result, subjects only completed 3 training sessions in week 4. Post-training testing was conducted 72 hours following the last training session of week 6 and was conducted in an identical manner as the baseline testing.

Study 2:

Pre-training VO_2-peak was assessed by a VO_2-peak incremental cycling test on an electronically braked cycle ergometer to exhaustion as described previously (25). Eight days later, subjects reported to the lab in the morning following an overnight fast (≥10 h) for baseline testing, and subsequently a resting skeletal muscle biopsy was obtained, from the *vastus lateralis* under local anesthesia (1% lidocaine) using a Bergstrom needle technique adapted for suction (57). The post-training muscle biopsy was obtained 72 hours following the last training session and the post-training testing session was conducted four days later, in an identical manner as the baseline testing.

Training Intervention*:*

Study 1:

As described previously, all subjects completed training four days per week for six weeks (week 4 only had 3 training sessions due to the mid-training VO_2-peak test for a total of 23 sessions) (50). SIT-2 exercise protocol was performed as described previously (19, 55). Briefly, subjects completed eight 20-second intervals at 170% of VO_2-peak separated by 10 seconds of rest eight times, for a total of four-minutes. During rest periods subjects cycled against no load at a self-selected cadence. The MICT exercise protocol consisted of 30 minutes of continuous cycling at 65% of VO_2-peak. Each exercise bout was performed at the same time of day for all subjects. A standardized warm up of descending and ascending 4 flights of stairs was completed prior to all training sessions. Training intensity was monitored by revolutions per minute (RPM) data for each training session.

Study 2

All subjects completed 18 sessions over a six week training period (Monday, Wednesday, Friday, each week). Each SIT-1 session consisted of a two minute warm up at 50W followed by three 20s all-out cycling sprints against a load that corresponded to 0.05/kg/kg body mass, interspersed by two minutes of low intensity recovery cycling (50W), with a 3 minute cool down at 50W; a total time commitment of 10 minutes.

Immunofluorescence

Muscle cross sections (7µm) were prepared from unfixed OCT embedded samples, allowed to air dry for 15-45 minutes and stored at -80˚C. Samples were stained with antibodies against Pax7 (1:1; cell supernatant from cells obtained from the DSHB (Developmental Studies Hybridoma Bank) , USA), myosin heavy chain type I (clone A4.951 (slow isoform), neat; DSHB, USA); myosin heavy chain type II (1:1000; ab91506, fast isoform, Abcam, Cambride, MA, USA), laminin (1:1000;L8271, Sigma-Aldrich, Burlington, Canada and Abcam ab11575, Abcam, Cambridge, MA, USA); MyoD1 (1:30; anti-MyoD1, clone 5.8A, Dako, Burlington, ON, Canada); Desmin (ab6322, 1:500, Abcam, Cambridge, MA, USA); Myogenin (Clone F5D, neat; DSHB,USA); PCNA (ab15497, 1:40, Abcam, Cambridge, MA, USA) .

For colourmetric co-staining with F5D and Pax7 , a sequential colourmetic reaction was completed following the Vector Labs co-immunohistochemical protocols manual (www.vectorlabs.com/protocols.aspx; Vector Canada, Burlington, ON, Canada). Briefly, slides were fixed with 2% PFA and washed in PBST for 3x5min. A blocking solution containing 2% BSA, 5% FBS, 0.2% Triton X-100, 0.1% sodium azide and 10% GS was used for 30min. Following blocking, slides were incubated in Pax7 1:1 in PBS for 60min. Pax7 was visualized with a biotinylated secondary antibody, 1:200; Vector Canada, Burlington, ON, Canada, followed by the application of the VectaStain Elite ABC-AP kit (Vector Canada, Burlington, ON, Canada) according to the manufacturer's instructions. The stain was then visualized using the Vector Black-AP kit (Vector Canada, Burlington, ON, Canada) according to the manufacturers' instructions. Following completion of the reaction for Pax7 visualization the slides were washed in tap water and then blocked with an avidin/biotin blocking step (Vector Canada, Burlington, ON, Canada). Slides were then incubated in F5D (neat) overnight at 4◦C. F5D was visualized with a biotinylated secondary antibody, 1:200; Vector Canada, Burlington, ON, Canada followed by the application of the VectaStain Elite ABC kit (Vector Canada, Burlington, ON, Canada) according to the manufacturer's instructions. The stain was then visualized using the diaminobenzidine (DAB) reagent (Vector Canada, Burlington, ON, Canada)

according to the manufacturers' instructions. Nuclei were counterstained with Mayer's haematoxylin (Sigma-Aldrich, Oakville, ON, Canada).

For immunofluorescent detection secondary antibodies used were either goat-anti-mouse IgG, goat-anti-rabbit IgG, and/or goat-anti-rabbit IgG based on the primary antibody used; specific antibodies were: Pax7 (Alexa Fluor goat-anti-mouse IgG 594/488, 1:500, Invitrogen, Molecular Probes, Carlsbad, CA, USA); MyoD1(biotinylated goat anti-mouse secondary antibody, 1:200; Vector Canada, Burlington, ON, Canada; and streptavidin-594 fluorochrome, 1:250; Invitrogen, Molecular Probes); laminin (Alexa Fluor goat-anti-rabbit IgG 647, 1:500, Invitrogen); Desmin (Alexa Fluor goat-anti-mouse IgG 488, 1:500, Invitrogen, Molecular Probes, Carlsbad, CA, USA); PCNA (biotinylated goat anti-rabbit secondary antibody, 1:200; Vector Canada, Burlington, ON, Canada; and streptavidin-594 fluorochrome, 1:500; Invitrogen, Molecular Probes). Nuclei were labelled with DAPI (4',6-diamidino-2-phenylindole) (1:20000, Sigma-Aldrich, Oakville, ON, Canada), prior to cover slipping slides with fluorescent mounting media (DAKO, Burlington, ON, Canada). Immunofluorescence staining methods were adapted from previously published methods (35, 36, 54). Briefly, for co-immunofluorescence staining, sections were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min followed by multiple washes in PBS. Sections were then covered for 90 min in a blocking solution containing 2% BSA, 5% FBS, 0.2% Triton X-100, 0.1% sodium azide and 5% GS. Following blocking, sections were incubated in the primary antibodies (i.e. cocktail of Pax7 and Laminin) at 4◦C overnight. Following washes, sections were then incubated in the appropriate secondary antibodies. To prevent migration of the secondary antibodies, sections were then re-fixed in 4% PFA and re-blocked in 10% GS in PBS. Following this, sections were incubated sequentially in the second primary antibodies, either MHCI and MHCII for the fiber specific SC quantification or MyoD1 for the quantification of quiescent, proliferating or differentiating SC. This was followed by incubation in the appropriate secondary antibody (see above). Nuclei were labeled with DAPI prior to cover slipping. The staining procedures were verified using both positive and negative controls, in order to ensure appropriate specificity of staining. Following

staining, slides were viewed with the Nikon Eclipse *Ti* Microscope (Nikon Instruments, Inc. USA), equipped with a high-resolution Photometrics CoolSNAP HQ2 fluorescent camera (Nikon Intruments, Melville, NY, USA). Images were captured and analyzed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments, Inc., USA). SC, CSA and MyoD1 quantification was conducted on ≥200 muscle fibers/subject/time point, and images were obtained with the 40x objective. Slides were masked for both age and time point.

SC quantification from our co-staining methods were verified against a Pax7/Laminin/Desmin stain which specifically demonstrates the anatomical location of the Pax7 stain (the anatomical niche between the basal lamina and the sarcolemma) to ensure the validity of the Pax7 stain during the co-stain with multiple antigen labeling (Fig 1 A-E). The anatomical location of a MyoD+ nuclei was also confirmed with a MyoD/Laminin/Desmin stain. This stain confirms that MyoD+ cells were located within the SC niche, between the basal lamina and the sarcolemma (Fig 1 F-J). The number of myonuclei were determined in muscle sections stained for Pax7/MHCI/MHCII /laminin/DAPI. Myonuclei were quantified as Pax7- nuclei located beneath the basal lamina, which was identified with laminin. The number of myonuclei per myofibre was determined for ≥ 100 myofibers per section, per subject, per time point.

To determine whether the proportion of cells identified as Pax7+/MyoD+ (activated SC) were similar to the number of Pax7+/PCNA+ cells, serial muscle cross sections of 3 subjects from both the SIT-1 and the SIT-2 group were quantified (Fig 5). Due to lack of tissue the analysis was restricted to only a small subset of subjects.

To identify newly formed fibres and/or regenerating fibres muscle cross sections were stained with neonatal myosin heavy chain (nMHC1:10; VP-M666; Vector Laboratories, Burlingame, CA, USA). Immunofluorescent staining resulted in no fibres staining positive for nMHC.

Statistical Analysis

Statistical analysis was performed using Sigma Stat 3.1.0 analysis software (Systat Software, Chicago, IL, USA). Study 1 and 2 were run separately and subjects in each group (SIT-1, SIT-2, MICT) were not matched on any previous baseline physiological measures and therefore between group comparisons between the three groups were not completed. Subjects from the SIT-2 and MICT groups ('study 1') were all recreationally active and VO_2 peak matched and assigned to either group. Therefore, between groups comparisons were completed between the SIT-2 and MICT groups as were within subject comparisons between time points; whereas only between subjects comparisons were completed in the SIT-1 group. Specifically, for *Study 1* a 2-way repeated measures ANOVA with one factor for time (pre/post) and one factor for group (SIT-2/ MICT) was conducted for all analyses. For *Study 2* a paired 2-tailed t test was conducted for pre/post comparisons. $P \leq .05$ was considered statistically significant. All results are presented as means ± standard deviation.

Results

Fibre Cross Sectional Area and Myonuclear Domain

Fibre CSA for both type I and II fibres was determined based on immunoflurescent staining for myosin heavy chain type I (MHCI) and myosin heavy chain type II (MHCII) of muscle cross sections. Six weeks of training did not lead to a significant increase of CSA of either type I or II fibres with SIT-1 (type I pre: 5588±1373 μm^2, post: 4996±1138 μm^2 ; type II pre: 6202±1552 μm^2, post: 5709±1445μm^2), SIT-2 (type I pre:4596±689 μm^2, post: 4926±1050 μm^2 ; type II pre: 5172±1545 μm^2, post: 5439±1547μm^2) or MICT (type I pre: 4417±1019 μm^2, post: 4480±856 μm^2 ; type II pre: 5037±1500 μm^2, post: 5078±933 μm^2) (Fig 2 A-B).

Training did not result in an increase in the number of nuclei per fibre for either the SIT-1 (pre: 3.7±1.2, post: 3.6±1.2), SIT-2 (pre: 3.2±0.2, post: 3.5±0.3) or MICT (pre: 3.3±0.4, post: 3.4±0.2 μm^2) groups ($p > .05$) (Fig 2 C). Additionally the myonuclear domain, defined as the CSA per myonuclei was determined, and consistent with CSA and nuclei per fibre it remained unchanged following training for all three groups (SIT-1: pre:

1575±275, post: 1474±276; SIT-2: pre: 1638±359, post: 1677±352; MICT: pre: 1576±407, post: 1593±281) ($p>.05$) (Fig 2 D).

Fibre type specific SC response to training

SC were quantified based on immunofluorescent staining of muscle cross section with MHCI and MHCII to determine fibre type, Pax7 as a marker of SC and DAPI and laminin to assure accurate anatomical location (Fig 3 A). Six weeks of training did not increase the number of SC associated with either type I (SIT-1: pre: 9.4±5.1, post: 9.6±5.8; SIT-2: pre: 10.7±5.5, post: 11.5±6.7; MICT: pre: 9.2±3.7, post: 7.5±4.2 Pax7+ cells/100 type I fibres) ($p>.05$) (Fig 3 B) or II fibres (SIT-1: pre: 10.0±3.4, post: 10.6±5.0; SIT-2: pre: 7.2±2.8, post: 9.2±5.7; MICT: pre: 8.8±3.5, post: 9.3±3.5 Pax7+ cells/100 type II fibres) ($p>.05$) (Fig 3C).

SC activity following training

To further describe the SC pool we determined the proportion of active SC at baseline and following training. Muscle cross sections were stained with Pax7, MyoD, laminin and DAPI (Fig 4 A). Activated SC were identified as expressing both Pax7 and MyoD (Pax7+/MyoD+), while differentiating SC were identified as SC only expressing MyoD (Pax7-/MyoD+). A significant increase in activated SC was observed following SIT-1 (pre: 1.5±1.7, post: 2.9±2.2 Pax7+/MyoD+ cells/100 fibres) SIT-2 (pre: 3.0±1.4, post: 6.9±1.3 Pax7+/MyoD+ cells/100 fibres) and MICT training (pre: 2.4±1.4, post: 6.7±1.9 Pax7+/MyoD+ cells/100 fibres ($p\leq.05$) (Fig 4 B). Additionally a significant increase in the number differentiating SC was observed following 6 weeks of either SIT-1 (pre: 2.0±2.7, post: 3.3±1.9 Pax7-/MyoD+ cells/100 fibres), SIT-2 (pre: 0.4±0.9, post: 3.1±1.6 Pax7-/MyoD+ cells/100 fibres) and MICT (pre: 1.4±0.7, post: 3.4±1.4 Pax7-/MyoD+ cells/100 fibres) training ($p\leq.05$) (Fig 4 C).

To verify that the proportion of MyoD+/Pax7+ cells were similar to the proportion of PCNA+/Pax7+ cells, serial muscle cross sections were stained for either Pax7/MyoD/laminin or Pax7/PCNA/laminin in a small subset of subjects (SIT-1, n=3;

SIT-2, n=3) (Fig 5 A, a-d). In the subset of subjects from the SIT-1 group the proportion of Pax7+ cells expressing MyoD (Pax7+/MyoD+) was 19.6% whereas the proportion of Pax7+ cells expressing PCNA (Pax7+/PCNA+) was 20.0% (Fig 5 B). In the subset of subjects from the SIT-2 group the proportion of Pax7+ cells expressing MyoD (Pax7+/MyoD+) was 60.8% and the proportion of Pax7+ cells expressing PCNA (Pax7+/PCNA+) was 50.1%. Although the following stain was completed in a small subset of subjects it did confirm that the proportion of Pax7+ cells identified as activated in serial sections with either MyoD or PCNA are similar.

Muscle sections of the SIT-2 and MICT groups were also stained for Myogenin (Fig 6 A), a MRF expressed during terminal differentiation (Fig 6 B). In accordance with the increase in differentiating SC assessed with MyoD staining, an increase in the number of cells stained positive for myogenin was observed following SIT-2 (pre: 0.5±0.4, post: 1.0±0.7) and MICT (pre: 0.6±0.5, post: 1.0±0.6) training.

Discussion

This study demonstrates, for the first time, that a non-hypertrophic training stimulus leads to an increase in SC activity without an appreciable expansion of the SC pool in humans. More specifically, we describe an increase in the proportion of active SC following three different types of aerobic exercise despite different exercise intensities and fitness status of subjects. Similar increases in each group despite different training protocols and baseline fitness measures further supports the likelihood that may SC play a role in skeletal muscle adaptations other than increases in fibre size. Our results suggest that describing the contribution of SC to a training stimulus, such as resistance and/or endurance (traditional or sprint interval) exercise, via enumeration of the SC pool alone may not be sufficient to fully appreciate the contribution of SC to muscle adaptation. General practice has been to describe the expansion of the SC pool to determine whether SC contributed to adaptation in response to a given stimulus, however data in the current manuscript provides evidence for the presence of activated SC while the SC pool remained constant. This may reflect a slow and consistent contribution of SC to muscle

fibres where proliferation and differentiation are virtually matched so that no appreciable expansion can be detected. Alternatively, it is possible that the SC pool remains constant because of a loss in activated SC which fail to fuse to existing myofibers as previously shown in denervated rodent models (13). The inclusion of additional acute muscle biopsies following the different types of exercise stimuli may have provided further information on the progression of the SC through the myogenic program. Determining whether intensity of the stimulus or the individuals training status may impact the initial expansion of the SC pool immediately following exercise or a change in activity status of the SC pool is yet to be determined.

Consistent with other reports, we did not observe an increase in CSA of either type I or II fibres following any of the interventions (Fig 2 A-B) (7, 18, 23, 54). Additionally, training interventions did not lead to nuclear accretion (Fig 2 C). In accordance, the myonuclear domain, defined as the cytoplasmic volume governed by each nucleus remained unchanged (Fig 2 D). Taken together these data suggest that all three training interventions were non-hypertrophic regardless of training intensity, volume or subject pool.

Several studies have examined expansion of the SC pool following various training interventions in humans. An increase in SC content following resistance training is well documented, which is usually associated with an increase in lean mass (21, 28, 43, 44, 60). The data regarding SC pool expansion following endurance training is equivocal with some studies reporting an increase in the SC pool (6, 61) while others report no change (18, 54). Importantly, the studies reporting an expansion in the SC pool following aerobic training also reported an increase in muscle fibre CSA, suggesting that the intensity of exercise was sufficient to be hypertrophic in nature or that the subject pool was sufficiently sedentary to require hypertrophic adaptation in response to the endurance training protocol. Taken together with our data, this suggests that for an expansion of the quiescent SC pool to occur an increase in fibre size is required. However, the observed increase in SC pool activity may support skeletal muscle adaptation observed during

endurance exercise interventions regardless of exercise intensity or training status of the individual.

Sprint interval training, much like endurance training, leads to numerous skeletal muscle adaptations (12). Although an expansion of the SC pool was not observed following the different training modalities used herein, it is possible that SC play a role in non-hypertrophic muscle remodeling that occurs with this type of training. We report that all training interventions led to an increase in activated and differentiating SC, despite no appreciable expansion in the SC pool. It is well established that MyoD is an important regulator of myoblast proliferation (22, 40), however the presence of MyoD in SC may not be sufficient to identify a cell as proliferating since MyoD is simply a transcritpion factor and not a bona fide marker of proliferation. Other specific markers of cell proliferation such as Ki67 or PCNA have been used to specifically identify proliferating SC (29). Even among these markers, however there exist discrepancies in the proportion of SC expressing Ki67 and PCNA 24 hours following eccentric exercise in muscle cross sections. These discrepancies are likely explained by the difference in protein half-life of Ki67 versus PCNA (4). We determined the proportion of Pax7+/MyoD+ and the number of Pax7+/PCNA+ cells in corresponding serial sections in a subset of subjects. Although the analysis was completed in a small number of subjects, due to limited tissue samples, we observed a similar proportion of Pax7+ cells co-expressing either MyoD or PCNA. These data suggest that the identification of a cell expressing both Pax7 and MyoD may be classified as an activated/proliferating SC as the proportion of PCNA positive cells is similar to the proportion of MyoD positive cells in serial muscle cross sections (Fig 5 B). Furthermore, as discussed by Rudnicki et al. (2008) (48), MyoD remains upregulated while Pax7 is downregulated at the onset of differentiation. On this basis, we indicated that the cells expressing both Pax7 and MyoD (Pax7+/MyoD+) were activated SC while cells expressing only MyoD (Pax7-/MyoD+) were cells that originally expressed Pax7 but initiated the process of differentiation. To ensure that the MyoD+ cells identified were in fact SC and not existing myonuclei we qualitatively assessed the anatomical location of MyoD+ nuclei within muscle cross sections (Fig 1, F-J). Several muscle cross sections

were stained accordingly and MyoD+ nuclei were consistently found to reside within the SC niche (between the basal lamina and the sarcolemma). Several rodent studies have reported that myonuclei and not only SC express MyoD (16, 17). Based on our observations that MyoD positive nuclei were almost exclusively located within the SC niche we suggest that nuclei expressing MyoD but not Pax7 were differentiating SC and not myonuclei based upon the anatomical localization of MyoD+ nuclei.

No change in fibre type distribution was observed following 6 weeks of SIT-1, however a greater proportion of type I fibres was observed following both SIT-2 and MICT training, which is consistent with previous reports (50). Irradiation studies have demonstrated that when SC are ablated, a fibre type shift can still be achieved suggesting that SC may not be necessary for fibre type shifting (45, 46). However, while not necessary, SC may be sufficient to induce fibre type transitions as we have previously shown that following training hybrid fibres were associated with a greater number of SC as compared to either type I or II fibres (18). The increase in mitochondrial content and transition in myosin heavy chain protein expression often observed following non-hypertrophic aerobic training may require a contribution of the SC as is evidenced by an increase in active SC following 3 distinct types of aerobic training (Fig 4 B-C).

Skeletal muscle is a highly plastic tissue. Muscle fibres are able to change in size (atrophy/hypertrophy) and alter their metabolic characteristics when challenged with appropriate stimuli. Adaptation of skeletal muscle occurs through changes in the translational capacity of resident myonuclei. It is commonly believed that nuclei are re-programmed in order to modulate the transcriptional potential of muscle fibres. It is, however, also possible that nuclei are eliminated from the muscle fibre through enucleation, or a process similar to pyknosis and karyorrhexis and replaced with new nuclei to further support muscle remodeling – the theory of myonuclear turnover (39). Here, we report an increase in proliferating and differentiating SC (Fig 4 B-C) without an appreciable expansion of the SC pool (Fig 3 B-C). Additionally, an increase in the number of nuclei per fibre was not observed (Fig 2 C) suggesting, in combination with an increase in SC activity (Fig 4 B-C), that there may be an elimination of myonuclei

throughout the training process and an addition of new myonuclei. Studies in rodent models have described a reduction in the number of nuclei per fibre and an increase in apoptotic nuclei in various atrophic conditions (1). Although this notion has been challenged (14), this suggests the nuclear content in skeletal muscle may fluctuate in response to different stimuli.

It is generally believed that SC contribute to muscle hypertrophy induced through resistance exercise training via nuclear addition but here we demonstrate that when faced with a non-hypertrophic stimuli SC may still play a role, albeit likely different than that associated with resistance training as training did not lead to an increase in nuclear content. Additionally, our results highlight the importance of considering the activation status of the SC pool when describing their contribution to various stimuli in humans, as simply enumerating SC content using various markers of SC such as Pax7 or NCAM may not be sufficient in fully describing the response of the SC to a given stimulus.

Figure legends

Figure 1. Representative image of Pax7/Desmin/DAPI/Laminin stain (A). Co-staining of Desmin/Laminin (B), Pax7/ Laminin (C), Pax7/Desmin (D), and Pax7/DAPI (E). Representative images of MyoD/Desmin /DAPI/Laminin (F). Co-staining of Desmin/Laminin (B), MyoD/Laminin (H), MyoD/Desmin (I), and MyoD/DAPI. To assure accurate anatomical location of the Pax7+ and MyoD+ cells within the SC niche.

Figure 2. Training does not lead to skeletal muscle hypertrophy. Skeletal muscle fibre cross sectional area of type I (A) and type II (B) fibres were measured pre and post training for each training intervention. The number of nuclei per fiber was determined (C) and the myonuclear domain (D) was calculated as fibre area μm^2/nucleus.

Figure 3. The fibre type specific SC response to training. Representative image of a Pax7/laminin/ MHCI/MCHII/nuclei immunofluorescent stain on a muscle cross section (A). MHCII and Pax7 (a), Pax7, MHCI and laminin (b), Pax7 (c), and nuclei (DAPI; d).

SC response of type I (B) and type II (C) fiber-associated SCs pre- and post SIT-1, SIT-2 and MICT training, expressed per 100 fibers. Bars represent means ± SE.

Figure 4. MyoD response of SC to training. Representative image of a Pax7/MyoD1/laminin/ nuclei immunofluorescent stain on a muscle cross section (A). MyoD1 and laminin (a), Pax7 and laminin (b), MyoD1 and Pax7 (c), and nuclei (DAPI, d). Pax7/MyoD1 response to training, active (Pax7+/MyoD1+) (B) and differentiating (Pax7-/MyoD+) (C) pre- and post SIT-1, SIT-2 and MICT training, expressed per 100 fibres. Bars represent mean ± SE. * $p \leq .05$ main effect for time following a 2 way repeated measures ANOVA. # $p \leq .05$ different from pre following a paired t test.

Figure 5. Representative image of PCNA/Pax7/DAPI/Laminin (A). Co-staining of Pax7/Laminin (a), PCNA/Laminin (b), Pax7/DAPI (c), and PCNA/DAPI (d) of muscle cross sections to determine the proportion of activated SC (Pax7+/PCNA+). The proportion of Pax7+ co-expressing PCNA or MyoD was determined in subset of subjects from the SIT-1 (n=3) and the SIT-2 (n=3) groups.

Figure 6. Myogenin response to training. Representative image of a Myogenin/ Pax7 immunohistochemical stain (A). The black arrow denotes perinuclear staining, red arrows denote myogenin positive cells. Myogenin response to pre- and post- SIT-2 and MICT training (B). * $p \leq .05$ main effect for time following a 2 way repeated measures ANOVA.

Acknowledgments

The Pax7 hybridoma cells developed by Dr.A. Kawakami, the A4.951 developed by Dr. H. Blau and F5D developed by Dr. W.E. Wright were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Grants

This study was funded by a Natural Sciences and Engineering Research Council Discover Grant awarded to G.P.

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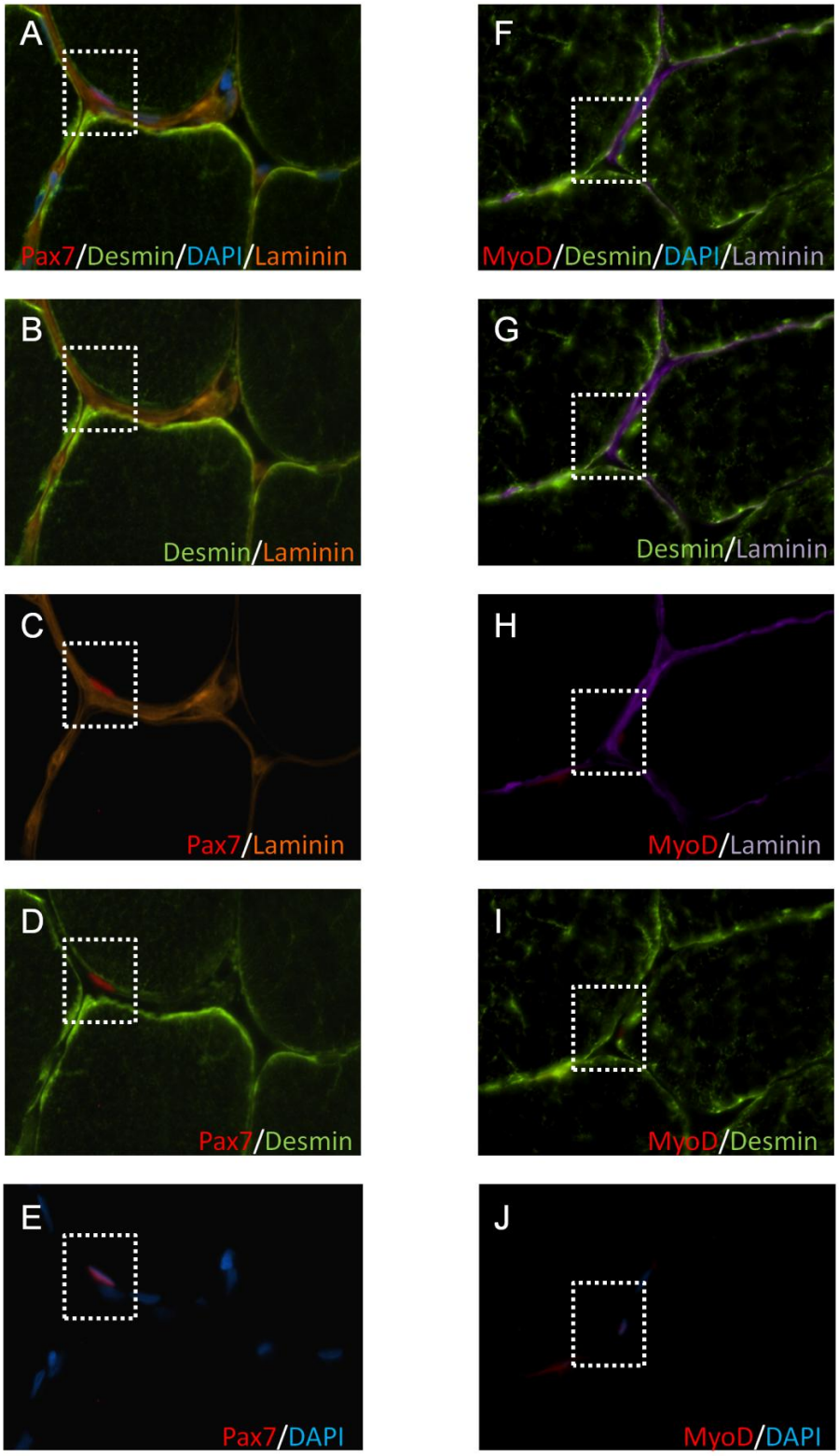
Figure 1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F
MyoD/Desmin/DAPI/Laminin
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Desmin/Laminin
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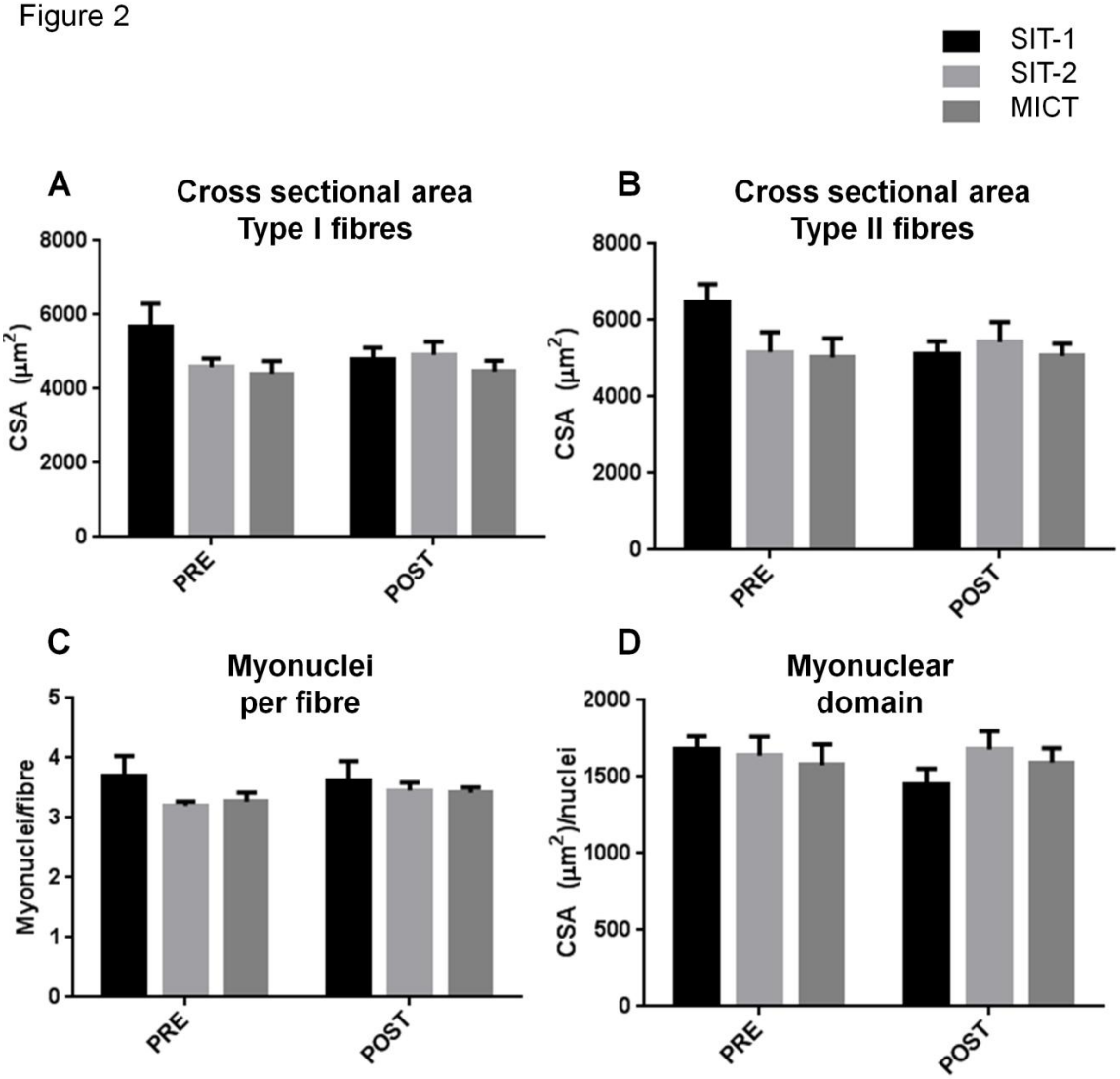
Figure 2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SIT-2
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Type I fibres
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Cross sectional area
Type II fibres
CSA (µm²)
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6000
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2000
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PRE
POST
C
Myonuclei
per fibre
Myonuclei/fibre
5
4
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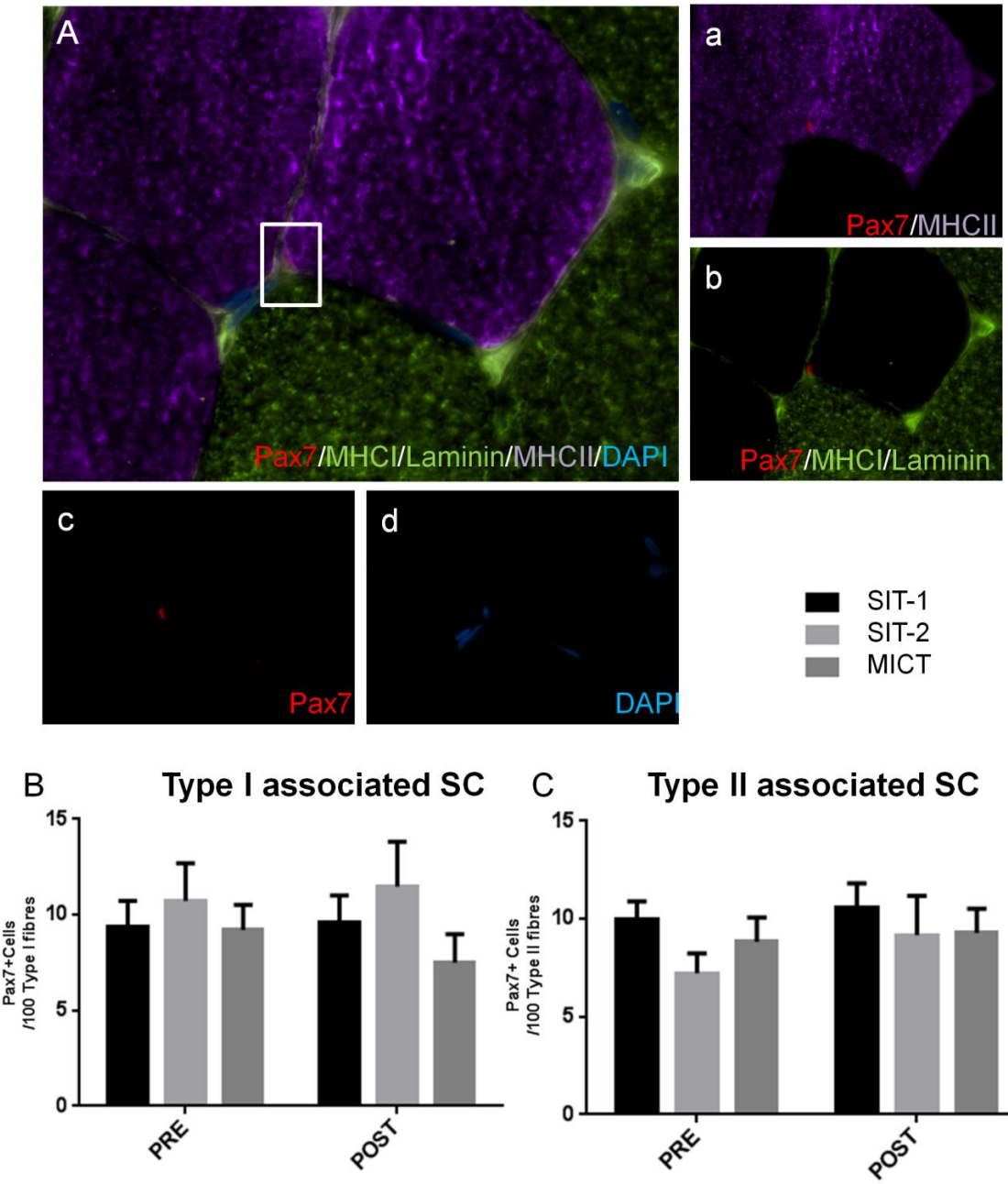
PRE
POST

Figure 3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Type I associated SC
Pax7+Cells /100 Type I fibres
15
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C
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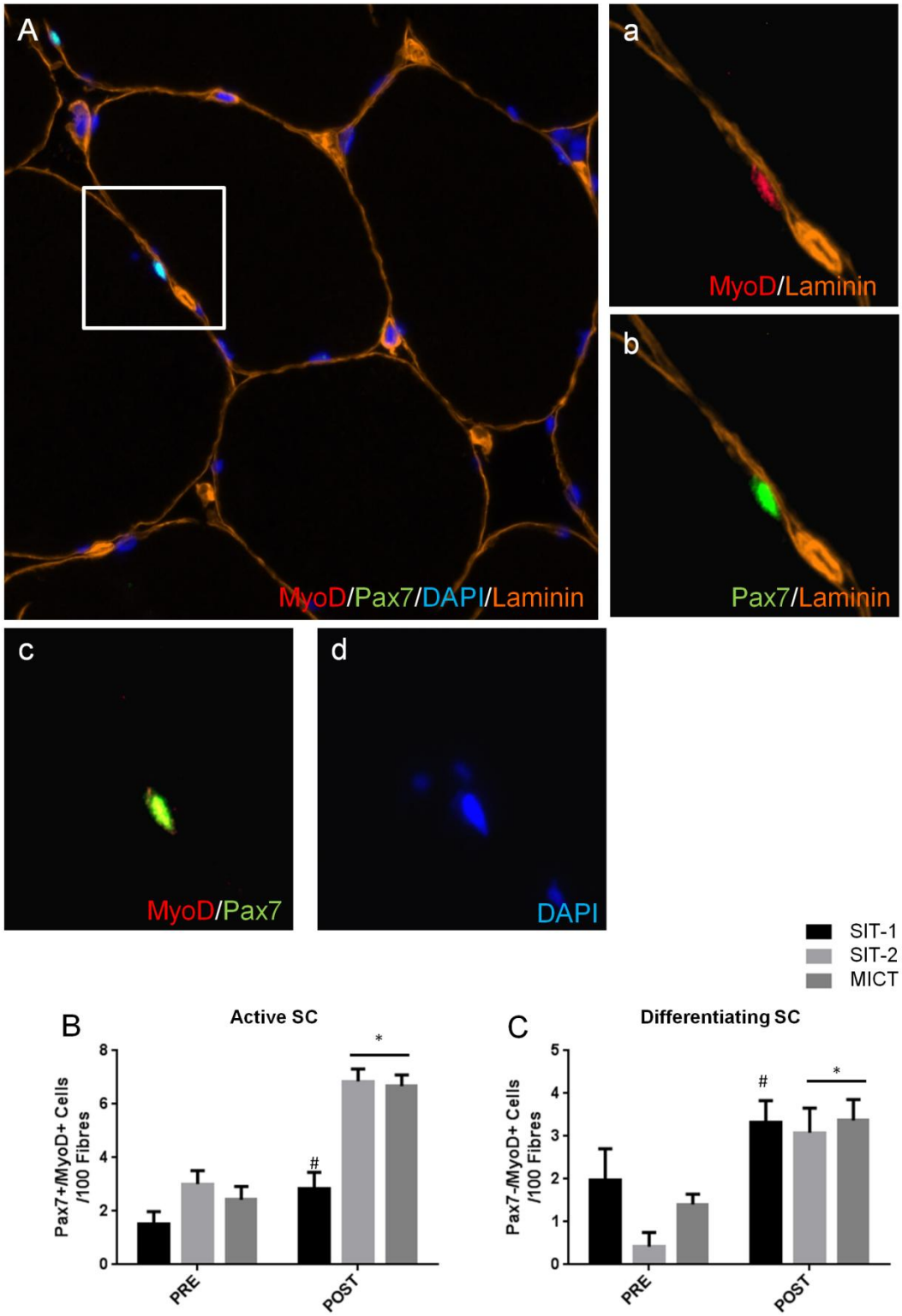
Figure 4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SIT-2
MICT
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Differentiating SC
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PRE
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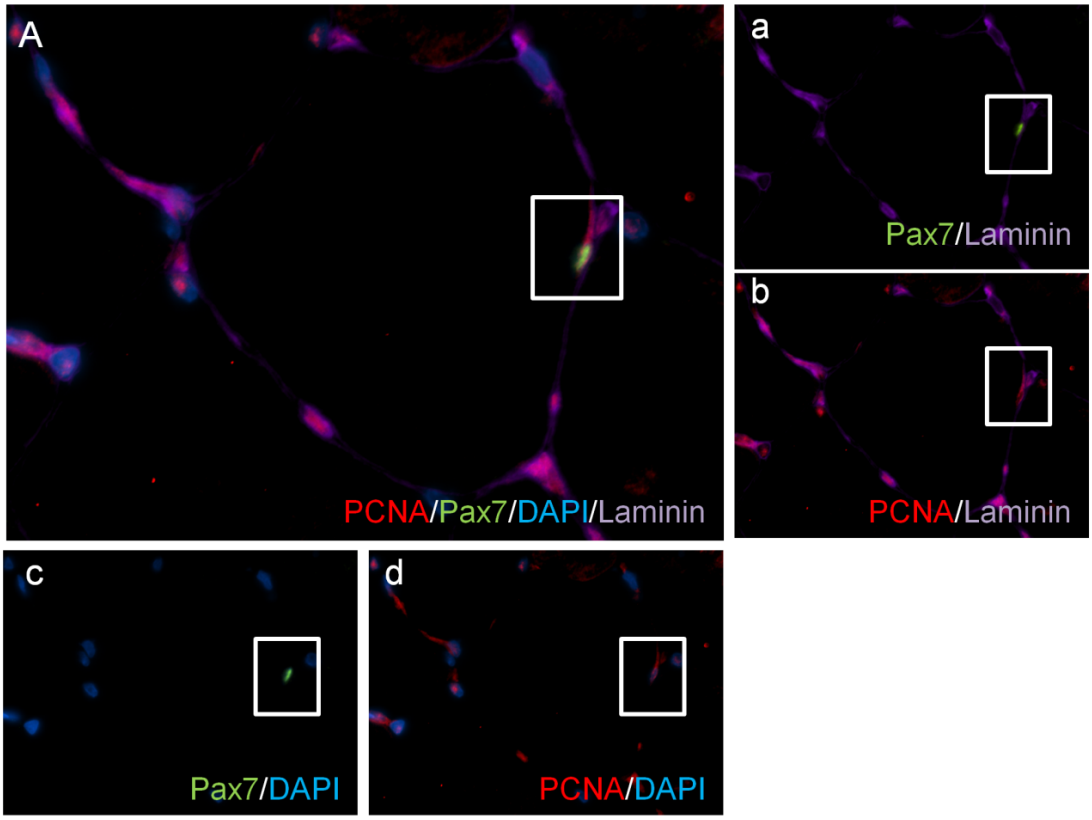
Figure 5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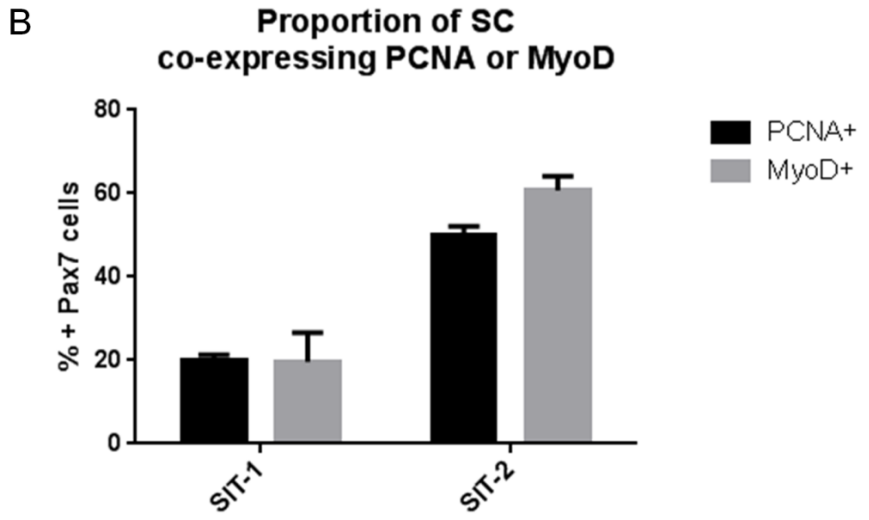
B
Proportion of SC
co-expressing PCNA or MyoD
% + Pax7 cells
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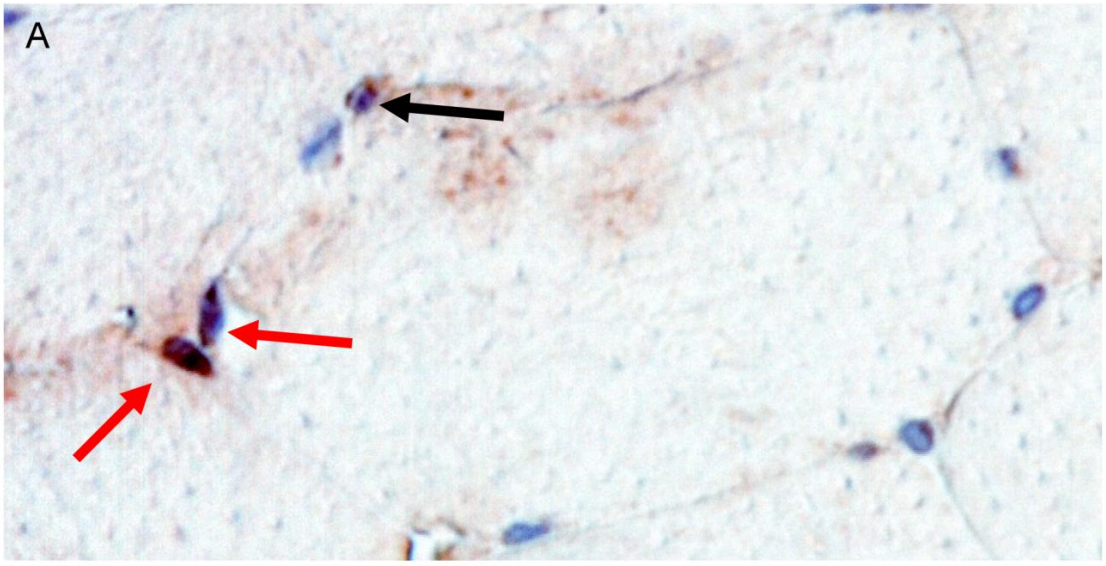
60
40
20
0
SIT-1
SIT-2
PCNA+
MyoD+

Figure 6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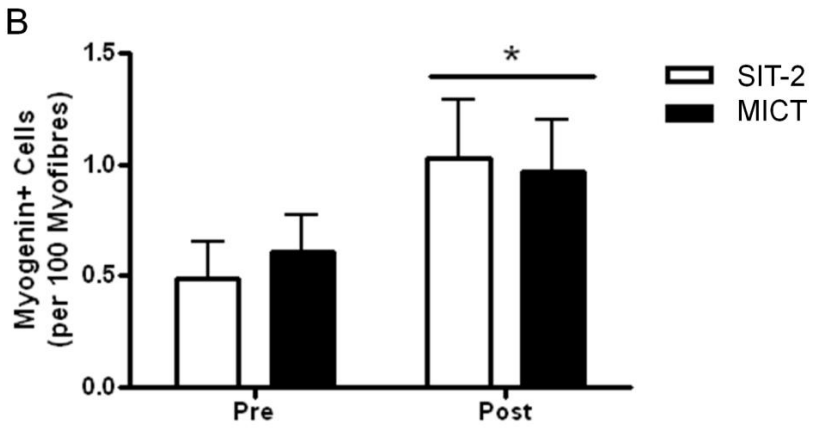
B
Myogenin+ Cells
(per 100 Myofibres)
1.5
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*
Pre
Post
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MICT

Chapter 4:

Exercise conditioning in old mice improves skeletal muscle regeneration

The following manuscript is in preparation for submission to the FASEB journal.

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Short title: Exercise improves regeneration

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List of non standard abbreviations

C/F*i*, capillary-to-fiber ratio on an individual fiber basis

CSA, cross sectional area

CTX, cardiotoxin

DSHB, Developmental Studies Hybridoma Bank

EDL, extensor digitorum longus

MRF, myogenic regulatory factors

OCT, optimum cutting temperature

PFA, paraformaldehyde

SC, muscle stem cell (satellite cell)

TA, tibialis anterior

Abstract

Skeletal muscle possesses the ability to regenerate following injury but is impaired or delayed with aging. Regardless of age, muscle retains the ability to positively respond to stimuli like exercise. We examined whether exercise is able to improve the regenerative response in skeletal muscle of aged mice. Twenty two month old male C57Bl/6J mice (n=20) underwent an 8wk progressive exercise training protocol (O-Ex) and were compared to old sedentary (O-Sed) and young sedentary (Y-Ctl) mice. Mice received injections of cardiotoxin into their tibialis anterior muscle. The TA were harvested prior to (O-Ex/O-Sed/Y CTL n=6), 10 days (O-Ex/O-Sed/Y D10 n=8) and 28 days (O-Ex/O-Sed/Y D28 n=6) post-injection. The average fibre cross sectional area (CSA) was reduced in all groups at D10 (CTL: O-Ex: 2499±140, O-Sed:2320±165, Y:2474±269; D10: O-Ex:1191±100, O-Sed:1125±99, Y:1481±167μm^2; all $p<0.05$) but was restored to control values in O-Ex and Y-Ctl groups at D28 (O-Ex:2257±181, Y:2398±171 μm^2, both $p>0.05$). Satellite cell content was greater at CTL in O-Ex (2.6 ± 0.4 SC/100 fibres) compared to O-Sed (1.0 ± 0.1 SC/100 fibres) ($p<0.05$). Exercise conditioning appears to improve the skeletal muscle's ability to regenerate following injury in aged mice.

Key words: skeletal muscle, regeneration, exercise, aging

Introduction

The loss of muscle mass and strength associated with advancing age (sarcopenia) can ultimately become debilitating (1). Developing interventions that target the mechanisms of age-related impairments in skeletal muscle growth/adaptation/repair is essential in improving the quality of life of the elderly. Satellite cells (SC) are resident skeletal muscle stem cells and play an important role in early postnatal muscle growth, formation of new fibers and maintenance of muscle mass (2). Historically, it was postulated that SC were necessary for skeletal muscle hypertrophy (3) however, more recent work highlights their redundancy in mediating this process in rodents (4). Although the role of SC in muscle remodelling remains debatable, they are essential in skeletal muscle regeneration (4,5). Depletion of SC in rodents leaves them able to hypertrophy to the same extent as wild type animals but their ability to regenerate skeletal muscle following injury is severely impaired (4). This points to a fundamental difference in SC with aging relevant to their roles in hypertrophy and regeneration following damage.

The regenerative process of skeletal muscle following injury is highlighted by several overlapping phases: the destructive/inflammatory phase, the repair phase and the remodeling phase (6). During the first phase of regeneration, the destructive phase, there is a degeneration of damaged fibres along with an influx of inflammatory cells (7). The influx of inflammatory cells not only leads to a clearance of muscle debris but also has a chemotactic role in SC migration to the site of injury (7). During the second or 'regenerative' phase, SC fuse to injured muscle fibres contributing to their repair, or fuse to each other to form new fibers (6). In the final phase, newly formed fibers or repaired fibers grow and ultimately re-establish contractility (6). In mice, regeneration of the tibialis anterior (TA) following injury, to control levels, has been consistently reported after ~21 days in young mice (8,9). However, existing evidence on whether old mice have the ability to fully regenerate their skeletal muscle following a similar injury is equivocal. Complete regeneration of the muscle following injury in old mice has been observed (8,10,11) while others report incomplete or delayed regeneration (12–14). More

specifically, some studies demonstrate that although early regeneration is impaired in old mice, regeneration occurs to the same extent in both young and old when examined at later time points (8,10,11). These studies underpin the notion that delays in the inflammatory response and angiogenesis account for the early impaired regenerative response observed in old mice (10,11).

Although the extent to which aged skeletal muscle is able to regenerate following injury is debatable although it is widely accepted that SC content and function decreases in aged rodents. The decrease in SC content is thought to, at least in part, play a role in skeletal muscle atrophy associated with aging (15) and the impaired/delayed regeneration in aged skeletal muscle (8,16–19). Age-related contraction of the SC pool likely contributes to impaired regeneration, but there is mounting evidence suggesting that the systemic environment of old mice also plays a role in the loss of SC function (13,14,20). Models of parabiosis have elegantly demonstrated that when old skeletal muscles are exposed to a young systemic circulation, regeneration and SC proliferation is restored (14,20) and the deposition of fibrotic tissue is reduced (14). These data highlight the importance of extrinsic cues from the systemic environment in mediating improvements in skeletal muscle regeneration.

Several studies have explored the adaptive potential of aged mice to exercise stimuli. Following endurance exercise training in old mice there are improvements in spontaneous locomotion (17) and an increased life-span (21). In addition to improvements in more functional outcomes, an increase in SC content has consistently been reported following endurance training in young (22,23) and old rodents (17,19). Moreover, resistance training impedes the progressive loss of muscle associated with aging in rats (24) and prevents the age associated shift in fibre type distribution (25).

As SC are essential in muscle regeneration and endurance training results in an increase in SC content it stands to reason that exercise may result in improved skeletal muscle regeneration in old mice. Additionally, as described above, exposing old mice to a young systemic environment resulted in improved muscle regeneration. In a human

model, exercise is also known to have pronounced systemic effects even in non-contracting muscles (26) and may promote a more youthful systemic environment contributing to improved muscle regeneration.

As described above, aged rodents maintain the ability to positively respond to exercise (17,19,21). Therefore the purpose of the current study was to determine the impact of endurance exercise training on skeletal muscle regeneration in old mice. The study design also enabled us to describe the effects of endurance exercise on the skeletal muscle of old mice and describe the effect of aging on skeletal muscle regeneration.

Materials and methods

Animals

Twenty-two month old adult male C57Bl/6J mice (n=10 Charles River, USA; n=10 Jackson Laboratories, Bar Harbor, ME, USA), were subjected to the 8 week exercise training protocol (O-Ex; n=20), mice were sacrificed at 24 months of age. Twenty-four month old adult male C57Bl/6J mice (Charles River, USA n=10; Jackson Laboratories, Bar Harbor, ME, USA n=10), were used as a sedentary group (O-Sed; n=20) and did not take part in exercise training. 8 wk old young adult male C57Bl/6J mice (Charles River, USA n=10; Jackson Laboratories, Bar Harbor, ME, USA n=10) were used as a young control group (Y-Ctl, n=20). All old mice were housed together in cages with no more than 4 animals; O-Sed and O-Ex animals were not housed separately. Old mice were housed in HEPA filtered clean cages. All young animals were housed together with no more than 5 animals per cage. Mice were provided with food and water *ad libitum*. Mice were kept on a 12-h light-dark cycle. Ethics approval was granted by the McMaster University Animal research Ethics Board and conformed to the standards established by the Canadian Council on Animal Care.

Exercise training protocol

Old mice were exercised trained (n=20) on an Exer 6M treadmill (Columbus Instruments, Columbus, OH, USA) 40 minutes/session, 3days/week for 8 weeks. Every exercise session was preceded by a 10 minute warm-up at 6 meters/minute followed by a 5 minute cool-down at 6 meters/minute. Exercise training was progressive and began at 8.5 meters/minute (week 1) and increased to 15 meters/minute (week 8). Mice were encouraged to run on the treadmill with light electric shock and hindlimb stimulation when they stopped running.

CTX injection

To determine the effect of aging and exercise on aged skeletal muscle, animals from each group Y-Ctl, O-Ex and O-Sed were subjected to bilateral injections of 50 µl (10µM) of cardiotoxin (CTX) (Latoxan, Valence, France) into their TA muscle.

Animal sacrifice

Mice were briefly anesthetized with isoflurane (Abraxis Bioscience, Summit, NJ, USA), then euthanized via cervical dislocation. Both TA muscles were removed, one was frozen immediately in liquid nitrogen for RNA analysis while the other was mounted in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) then frozen in liquid nitrogen pre-cooled isopentane for histology experiments. For baseline comparisons (CTL) mice not subjected to CTX injections were euthanized from each group, Y-Ctl, O-Ex and O-Sed (n=6, per group). Mice from the O-Ex group were euthanized 3 days following their last bout of exercise to minimize the possibility of observing acute exercise effects. To observe skeletal muscle regeneration, mice from each group were euthanized 10 (D10) (n=8, per group) and 28 days (D28) (n=6, per group) following CTX injection.

RNA isolation, reverse transcription and quantitative RT-PCR reaction

Total RNA was isolated from TA muscles using a combination of TRIzol (Invitrogen) and E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA).

Whole TA muscles were homogenized using 1 ml of TRIzol reagent in Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA),with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) twice for 40 s at 6 m/s. Samples were stored at -80°C until further processing. Samples were thawed and 200 µl of chloroform was added to each sample and mixed vigorously for 15 s, incubated for 5 min at room temperatures and then centrifuged at 12 000g for 10 min at 4°C. The upper aqueous phase was removed and RNA was isolated following the E.Z.N.A. Total RNA Kit 1 manufacturer's instructions. RNA was reverse transcribed using a commercially available kit (high-capacity cDNA reverse transcription kit; Applied Biosystems, Carlsbad, CA, USA) following manufacturer's instructions using an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Mississauga, ON, Canada). Quantitative RT-PCR reactions were prepared using the epMotion 5075 Eppendorf automated pipetting system (Eppendorf) and conducted in duplicates in an Eppendorf realplex^2 Master Cycler ep gradient S (Eppendorf). All samples were normalized to RPS11 fold changes in gene expression were calculated using the ΔΔCt method and expressed in relation to Y-Ctl, CTL values. The primer sequences are as follows RPS11: forward 5'-*CGTGACGAAGATGAAGATGC*-3', reverse 5'-*GCACATTGAATCGCACAGTC*-3'; VEGF: forward 5'-*TTACTGCTGTACCTCCACCA*-3', reverse 5'-*ACAGGACGGCTTGAAGATGTA*-3'.

Immunohistochemistry

7µm thick TA muscle cross sections were prepared from OCT embedded muscle. To determine SC content, fibre cross sectional area (CSA) and proportion of regenerating fibres identified as fibres with centrally located nuclei, TA muscle cross sections were stained using the Vector Laboratories (Burlington, ON, Canada) mouse-on-mouse immunodetection kit as per the manufacturer's instructions, with minor modifications (overnight blocking step). Slides were treated with primary antibodies for Pax7 [neat; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA] and laminin (1:1000; ab11575 Abcam). Pax7 was detected using streptavidin-594 fluorochrome,

1:500; Invitrogen, Molecular Probes and laminin was detected using Alexa Fluor 647goat anti rabbit (1:500). Nuclei were visualized with DAPI. Images were taken at 20x with a CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA). Images were analyzed using Nikon NIS elements AR software. CSA and the proportion of regenerating fibres were determined on an average of >200 fibres/animal, SC content was determined on an average of >700 fibres/animal.

To determine capillarization, TA muscle sections were stained with CD31 and laminin. Slides were treated with PFA for 10 min, washed in PBST then incubated in block (1% BSA and 10% goat serum) for 90 min. Slides were incubated in CD31 primary antibody (Abcam 28364) overnight at 4ºC. Slides were washed and CD31 was detected using Alexa Fluor 488 goat anti rabbit (1:500), slides were re-fixed in 4% PFA washed and incubated with laminin primary antibody (1:1000; ab11575 Abcam) for 2 h at room temperature. Nuclei were detected using DAPI. The capillary-to-fiber ratio on an individual fiber basis (C/F*i*) was determined as previously described (27). Images were taken at 20x with a CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY, USA). Images were analyzed using Nikon NIS elements AR software. C/Fi was determined on an average of 50 fibres/animal.

Masson's Trichrome stain was used to determine collagen content in TA muscle cross sections. Sections were fixed in 4% PFA for 1 h then incubated in Bouin's fixative (Sigma, Oakville, ON, Canada) overnight at room temperature. Slides were rinsed in water, then incubated in Weigert's Iron hematoxylin for 5 min, washed again and incubated in biebrich scarlet - acid fuchsin for 15 min. Slides were then rinsed in water, incubated in phosphomolybdic-phosphotungstic acid 3x3min, incubated in aniline blue, dipped in water and incubated in 1% glacial acetic acid for 2 min. Slides were treated with graded ethanol washes then cover slipped. The area occupied by collagen (stained blue) was determined and represented as a percentage of total area. Images were taken at 20x using the Nikon DS-Fi1. Images were analyzed using Nikon NIS elements AR software.

Oil-red-o stain was used to stain lipid in TA muscle cross sections. Slides were fixed for 1 h in 4% PFA, rinsed in water and incubated in oil red o working solution prepared with isopropanol for 30 min. Sections were rinsed in running water and cover slipped. Images were taken at 40x using the Nikon DS-Fi1. To quantify staining intensity was determined using using Nikon NIS elements AR software.

Statistical Analysis

To determine the impact of exercise training in old mice student's t-tests were run on all outcome measures between the O-Ex and the O-Sed groups at the CTL time point. To determine the impact of aging on muscle regeneration on all outcome measures a one way ANOVA was used to determine if there were differences between Y-Ctl and O-Sed at each time point (CTL, D10 and D28). To determine the impact of aging and exercise on all outcome measures a one way ANOVA was used to determine if there were differences between each group, Y-Ctl, O-Ex and O-Sed at each time point (CTL, D10, D28). Statistical significance was accepted at $P \leq 0.05$. Any significant main effects were analyzed using the Tukey's post-hoc test, alpha was adjusted based on the number of planned comparisons. All results were presented as means ± standard error of the mean (SEM).

Results.

1. Experimental Approach

The present study was designed to investigate whether exercise was able to improve muscle regeneration in old mice. Additionally, the experimental design allowed us to determine the impact of exercise on old mice and the impact of age on muscle regeneration. Mice were 22 months old at the beginning of training (O-Ex). Mice trained 3 days/week for 8 weeks; mice were 24 months old at the end of the training protocol. A subset of mice (n=6) were sacrificed following training and their tibialis anterior (TA) muscles were isolated and frozen appropriately for further experiments.

To determine the effect of exercise in old mice, TA muscles from O-Ex mice were compared to 24 month old sedentary mice that did not undergo any training (n=6). To determine the effect of aging on muscle regeneration, O-Sed animals were compared to 8 month old young mice (Y-Ctl). Mice were compared at baseline, prior to muscle injury (CTL), 10 d following injury (D10) and 28 d following injury (D28).

To determine the effect of exercise training on muscle regeneration TA muscles isolated from Y-Ctl, O-Ex and O-Sed were compared at baseline, 10 and 28 days following injury.

2. The effect of exercise in old mice.

Fibre CSA was determined using immunofluorescent staining for laminin (FIG 1 A-I). Eight weeks of endurance exercise training did not lead to a change in fibre CSA of old mice (CTL: OEx: 2486 ± 125 μm^2; O-Sed: 2275 ± 155 μm^2) ($p>.05$) (FIG 1J).

The extent of muscle regeneration was determined by the number of fibers containing centrally located nuclei (FIG 1 A-I), a hallmark of regenerating fibres (28). Old mice that underwent exercise training had a greater number of regenerating fibers (10 ± 2%) compared to old sedentary mice (4 ± 1%) ($p<.05$) (FIG 1 K). Although the exercise training protocol did not lead to an increase in CSA it did induce fibre regeneration.

SC content was determined with immunofluorescent staining of laminin, Pax7 and DAPI (FIG 1 A-I). Old mice that underwent exercise training had significantly more SC than old sedentary mice (CTL: O-Ex: 2.6 ± 0.4; O-Sed: 1.0 ± 0.1 SC/100 fibres) ($p<.05$) (FIG 1 L).

3. The effect of age on regeneration.

Regeneration is impaired in old mice

Fibre CSA was significantly reduced in both Y-Ctl and O-Sed mice 10 days following injury compared to CTL (CTL: Y-Ctl: 2475 ± 469, O-Sed: 2275 ± 155 μm^2; D10: Y-Ctl: 1482 ± 167, O-Sed: 1086 ± 79 μm^2) ($p<.05$). However, 28 days following injury CSA was re-established in Y-Ctl (D28: 2551 ± 226 μm^2), whereas it remained

reduced in O-Sed (1351 ± 121 μm^2) ($p<.05$) (FIG 1 M). Additionally, the percentage of regenerating fibres was significantly smaller in O-Sed (53 ± 6%) 28 days following injury compared to Y-Ctl (76 ± 5%) ($p=.05$) (FIG 1 N).

The SC response to regeneration is impaired in old mice

SC content was greater 10 days following injury compared to CTL in both Y-Ctl (CTL: 2.6 ± 0.4; D10: 9.1 ± 1.3 SC/100 fibres) and O-Sed (CTL: 1.0 ± 0.1; D10: 5.7 ± 0.8 SC/100 fibres) ($p<.05$). Twenty-eight days following injury SC content remained elevated in Y-Ctl compared to CTL (CTL: 2.6 ± 0.4; D28: 6.7 ± 1.1 SC/100 fibres) ($p<.05$). No difference in SC content was observed in O-Sed between CTL (1.0 ± 0.1 SC/100 fibres) and D28 (2.4 ± 0.5 SC/100 fibres) ($p>.05$) (FIG 1 O).

The revascularization process during regeneration is impaired in old mice

The number of capillaries per individual fibre (C/Fi) was determined via immunofluorescent staining of TA muscle cross section for laminin and CD31 (FIG 3 E-F). C/Fi was not different between Y-Ctl and O-Sed mice at CTL and 28 days following injury ($p>.05$) (FIG 3 I). VEGF mRNA expression was increased in both Y-Ctl and O-Sed groups 10 days following injury approximately 3- and 5-fold respectively group ($p<.05$) (FIG 3 J).

Fibrosis is increased in old mice during regeneration

In O-Sed the fibrotic index was greater 10 days (8.1 ± 0.9%) following injury compared to CTL (3.4 ± 1%) ($p<.05$). The fibrotic index was greater in the O-Sed group (8.1 ± 0.9%) compared to the Y-Ctl (4.6 ± 1%) group 10 days following injury ($p<.05$). No differences in fibrotic index were observed in the Y-Ctl group across any time point. Aging did not affect fibrosis as no differences existed at CTL between the Y-Ctl and O-Sed group at baseline ($p>.05$) (FIG 4K).

4. The effect of exercise on regeneration in old mice.

Exercise rescues impaired regeneration in old mice

CSA was reduced in all groups 10 days following injury compared to CTL ($p<.05$). CSA was re-established to CTL values 28 days following injury in Y-Ctl and O-Ex but not in O-Sed (CTL: Y-Ctl: 2475 ± 265 μm^2; OEx: 2486 ± 125 μm^2; O-Sed: 2275 ± 155 μm^2; D28: Y: 2551 ± 226 μm^2; OEx: 2208 ± 169 μm^2; O-Sed: 1351 ± 121 μm^2) (FIG 2 A). The percentage of regenerating fibers was greater in all groups 10 days following injury ($p<.05$). Additionally, the percentage of regenerating fibres was smaller at D28 in the O-Sed group (53 ± 6%) compared to the Y-Ctl group (76 ± 5%) ($p=.05$) (FIG 2 B).

The SC response during regeneration in old mice

SC content was greater 10 days following injury in all groups: Y-Ctl (CTL: 2.6 ± 0.4; D10: 9.1 ± 1.3 SC/100 fibres), O-Ex (CTL: 2.3 ± 0.3; D10: 7.9 ± 1.7 SC/100 fibres), and O-Sed (CTL: 1.0 ± 0.1; D10: 5.7 ± 0.5 SC/100 fibres) ($p<.05$) (FIG 2 C).

Exercise improves revascularization during regeneration in old mice

C/Fi was greater 28 days following injury in the O-Ex group only (CTL: 2.0 ± 0.1; D28: 2.6 ± 0.2 cap/individual fibre) ($p<.05$) (FIG 3 K). Additionally, C/Fi was greater in the O-Ex (2.6 ± 0.2 cap/individual fibre) 28 days following injury compared to both the O-Sed (1.8 ± 0.17 cap/individual fibre) and the Y-Ctl group 1.9 ± 0.1 cap/individual fibre) ($p<.05$) (FIG 3 K). VEGF mRNA expression was increased 10 days following injury in both the Y-Ctl and O-Ex group ($p<.05$) (FIG 3 L) and returned to CTL levels 28 days following injury.

Exercise does not impact lipid or collagen content during regeneration in old mice

Aging and/or exercise conditioning during regeneration did not significantly impact lipid content as assessed by oil red o staining in muscle cross section (FIG 5). Additionally aging and/or exercise during regeneration did not affect collagen expression as assessed via Mason's trichrome staining of muscle cross sections (FIG 4).

Discussion

For the first time, we demonstrate that exercise conditioning can rescue age-associated impaired muscle regeneration. Specifically, exercise-conditioned old mice re-established muscle fibre CSA 28 days following injury to the same extent as young animals. Consistent with previous reports, the exercise training protocol employed in this study resulted in an increase in SC content in old mice (FIG 1 L) without leading to an increase in muscle fibre CSA (FIG 1 J) (17,19). Although there is no clear consensus on the ability of skeletal muscle from old mice to regenerate following injury, we observe incomplete regeneration in O-Sed animals 28 days following injury (FIG 2 A).

Exercise training results in remodelling of skeletal muscle tissue. These adaptations may, in part, explain the improvements observed in muscle regeneration in the O-Ex group. Here, we demonstrate that exercise resulted in an increase in SC content in old animals (FIG 1 L). SC are indispensible for skeletal muscle regeneration (4,5) and the increase in SC content in the O-Ex group as compared to that of the O-Sed group likely contributed to the similar regenerative pattern observed in both the O-Ex and Y-Ctl groups. The increased SC response in the Y-Ctl group persisted throughout the regeneration timeline, whereas this was not observed in the O-Sed group (FIG 1 O). Upon activation, SC proliferate; some differentiate and donate their nuclei to repair damaged fibers or fuse to one another to establish new fibers, while others revert to quiescence in order to maintain the SC pool (6,28). The greater SC content of young animals 28 days following injury that was not observed in the old sedentary group further demonstrates that skeletal muscle from old animals does not respond to injury in the same manner as young animals. The difference in SC content 28 days following injury likely contributed to the inability to completely restore CSA in old sedentary animals.

A hallmark of regenerating muscle is the presence of centrally located nuclei (28). A greater proportion of regenerating fibers were observed at CTL in O-Ex compared to O-Sed animals (FIG 1 K) suggesting that the exercise protocol was demanding enough to require some degree of skeletal muscle adaptation/repair. Furthermore, we reported incomplete regeneration, as assessed by fibre CSA, in O-Sed animals. However the proportion of regenerating fibers 28 days following injury was smaller in the O-Sed group

compared to the Y-Ctl group (FIG 1 B) This difference was not observed between the O-Ex and the Y-Ctl group. Fewer regenerating fibres at D28 would suggest that the regenerative process was slowing in the O-Sed compared to the Y-Ctl group although fibre CSA was yet to be re-established to CTL group levels. Therefore, it is possible that the smaller fibre CSA observed in the O-Sed was not simply due to a delay in muscle regeneration but also that it may never be fully re-establish in light of the slowing indices of regeneration observed in this group. These findings are contradictory to previous work that reported a re-establishment of fibre CSA following injury in old mice (8). These authors induced injury via notexin, which causes degeneration via a similar mechanism as CTX (29). However, the use of different protocols may explain the differences observed between studies as, unlike the current study, injury was induced in the extensor digitorum longus (EDL) of female C57BL/6J mice.

Revascularization is an important process during muscle regeneration (30), however, reports evaluating capillarization in old rodents are conflicting. A reduction (31,32), no change (31) and even an increase (31,33) in capillarization during muscle fibre regeneration have all been reported. These differences may, in part, be attributed to the difference in rodent model used (mouse versus rat) and muscle examined. Exercise is able to increase capillary content, albeit to a lesser extent, in older animals compared to young (34). Here, we report no difference in C/Fi between each group at baseline (FIG 3 K). However, we report a greater C/Fi in O-Ex compared to both the O-Sed and the Y-Ctl group 28 days following injury (FIG 3 K). Enhanced vascularization 28 days following injury in the O-Ex group presumably provides improved support during myogenesis, which was impaired in the O-Sed group. Improved perfusion of the skeletal muscle may allow for enhanced exposure to the circulating systemic environment resulting in improved support of muscle regeneration in exercise-conditioned animals. In contrast, we do not observe an increase in C/Fi in the Y-Ctl group following injury. It is possible that the last time-point (28 days) of our timeline was too late to observe an increase in the Y-Ctl group. Additionally, it is possible that the increase in C/Fi of the O-Ex group may be a compensatory mechanism. Muscle from old animals may require an even greater

perfusion as compared to that of young animals to fully regenerate and we demonstrate in the current study that up-regulation of the angiogenic factor VEGF following injury is impaired in old sedentary animals (FIG 3 L). Our findings are supported by data that demonstrated impaired angiogenesis associated with reduced expression of VEGF following ischemic injury in old compared to young mice (32).

When animals are treated with a VEGF neutralizing antibody the associated increase in vascularisation following exercise is not observed (35), suggesting a role of VEGF in neovascularization. An increase in VEGF mRNA expression was observed 10 days following injury in the O-Ex and the Y-Ctl group, suggesting that muscle from these animals was expressing the appropriate cues to re-establish vascularisation following injury. Due to the profound injury and incomplete muscle fiber formation, C/Fi was not analyzed 10 days following injury.

In addition to muscle fibre CSA we also aimed to determine the composition of skeletal muscle. Previous work has reported an increase in the fibrotic index in the early days following muscle injury in old mice (14). However, when regeneration is followed to later time points (i.e., 30 days following notexin injury) there were no observed differences in fibrotic index between young and old animals (8). Our results are in accordance with both of these studies. When determining the effect of aging on fibrosis during regeneration a greater fibrotic area was observed 10 days following injury in the O-Sed group compared to baseline (CTL) whereas the difference was no longer apparent 28 days following injury (FIG 4 K). However, when analyzing whether aging and exercise had an effect on fibrosis during regeneration, post-hoc tests revealed no differences between groups or time points. This may be due to lack of statistical power. We also determined lipid content as lipid infiltration of skeletal muscle increases with age in humans (36). Lee et al 2013 reported an increase in lipid deposits in old and senescent mice 30 and 21 days respectively following injury (8). Although not significant, lipid staining intensity was 80% greater in the O-Sed group 28 days following injury compared to baseline (CTL) whereas it was 14 and 37% greater in the Y-Ctl and O-Ex group's respectively (FIG 5 L). The content of non-muscle tissue (i.e. lipid and collagen) in

skeletal muscle may impact regeneration and ultimately impair the re-establishment of fibre CSA and this seems to be especially true in O-Sed animals. Determining if the functional capacity of O-Ex mice was improved as compared to O-Sed mice would have been beneficial in determining if the re-establishment of fibre CSA translated to functional measures like strength and fatigability. In addition, an excess of non-contractile tissue such as adipose and connective tissue in skeletal muscle could also impact tissue contractility and result in reduced functional capacity.

Skeletal muscle has an outstanding ability to fully regenerate following traumatic injury. However, the ability of skeletal muscle to completely regenerate following injury has, on occasion, reported to be delayed or impaired in old animals (12–14). These findings are confirmed in the present study with O-Sed animals displaying impaired muscle fibre regeneration 28 days following injury. Here, however, we demonstrate that 8 weeks of progressive endurance training is able to rescue impaired muscle regeneration of old mice following CTX injury. Rescued regeneration was likely a result of increased SC content following exercise. In addition, exercised animals appeared to have an improved re-establishment of the vascular network in response to exercise, which may have also contributed to complete skeletal muscle regeneration observed in this group. In conclusion, exercise-conditioning rescues delayed skeletal muscle regeneration observed in advanced age.

Acknowledgments

The Pax7 hybridoma cells developed by Dr. A. Kawakami were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA52242 USA.

Figure legend

Figure 1. Representative images of TA muscle cross sections from O-Ex (A-C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) stained for laminin (green), Pax7 (red) and nuclei (DAPI-blue). White circles indicate SC (Pax7 positive nuclei). The effect of exercise on CSA (J), the percentage of regenerating fibres (K) and on SC content (L) are presented at CTL in the O-Ex and O-Sed group. To determine the effect of age on regeneration CSA (M), the percentage of regenerating fibres (N) and SC content (O) are presented at CTL, D10 and D28 in the Y-Ctl and O-Sed groups. * significantly different from O-Ex, ** significantly different from Y-Ctl, *** significantly different that CTL within the same group.

Figure 2. The effect of exercise and aging on CSA, the dotted line represents average CSA at CTL of Y-Ctl, O-Ex and O-Sed, (A), the percentage of regenerating fibres (B) and on SC content (C) following regeneration are presented in Y-Ctl, O-Ex and O-Sed. Graphs of fibre CSA distribution at CTL (D), D10 (E) and D28 (F). Distribution shows a leftward shift of the curve in all groups at D10 indicating a greter number of smaller fibers, at D28 there is a rightward shift of the curve in the Y-Ctl and O-Ex group indicating re-establishment of CSA to CTL values. *** significantly different that CTL within the same group, b significantly different that O-Sed and D10 within the same group, ** significantly different from Y-Ctl.

Figure 3. Representative images of TA muscle cross sections from O-Ex (A,B), O-Sed (C,D) and Y-Ctl (E,F) stained for laminin (purple) and CD31 (green). The effect of exercise on C/Fi (G) and VEGF mRNA expression (H) are presented at CTL. The effect of aging and regeneration on C/Fi (I) and VEGF mRNA expression (J) are presented at CTL and D28 and CTL, D10 and D28 respectively. The effect of exercise and aging on C/Fi (K) and VEGF mRNA expression (L) during regeneration are presented in Y-Ctl, O-Ex and O-Sed. *** significantly different that CTL within the same group, c significantly different than D10.

Figure 4. Representative Masson trichrome images of TA muscle cross sections, from O-Ex (A-C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) from collagen is stained blue. The effect of exercise (J), aging and regeneration (K) and exercise and aging during regeneration (L) on collagen content are represented. *** significantly different that CTL within the same group, ** different than Y-Ctl.

Figure 5. Representative Oil-red-o images of TA muscle cross sections, from O-Ex (A-

C), O-Sed (D-F) and Y-Ctl (G-I) at CTL (A,D,G), D10 (B,E,H) and D28 (C,F,I) lipid is stained red. The effect of exercise (J), aging and regeneration (K) and exercise and aging during regeneration (L) on collagen content are represented.

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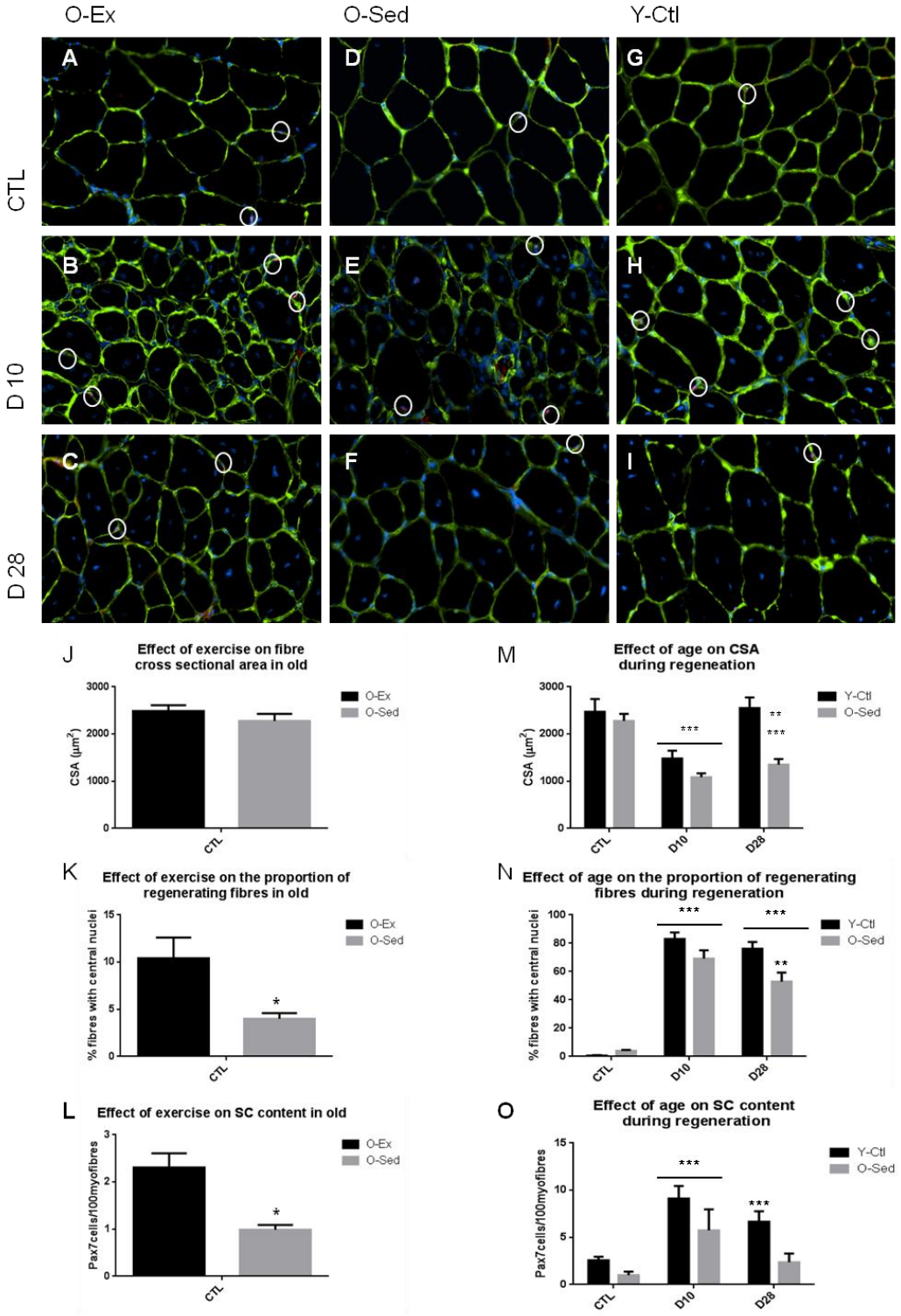
Figure 1


O-Ex
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Effect of exercise on fibre cross sectional area in old
CSA (µm²)
O-Ex
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Effect of age on CSA during regeneation
Y-Ctl
O-Sed
CTL
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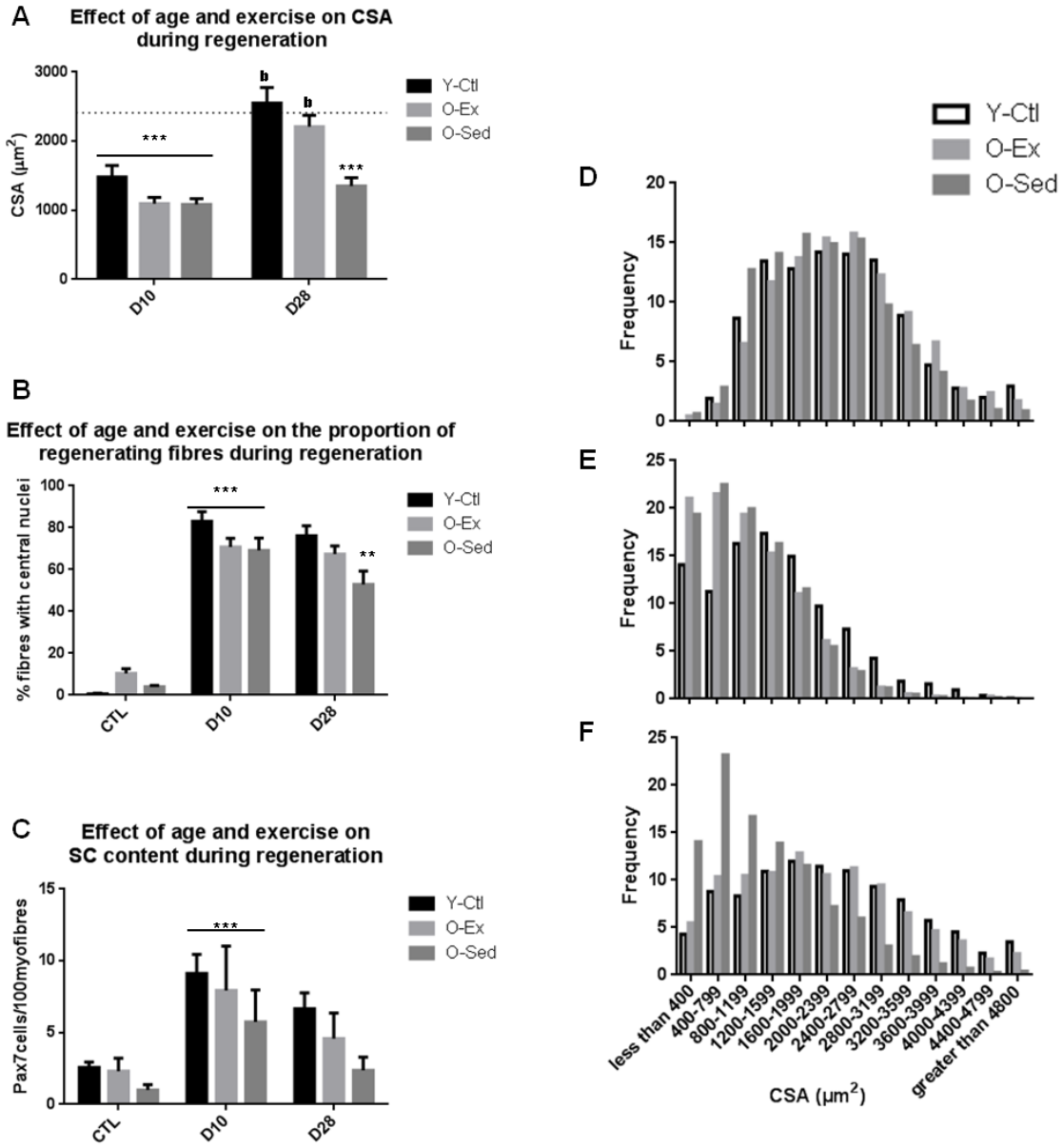
K
Effect of exercise on the proportion of regenerating fibres in old
% fibres with central nuclei
N
Effect of age on the proportion of regenerating fibres during regeneration
L
Effect of exercise on SC content in old
Pax7cells/100myofibres
O
Effect of age on SC content during regeneration

Figure 2


A
Effect of age and exercise on CSA during regeneration
CSA (μm²)
3000
2000
1000
0
***
b
b
***
D10
D28
Y-Ctl
O-Ex
O-Sed
B
Effect of age and exercise on the proportion of regenerating fibres during regeneration
% fibres with central nuclei
100
80
60
40
20
0
***
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CTL
D10
D28
Y-Ctl
O-Ex
O-Sed
C
Effect of age and exercise on SC content during regeneration
Pax7cells/100myofibres
15
10
5
0
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CTL
D10
D28
Y-Ctl
O-Ex
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D
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F
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Frequency
20
15
10
5
0
25
less than 400
400-799
800-1199
1200-1599
1600-1999
2000-2399
2400-2799
2800-3199
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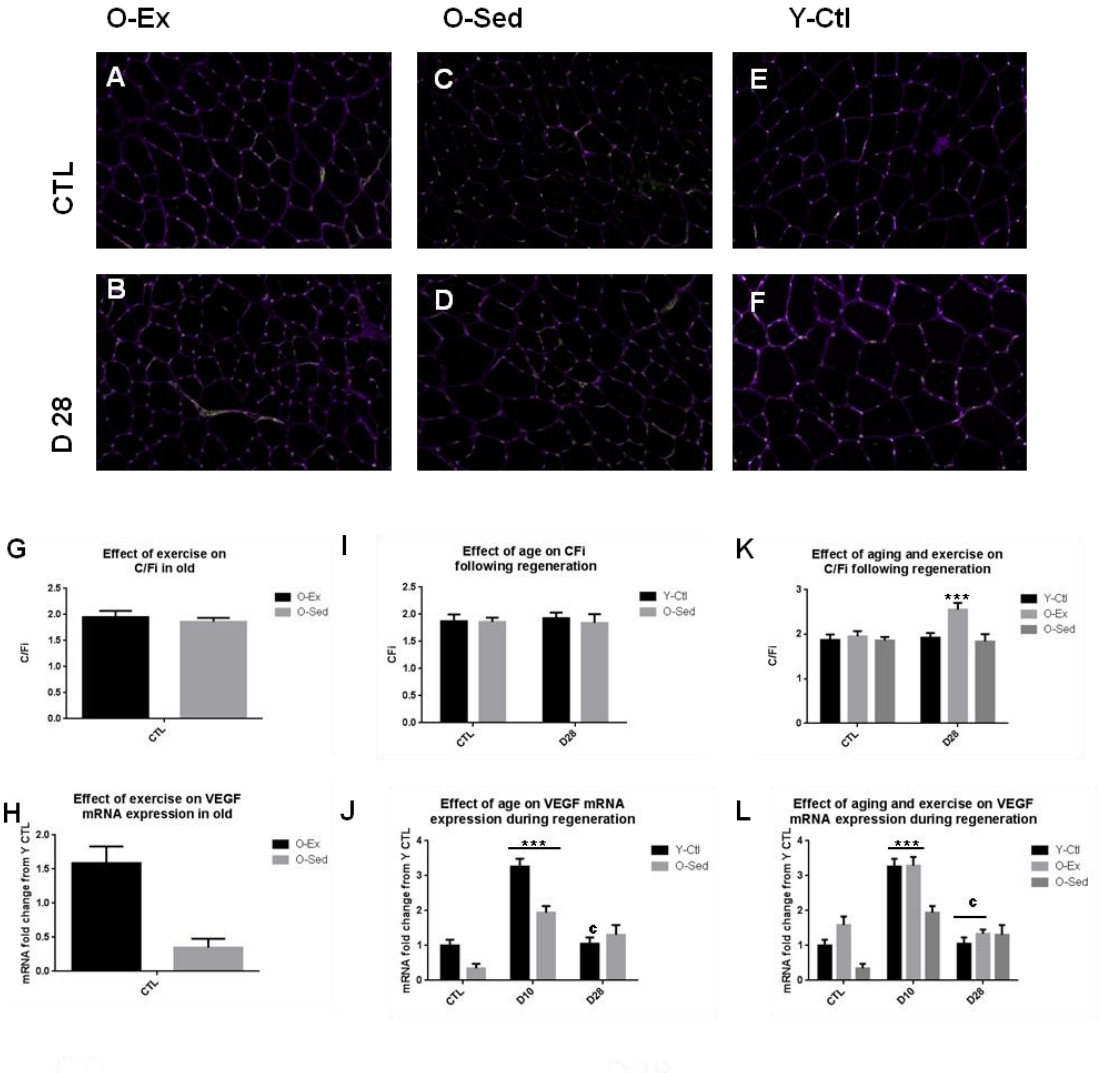
3600-3999
4000-4399
4400-4799
greater than 4800
CSA (μm²)

Figure 3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Effect of exercise on C/Fi in old
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Effect of age on CFi following regeneration
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Effect of aging and exercise on C/Fi following regeneration
C/Fi
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Y-Ctl
O-Ex
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CTL
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H
Effect of exercise on VEGF mRNA expression in old
mRNA fold change from Y CTL
O-Ex
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J
Effect of age on VEGF mRNA expression during regeneration
mRNA fold change from Y CTL
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c
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D28
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Effect of aging and exercise on VEGF mRNA expression during regeneration
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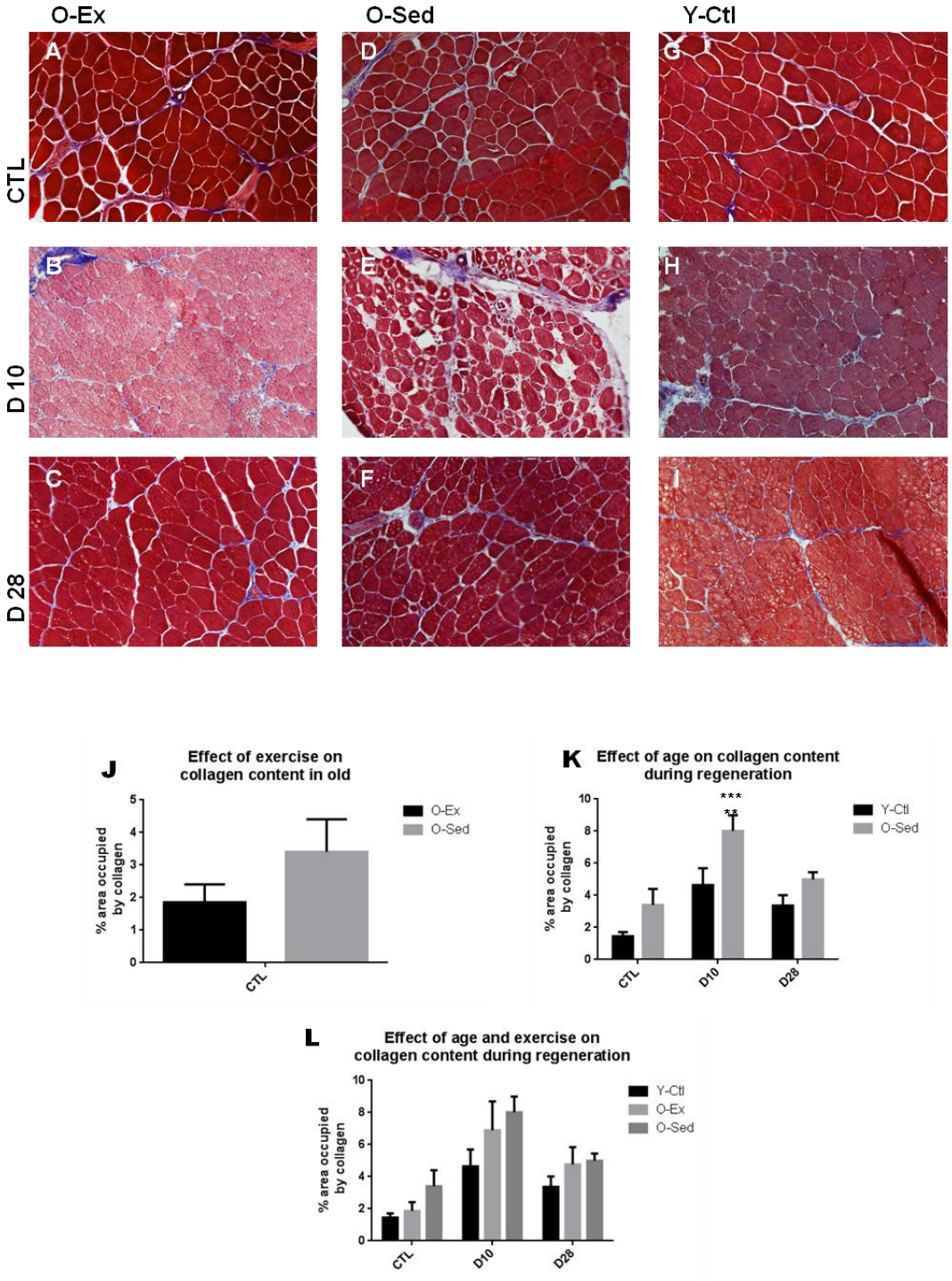
Figure 4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Effect of exercise on collagen content in old
% area occupied by collagen
O-Ex
O-Sed
CTL
K
Effect of age on collagen content during regeneration
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Y-Ctl
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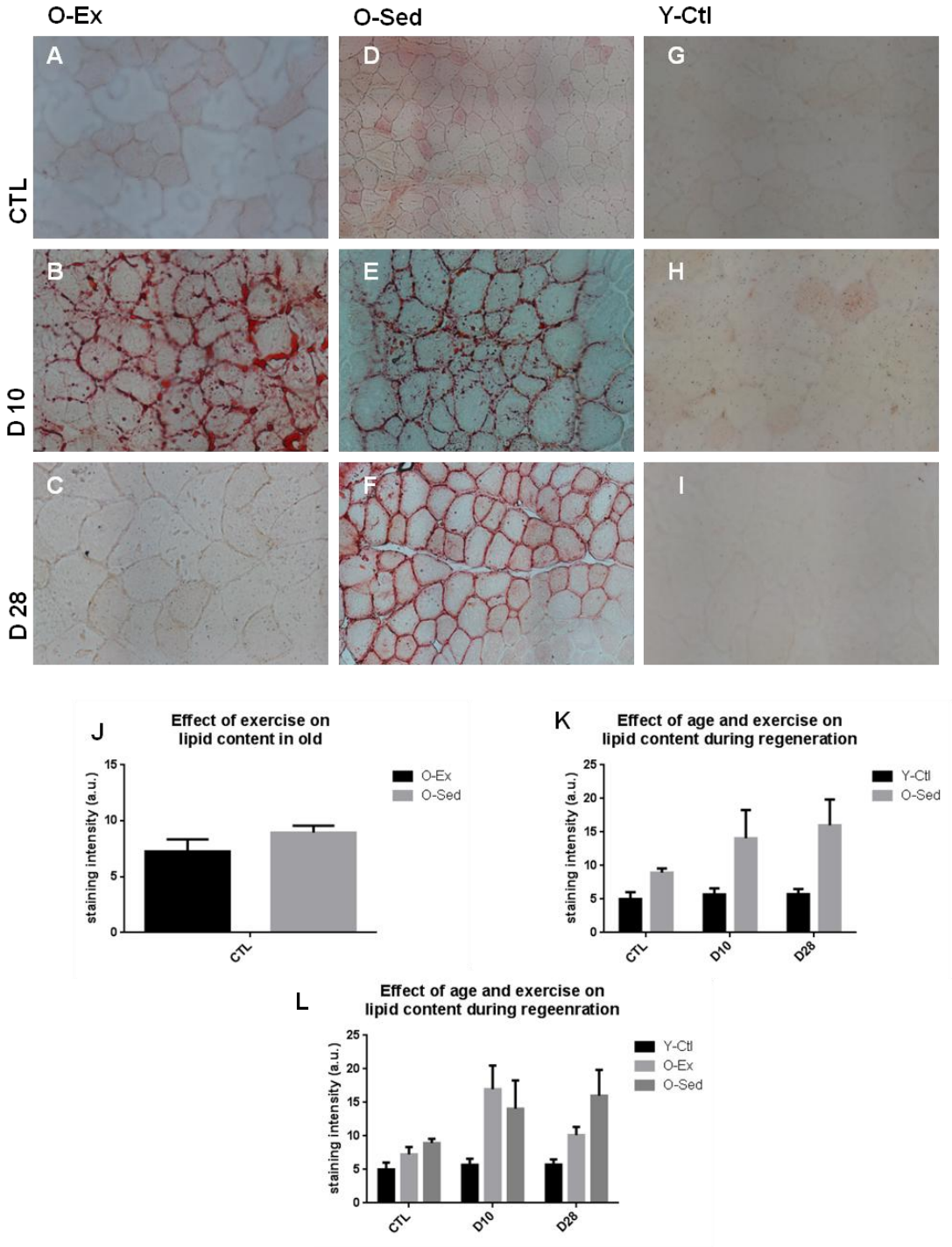
Effect of age and exercise on collagen content during regeneration
Y-Ctl
O-Ex
O-Sed

Figure 5


O-Ex
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D 10
D 28
J
Effect of exercise on lipid content in old
staining intensity (a.u.)
O-Ex
O-Sed
CTL
K
Effect of age and exercise on lipid content during regeneration
Y-Ctl
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Effect of age and exercise on lipid content during regeenration
Y-Ctl
O-Ex
O-Sed

Chapter 5: General Discussion

5.1 Introduction

The data presented in this thesis provides support for the role of SC in mediating adaptations induced via nonhypertrophic exercise training. We first sought to determine the effects of aerobic training on SC content. We demonstrated that interval-type aerobic training does not increase SC content but rather results in a greater number of active SC associated with remodelling fibres (Chapter 2) and that both traditional aerobic exercise training and various interval-type aerobic training models result in an increased SC pool activity without an increase in total SC content (Chapter 3). We then demonstrated that aerobic exercise conditioning in old mice improved muscle regeneration. This may be mediated by an increase in SC content due to training (Chapter 4). This thesis demonstrates for the first time that various modes of aerobic exercise training, not resulting in skeletal muscle fibre hypertrophy, do not result in increases in total SC content in young adults, these findings are presented in Figure 1. Rather, a specific increase in SC associated with hybrid fibres was observed.

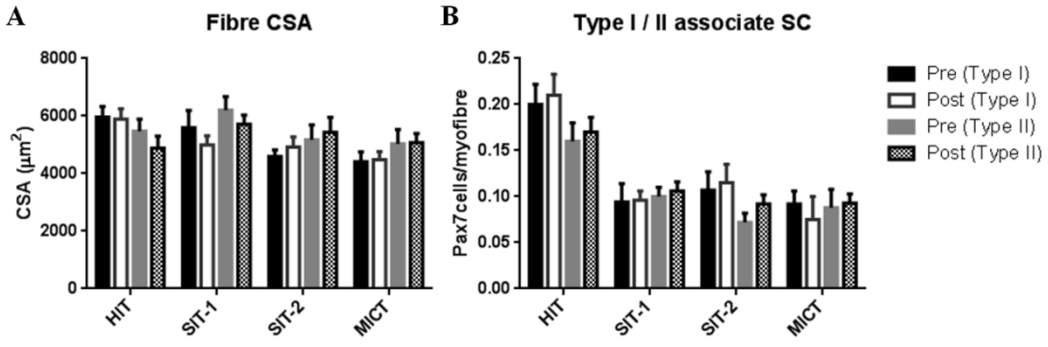

Figure 1. The effect of aerobic exercise training on muscle fibre CSA and fibre type specific SC content. Data from Chapter 2 and 3 are presented graphically. Data from panel (A) demonstrates that four different training paradigms did not result in an increase

in either type I or II fibre CSA. Panel (B) demonstrates that type I and type II associated SC content is also not affected by four different training paradigms.

Additionally, we report that aerobic exercise conditioning prior to inducing skeletal muscle injury in aged mice improves skeletal muscle regeneration. The following sections will collectively discuss the significance of the findings in this thesis.

5.2 The 'non-traditional' role of SC in aerobic exercise

5.2.1 Significance of the studies

Common dogma has long associated SC with adaptations observed following periods of resistance training resulting in an increased muscle fibre CSA (63). Several studies involving humans have reported associations with increases in SC content concomitantly with muscle hypertrophy, regardless of participant age (2, 34, 47, 61, 70). Accordingly, an increase in fibre size is often associated with an increase in myonuclear content (2, 34, 47, 61). The myonuclear domain theory suggest that each myonucleus is responsible for a set volume of cytoplasm and once this volume is exceeded additional nuclei need to be added to the muscle fibre (1). The addition of new nuclei would therefore maintain a relatively constant myonuclear domain even during periods of hypertrophy. As skeletal muscle is a post mitotic tissue it is believed that the incorporation of nuclei occurs via fusion of SC (Figure 3).

Although no causal link can be established with the above described data, it does suggest that SC play a role in skeletal muscle hypertrophy by 'donating' their nuclei to maintain the myonuclear domain. However, only one study in humans has reported an increase in SC content following training without an increase in fibre size (37). More

recent work has described an increase in fibre CSA without an observable increase in SC content (17). The necessity of SC for the induction of hypertrophy has been further challenged in rodent models. Indeed, mice that are SC-depleted maintain the ability to respond to hypertrophic stimuli and increase their muscle fibre CSA (29, 39). Therefore, it stands to reason that the role of SC in exercise-induced skeletal muscle adaptation is not restricted to exercise that elicits a hypertrophic response.

Previous work has described an increase in SC content following aerobic exercise training however, this training was accompanied by increases in muscle fibre CSA (8, 71). It is difficult to ascertain, whether the expansion of the SC pool occurred to support the increased fibre CSA or whether it is due to other adaptations occurring as a result of aerobic training, for example a shift towards a more oxidative phenotype, or perhaps both. In Chapters 2 and 3 of the current thesis, we suggest a role for SC following aerobic exercise training without an expansion of the SC pool (Figure 1). Specifically in Chapter 2, we report no increase in the overall SC pool following interval-type aerobic training (Figure 1). However, the number of SC associated with hybrid fibres increased 13-fold following training (Chapter 2, Figure 2). Further analysis revealed that there are a greater number of differentiating SC associated with these fibres (Chapter 2, Figure 5). We further report a potential role for SC in muscle remodelling with a high number of SC associated with fibres expressing neonatal myosin heavy chain (nMHC) (Chapter 2, Table 2). nMHC is a MHC isoform expressed by newly formed or regenerating fibres (72). In Chapter 3, the findings of Chapter 2 were supported by demonstrating that various nonhypertrophic training protocols do not lead to an overall expansion of the SC pool

(Figure 1). However, we consistently observed an increase in active SC following training (Chapter 3, Figure 4). The effects of resistance and aerobic training on the SC pool are summarized in Figure 2. Furthermore, an expansion of the SC pool is observed in old mice following aerobic exercise conditioning without an increase in CSA (Chapter 4, Figure 1).

The primary and novel finding of the thesis is that SC likely play a role in mediating adaptations observed following nonhypertrophic exercise training. This finding furthers our knowledge of SC biology and challenges traditional thought that SC simply mediate adaptations associated with increased muscle fibre size. Not only is a novel role for SC described, the results presented also suggest that simply enumerating the number of Pax7+ cells may not be sufficient in describing the response of SC to a specific stimulus. We suggest that it may be necessary to describe the activity of the SC pool by determining the proportion of SC that are quiescent, proliferating and differentiating in order to better understand the contribution of SC to muscle adaptation. Finally, the results from Chapter 4 demonstrate that nonhypertrophic exercise is capable of rescuing the impairment of muscle regeneration in aged mice (Chapter 4, Figure 2).

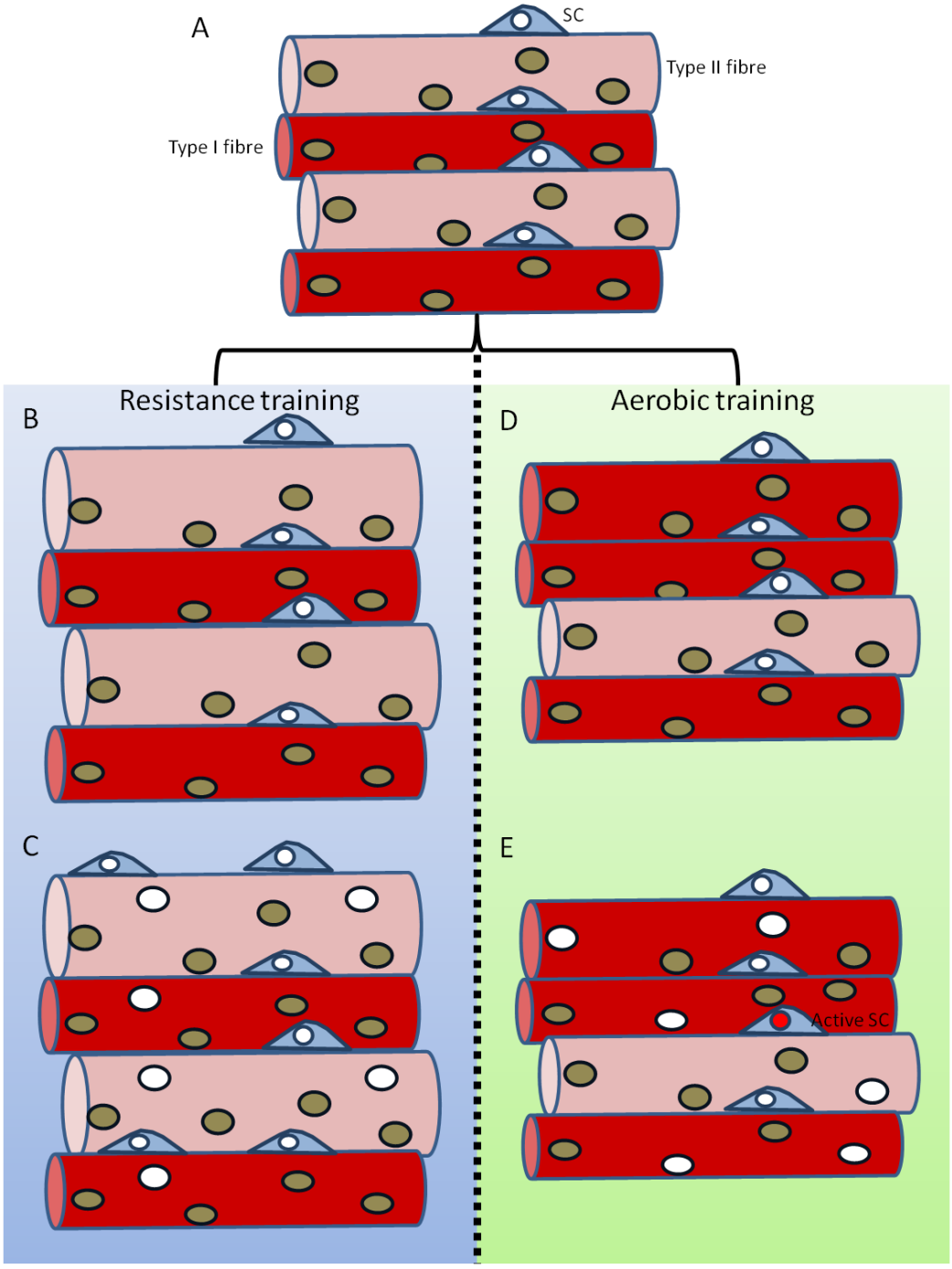

Figure 2. The proposed role of SC in resistance and aerobic exercise. In the basal state (A) each myonucleus is responsible for a set volume of cytoplasm, termed the myonuclear domain. Following resistance training an increase in muscle fibre CSA is observed (B). To maintain the myonuclear domain SC fuse with muscle fibres and

'donate' their nuclei, significant fibre hypertrophy results in an increase in SC content (C). Aerobic training does not result in an increase in fibre CSA rather a more oxidative phenotype, demonstrated by a greater number of type I fibres (represented as red fibres) (D). An increase in active SC is observed, while no increase in SC content or myonuclear domain is observed (E). We propose that SC are incorporated and contribute their nuclei to muscle fibres while other myonuclei are eliminated to maintain myonuclear content (E).

5.2.2 Potential role of SC in mediating exercise induced adaptations

There is considerable evidence that during resistance training SC contribute their nuclei to muscle fibres in order to support hypertrophy. However, this thesis demonstrates that SC are also likely contributing factors to nonhypertrophic muscle remodelling following aerobic-type training. Aerobic exercise training leads to many skeletal muscle adaptations. In addition, several studies have demonstrated similar adaptations following traditional endurance exercise and interval-type aerobic training (19, 21). Previous work in rats demonstrated that intensity rather than duration of exercise dictated the expansion of the SC pool following training (32). In Chapter 2, we hypothesized that intensity of exercise may be important in eliciting a SC response, similar to that observed in rats (32). Indeed, HIT resulted in an increased number of SC associated with hybrid fibres (Chapter 2 Figure 2). We directly addressed whether intensity mediated the SC response following aerobic training in Chapter 3. Training utilizing moderate intensity continuous exercise resulted in an increase in SC pool activation as did two other interval-type aerobic training programs (Chapter 3, Figure 4). However, these studies fail to determine whether intensity of exercise affects the acute response of SC.

Rodent studies have explored whether SC are necessary for a shift in fibre type to occur. When SC were ablated via gamma irradiation in rats and muscle was exposed to

overload-induced hypertrophy, a change in fibre-type distribution was observed similar to control animals (51, 52). Recent work in SC-deficient mice demonstrated that voluntary wheel running increased the proportion of oxidative fibres and increased mitochondrial function in the plantaris muscle to the same extent as that observed in control animals (28). These results would suggest that SC are not necessary in mediating changes in fibre type transitions. It is important to note that transgenic animals may respond to stimuli in a similar manner to that of wild type animals although, this response may be achieved by different compensatory mechanisms. However, in Chapter 2 we demonstrated that interval-type aerobic training resulted in a decreased proportion of type II fibres and a trend for an increase in the proportion of hybrid fibres (Chapter 2, Table 1). Additionally, an increase in the number of SC associated with hybrid fibres was observed following training (Chapter 2, Figure 2). Contrary to the reports in rodents, SC do seem to be involved in the transition of fibre types in humans evidenced by the findings presented in Chapter 2 and 3. Traditional endurance training and one of the interval-type aerobic training protocols used in this study resulted in an increase in the proportion of type I fibres (57). Although no fibre-type specific changes in SC were observed in Chapter 3, an approximate 2-fold increase in the number of active SC was observed and may have been involved in mediating the transition to an increased proportion of type I fibres following training (Chapter 3, Figure 4). In support of our results, chronic low frequency stimulation in rat EDL muscle resulted in an increase in the proportion of MHC I, IIa and developmental MHC with a concomitant increase in SC content (50). These data may suggest a potential role for SC in mediating fibre type transition. To further support the

role of SC in muscle remodelling there were more regenerating hybrid fibres compared to type I and II fibres (Chapter 2, Figure 3), determined by the proportion of fibres which had centrally located nuclei, following training. This was also associated with a fibre type specific SC increase (Chapter 2, Figure 2). Additionally, in Chapter 4 we described a 2.5-fold greater number of regenerating fibres following eight weeks of training in old mice, which was also associated with an increase in SC content (Chapter 4, Figure 1).

Aerobic exercise training not only results in structural remodelling of muscle fibres but also induces metabolic adaptations, such as mitochondrial biogenesis (20). Early work, *in vitro,* suggests that myotube formation is impaired when mitochondrial synthesis is inhibited, however expression of MyoD and myogenin mRNA remained unaffected (25). Following gastrocnemius muscle injury in rats, there was a marked reduction in mitochondrial functionality. However, by restoring mitochondrial function through RNA delivery there was an increase in SC proliferation which was accompanied by improved muscle regeneration (30), suggesting that mitochondrial function may have a considerable role in myogensis. Although not reported in this thesis, participants in Chapter 2 did demonstrate improvements in mitochondrial capacity following interval-type aerobic training (22) as did participants in Chapter 3 (23, 57). Taken together, there may be a relationship between mitochondrial function and the increase in the proportion of active SC following training described in Chapters 2 and 3. Following aerobic exercise training, muscle regeneration was rescued in aged mice in the face of CTX-induced injury, as described in Chapter 4. Considering the improvement in mitochondrial function observed in the human model following similar training style, one could presume that an

improvement in mitochondrial content or function may have facilitated an improvement in muscle regeneration based on the potential role of mitochondria in the myogenic programme. Specifically, the greater SC content in old exercised compared to sedentary animals may have in part be due to an increase in mitochondrial content and function observed in these animals ultimately improving the muscle's ability to regenerate. Endurance training is able to rescue the accelerated aging phenotype in a mouse model characterized by progeroid aging. Exercise was able to re-establish mitochondrial content and function to control levels (55). Therefore it is likely that old exercised mice did have improvements in mitochondrial content and function. Although speculative, the data described above suggests that a relationship may exist between mitochondrial function and SC content; whereby an increase in mitochondrial function results in an increased proportion of active SC. It is however important to note that increases in mitochondrial function following increased physical activity in SC-depleted mice have been reported (28). Even though SC are not necessary in mediating improvements in mitochondrial function, when they are present increased mitochondrial function may improve their proliferative capacity.

Adaptation in skeletal muscle occurs due to changes in the transcriptional capacity of myonuclei in addition to post-translational modifications. In response to a given stimulus, myonuclei are 'reprogrammed' to support muscle fibre remodelling. However, it may be possible that resident myonuclei are eliminated and replaced with new nuclei via fusion of SC. In Chapter 2, we describe a 12 and 15-fold greater proportion of active SC following training associated with hybrid compared to type I and II fibres respectively

(Chapter 2, Figure 5). Furthermore, in Chapter 3 an increase in active SC following training was described (Chapter 3, Figure 4). Importantly, the increases in active SC were observed in the absence of an overall expansion of the SC pool. This may be due to a slow and constant turnover of nuclei, where rates of SC proliferation and differentiation are virtually identical with no appreciable expansion of the SC pool. Nuclear turnover describes a process by which new nuclei are incorporated into muscle fibres while resident myonuclei are eliminated to ultimately maintain a constant myonuclear content and domain. SC from adult extraocular muscle have observed to be chronically active; however, myonclear content appears stable, therefore supporting the notion of nuclear turnover (42). A variety of atrophic conditions have been shown to result in a decrease in the number of myonuclei (1). Although this notion has been challenged (24), it does suggest that nuclear content of skeletal muscle is flexible and that nuclei may be eliminated and potentially replaced when challenged with the appropriate stimuli.

5.3 Exercise training and muscle repair/regeneration

5.3.1 Potential mechanisms leading to improved regeneration

Skeletal muscle repair/remodelling is essential in maintaining healthy muscle throughout life. Exercise results in improvements in various metabolic and structural aspects of skeletal muscle health. It is possible that exercised muscle may be able to better repair and regenerate following injury. In Chapter 4, we described that old exercised mice were able to re-establish muscle fibre CSA to control levels as did young mice 28 days following injury whereas fibre CSA remained reduced in old sedentary mice (Chapter 4, Figure 2). The following section will aim to describe the ways in which exercise training

may improve skeletal muscle regeneration. A summary of the findings of Chapter 4 are presented in Figure 2.

A reduction in SC content in old rodents is often reported and is summarized in Table 1 of Chapter 1. Aerobic exercise conditioning has consistently demonstrated an expansion of the SC pool in old rodents (59, 60). In accordance with previous studies, we report an expansion of the SC pool in old mice following training (Chapter 4, Figure 1). Previous data would suggest that SC are absolutely necessary for successful skeletal muscle regeneration (39, 56) and it is therefore likely that the exercise-induced increase in SC content contributed to improved regeneration.

Revascularization is an important part of the regenerative process in skeletal muscle (4). Exercise conditioning resulted in an increased expression of the angiogenic factor VEGF following injury in a pattern similar to what was observed in young animals. The extent of capillarization was also increased following injury in the exercised group (Chapter 4, Figure 3). Contrary to previous reports (18, 35) exercise training did not lead to an expansion of the capillary network at baseline. Previous work in humans has established that active SC reside closer to capillaries compared to quiescent SC (10). Additionally a link between endothelial cells and SC has previously been established (3, 9). Although no increase in capillarization was observed at baseline these results suggest that exercise enables an optimal revascularization of skeletal muscle during regeneration. This improvement in revascularization following muscle degeneration may directly impact SC activation. This notion is reflected in a greater VEGF expression 10 days

following injury in exercised and young mice, which was associated with an increase in the SC pool at this time point in both groups (Chapter 4, Figure 3).

Muscle fibre perfusion is critical to skeletal muscle maintenance, as inadequate muscle fibre perfusion could limit delivery of oxygen, nutrients and growth factors to muscle fibres (68). Exercise training induces changes in the systemic environment (45) and has the ability to impact the SC microenvironment thereby impacting their function (16). Exercise has been shown to result in increased circulating levels of various cytokines. The term 'myokine' has recently been coined and describes a cytokine released by skeletal muscle. Myokines are produced following exercise and can act in both a paracrine or endocrine manner altering the SC microenvironment (44). Improved muscle regeneration observed in exercised mice may, in part, be due to the changes in the systemic environment of these animals. The benefits of exercise are numerous and the results presented in Chapter 4 demonstrate that old mice retain the ability to positively respond to exercise training. This is reflected in improved muscle regeneration following injury compared to old sedentary animals. The increase in SC content may be a result of an improved systemic circulating environment, which may be linked to improved vascularisation during muscle regeneration. The findings presented in Chapter 4 are novel and may lend valuable insight into SC biology in humans.

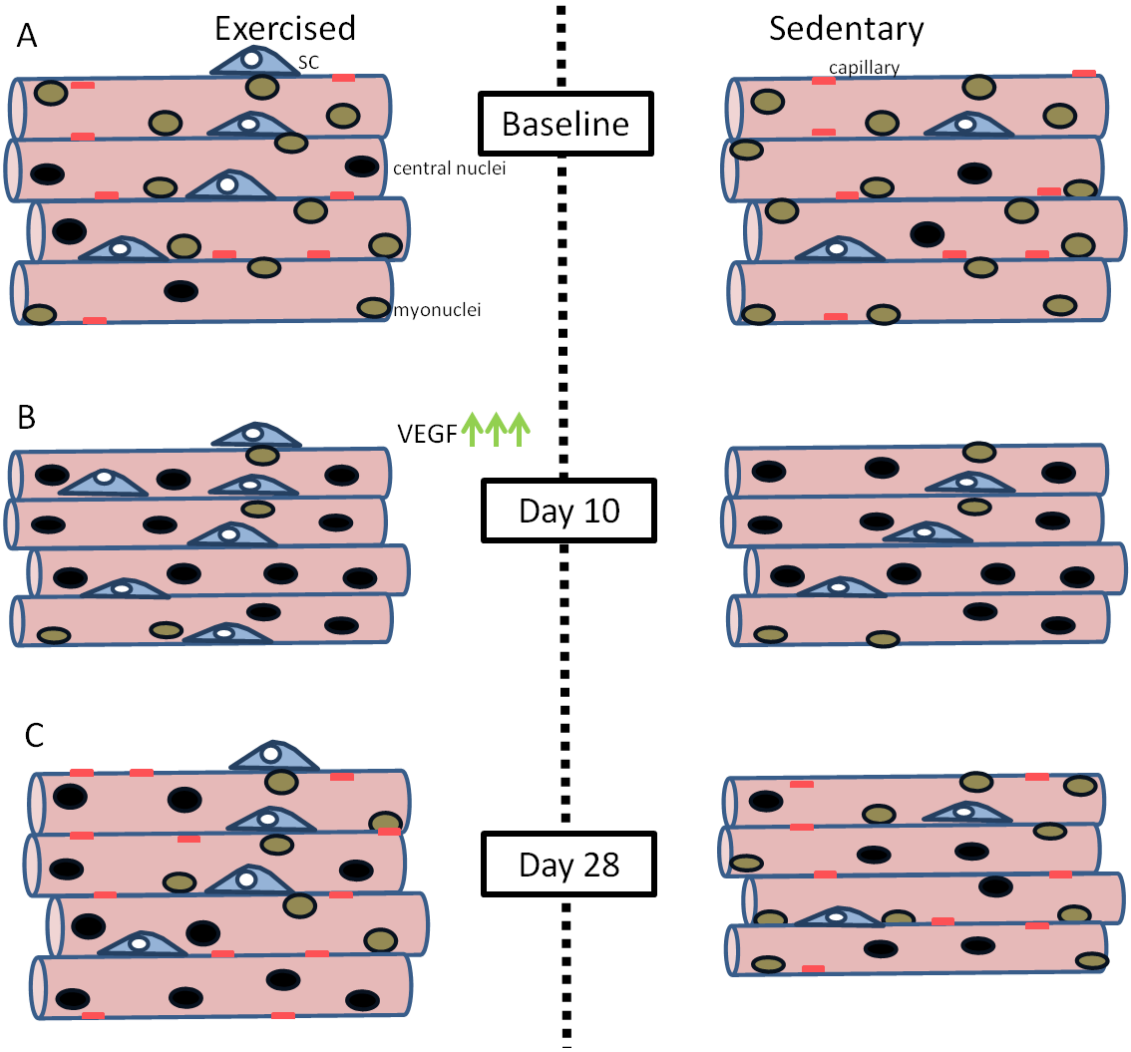

Figure 2. The effect of exercise on muscle regeneration in old mice. Exercise training increased SC content and the proportion of fibres with central nuclei in old mice (A). There is an increased expression of angiogenic factor VEGF in old exercised mice and SC content in both groups10 days following injury (B). Muscle fibre CSA was reduced whereas the proportion of fibres with central nuclei was increased in both groups 10 days after injury (B). Muscle fibre CSA remained reduced in sedentary mice but was re-established to baseline levels in exercised mice 28 days following injury; the proportion of fibres with central nuclei was reduced in sedentary compared to young animals (C). The number of capillaries per fibre was increased in exercised mice only 28 days following injury (C).

5.3.2 Aging, exercise and regeneration: Implications in humans

Given the negative consequences of a rapidly aging population, there has been considerable focus on identifying the underlying mechanisms of sarcopenia. An increased incidence of injury in older adults may result in increased periods of reduced mobility or bed rest. Importantly, short periods of reduced mobility leads to rapid declines in health and accelerated loss of muscle mass in older individuals (14). Therefore, determining the factors that may minimize loss of muscle and maximize the capacity for older individuals to re-establish muscle mass following periods of disuse should be a priority.

` Translating findings from studies employing animal models to humans must be done with caution. In Chapter 4, a model of skeletal muscle injury in old mice was employed to determine the effect of aging and exercise on muscle regeneration. There are methodological limitations to studying regeneration in humans, and for this reason a rodent model was used. This model was employed to further our understanding of SC biology with hopes of applying the findings to guide future work in humans. Injection of CTX causes widespread regeneration of skeletal muscle (26, 49) highlighted by degeneration, repair and remodelling (67). Although CTX-induced injury is an extreme model of injury that cannot be directly translated to humans, insights can be gained on the ability to re-establish muscle fibre CSA during the early phases of regeneration.

The results of Chapter 4 indicate that exercised-conditioned muscle is better able to regenerate following injury compared to muscle from sedentary animals. Work in humans demonstrated that there is an inability to re-establish muscle fibre CSA and that SC content is impaired following periods of disuse in old adults (66). The reduction in

CSA has been reported as soon as 7 days following the onset of immobilization (27). These results are similarly reflected in rodent models, which demonstrated impaired early muscle regeneration (5, 12). Impaired early regeneration may preclude the muscle to ever fully regenerate. Indeed, this is highlighted by the results of Chapter 4 describing fewer regenerating fibres in sedentary animals following injury compared to young animals even though CSA was not fully re-established. Importantly exercise conditioning was able to rescue impaired regeneration observed in sedentary older animals (Chapter 4, Figure 2).

Work in humans consistently demonstrates that older adults are able to positively respond to exercise training (64). Resistance exercise training is commonly studied as an intervention to prevent the onset of sarcopenia (31, 34, 46, 53, 71). Although resistance exercise is the gold standard for increasing muscle mass, aerobic exercise in older individuals may not only improve cardio metabolic health but may also improve skeletal muscle health and its ability to repair/regenerate following periods of disuse. In Chapter 2 and 3, we report an increase in SC pool activity without the expansion of the total pool. Considering the indispensable role of SC in muscle regeneration, it may be of interest to study the effect of exercise prior to immobilization and its subsequent effect on re-loading of skeletal muscle.

5.4 Implications and limitations

To determine SC contribution to skeletal muscle remodelling following exercise, researchers have consistently enumerated the number of quiescent SC associated to each fibre via immunohistochemical analysis of muscle cross sections. SC have been identified

by the expression of marker such as Pax7 (7, 38, 40, 41, 69) and NCAM (CD56) (13, 15, 36, 41, 43) as nuclei residing within the sub-laminar SC niche. If no increase in the number of Pax7 or NCAM positive nuclei were observed it was commonly reported that SC were not involved in muscle adaptation. However, more recent literature has sought to further describe the SC pool by identifying SC co-expressing various markers of proliferation (38, 40). Based on results from Chapter 2 and 3, where an increase in active SC was identified without an increase of overall SC content, we can conclude that it is not sufficient to evaluate the contribution of SC to a specific stimulus by simply enumerating the SC pool as the proportion of active SC may change without an expansion of the pool. Future studies should consider determining the proportion of quiescent and active SC to fully describe the impact of a stimulus on the SC pool.

Very few studies had examined the effect of aerobic exercise on SC in humans. Two studies reported an increase in SC content following interval-type aerobic training, which also induced increases in CSA (8, 71), while another study in type 2 diabetics reported no change following traditional endurance exercise training (65). Additionally, these studies were performed in older adults. In Chapters 2 and 3 we reported that in young healthy individuals the basal SC pool does not expand following various types of aerobic-style training (Figure 1). In addition it was postulated in Chapter 2 that intensity may play a role in mediating SC pool expansion as this was reported in rats (32). In Chapter 3, it was reported that intensity of training likely does not dictate expansion of the basal SC pool in response to training as 3 exercise training interventions of varying intensities were employed all of which lead to similar outcomes.

Whether aged skeletal muscle can fully regenerate following injury in old animals is debatable with some studies showing impairment (5, 12, 54) while others report complete re-establishment of muscle fibre CSA (33, 58, 62). In Chapter 4, we report impaired regeneration in old sedentary mice whereas regeneration was unaffected in old exercised mice. Together with the results from Chapters 2 and 3, aerobic exercise may improve repair/regeneration in older adults following periods of disuse induced muscle atrophy.

Based on results from Chapters 2 and 3, we conclude that the basal SC content is not affected following various types of aerobic exercise training in young healthy adults. Furthermore, we determined that unlike a single report using a rat model (32) intensity of aerobic training did not affect the SC response. The inclusion of acute muscle biopsies following the first bout of exercise in each training intervention would have provided more information on the progression of the SC through the myogenic programme and whether intensity of exercise would have any influence. Additionally, in Chapter 2 the proportion of active SC was only determined in muscle cross sections of post training biopsies to determine the fibre type differences. We were, however, not able to comment on whether the overall activation of the SC pool was affected by training as the proportion of active SC was not determined in the pre-training samples. The primary and novel finding of Chapter 3 was the increase in SC pool activity without an overall expansion of the pool following aerobic-type exercise. The notion of nuclear turnover was proposed, however it is impossible to assess this in humans. The presence of apoptotic nuclei determined via the terminal deoxynucleotidyl transferase (TdT) dUTP

nick-end labeling (TUNNEL) assay would have allowed us to evaluate whether the constant number of nuclei can be explained by the replacement of apoptotic nuclei with nuclei from the SC. Alternatively, the removal of myonuclei from fibres may not be an apoptosis-dependent process at all. In addition we were unable to determine if the increase in proliferating SC resulted in fusion of the SC to the muscle fibre or if activated SC were 'lost' and never actually fused to the fibre.

The beneficial effects of exercise in improving muscle regeneration were described in Chapter 4. Although numerous changes occur in skeletal muscle following aerobic training, the likely cause of improved muscle regeneration may be exposure to an enhanced circulating systemic environment. Exposing old mice to a young systemic environment was able to rescue impaired regeneration (5, 11, 12) implying a role for the systemic environment in promoting muscle regeneration. Exercise has pronounced systemic effects even in non-contracting muscles (6). Collecting serum from exercised and sedentary mice may have allowed us to identify candidate circulating factors underlying the reported adaptations.

The extent of muscle regeneration was determined via muscle fibre CSA. Although exercised mice re-established CSA to that of baseline levels following injury, functional outcomes were never measured. While re-establishing muscle mass is important, determining if this translated to functional outcomes such as grip strength, VO_{2peak} and fatigability would have provided more information on overall muscle health following injury and how that compared to young animals. In addition, animals completed aerobic exercise conditioning which likely resulted in improved mitochondrial

content and function. Determining whether mitochondrial function was restored to the same extent in old exercised mice compared to young mice following injury via CS activity, immunohistochemical analysis of CS and succinate dehydrogenase (SDH) or mRNA expression of various markers of mitochondrial biogenesis would have provided more information on overall muscle function.

The fundamental differences that exist in metabolic control between human and animal skeletal muscle have been described (48). Differences were directly observed in this thesis. Specifically, an increase in SC content was reported in old mice following a nonhypertrophic exercise stimulus, whereas 4 different nonhypertrophic exercise stimuli in humans did not result in an increase in SC content. Therefore caution must be used when translating results from rodent models to humans.

5.5 Conclusions

Common dogma is that SC pool expansion is necessary to mediate adaptations following resistance exercise training. The major findings of this thesis demonstrate that nonhypertrophic exercise training results in an increase in active SC without expansion of the pool. In addition, nonhypertrophic exercise training can improve the regenerative response in old mice and this may in part be due to an increased SC content. In conclusion, the studies discussed in this thesis have advanced our knowledge in understanding the 'non-traditional' role of SC following exercise training and provides new avenues for future studies examining aerobic training as a strategy to improve the impaired regenerative process in old adults.

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