MICROVASCULATURE AND MUSCLE STEM CELLS

THE RELATIONSHIP BETWEEN CAPILLARIES AND MUSCLE STEM CELLS: CONSEQUENCES FOR ADAPTATION, REPAIR AND AGING

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LAY ABSTRACT

Skeletal muscle health is, in part, maintained by a population of stem cells associated with individual muscle fibres. When muscle is damaged or stressed, these cells become activated, aid in muscle repair, and help drive adaptations to exercise. The central purpose of this thesis was to examine the relationship between muscle capillaries and muscle stem cells, and determine how that relationship impacts muscle stem cell function. We demonstrated that muscle stem cells and capillaries exist in close proximity to each other in skeletal muscle. We observed that a greater muscle capillarization is linked to improved muscle stem cell function during muscle repair. However, we also report that the distance between muscle capillaries and muscle stem cells becomes greater in aging, and may be a root cause of impaired muscle stem cell function in aging.

ABSTRACT

Skeletal muscle possesses a remarkable plasticity, able to repair, remodel and adapt to various stressors. A population of resident muscle stem cells, commonly referred to as satellite cells (SC), are largely responsible for skeletal muscle plasticity. The loss of muscle mass and plasticity typically observed in aging has been attributed to the deterioration of SC function. SC reside in a quiescent state, but following stimuli they become active, proliferate and eventually differentiate, fusing to existing muscle fibres. The progression of SC through this process, termed the myogenic program, is orchestrated by a complex network of transcription factors, termed myogenic regulatory factors. SC function is regulated by various growth factors and/or cytokines. The delivery of these signalling factors to SC is, in part, dependent on their proximity and exposure to local microvascular blood flow. The purpose of this thesis was to examine the relationship between skeletal muscle capillaries and muscle SC. We examined the effect of age on the spatial relationship between SC and muscle fiber capillaries, and observed that type II muscle fiber SC were located at a greater distance from the nearest capillary in older men as compared to their younger counterparts. We then examined the changes in SC activation status following a single bout of resistance exercise, prior to and following a 16wk progressive resistance training (RT) program. We observed that following RT, there was an enhanced SC activation in response to a single bout of resistance exercise. This enhanced response was accompanied by an increase in muscle capillarization following training. Furthermore, we investigated the impact of muscle fiber capillarization on the expansion and activation status of SC in acute response to

muscle damaging exercise in healthy young men. We observed that muscle capillarization was positively related to SC pool activation and expansion. Taken together, we demonstrate that muscle capillarization may be related to the SC response following acute resistance exercise or exercise-induced injury, and may be implicated in adaptation to RT. Furthermore, the spatial relationship between muscle capillaries and SC is negatively altered by aging.

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LIST OF ABBREVIATIONS

1RM	1 repetition maximum
ANOVA	Analysis of variance
BSA	bovine serum albumin
C/Fi	capillary to individual fibre ratio
C/F_i	individual capillary-to-fibre ratio
CC	capillary contacts
CD	capillary density
CD56/NCAM	neural cell adhesion molecule
CFPE	capillary-to-fibre perimeter exchange index
CK	creatine kinase
CSA	cross sectional area
DAPI	4',6-diamidino-2-phenylindole
DSHB	Developmental Studies Hybridoma Bank
DTA	diphtheria toxin A
ECM	extracellular matrix
FBS	Fetal bovine serum
FGF	fibroblast growth factor
GS	goat serum
HGF	hepatocyte growth factor
HGFA	HGF activator
IGF-1	insulin like growth factor 1
IL-6	interleukin 6
M-Cad	cell adhesion protein M-cadherin
MGF	mechano growth factor
MHC	myosin heavy chain
MHC	myosin heavy chain
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
Mstn	myostatin
OCT	optimum cutting temperature
Pax	paired box
PCNA	proliferating cell nuclear antigen
PDGF-BB	platelet derived growth factor
PFA	paraformaldehyde
SC	satellite cell
SF	sharing factor
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
VO_2max	maximal oxygen consumption

PREFACE
DECLARATION OF ACADEMIC ACHIEVEMENT

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies' Guide for the Preparation of Theses. It includes a general introduction, three overall studies prepared in journal article format, and an overall discussion. The candidate is the first author on all of the manuscripts. At the time of the thesis preparation, Chapters 2 and 3 were published in peer-reviewed journals; Chapters 4 and the related discussion were submitted to peer-review.

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PREFACE

The notion of tissue regeneration is as ancient and timeless as the Greek mythos. In Hesiod's *Theogeny*, as punishment for bringing the gift of fire to humanity, the titan Prometheus was chained to a rock amongst the Caucasus Mountains. Every day, his liver was devoured by eagles, only to be renewed every evening. The term regenerate derives its archaic roots from the Latin term *regenerare*; which means 'to make over, generate again' or 'a being born again'. The word conjures up the imagery of the chained titan's organs rapidly repairing themselves at dusk, in this case, a curse of his immortality. In this way, Prometheus serves as a metaphor for bringing scientific enlightenment to humanity, but also provides us with a poignant example of the seemingly godlike quality of regeneration.

While the story of biology is considerably less fanciful, the remarkable plasticity and the extraordinary abilities of human skeletal muscle to regenerate and repair are no less fascinating than the myth. In 1961, Dr. Alexander Mauro discovered a cell that was 'wedged between the plasma membrane of the muscle fibre and basement membrane'. Mauro asserts that these cells, "intimately associated with the muscle fiber…we have chosen to call satellite cells". With considerable foresight, Mauro predicted that these cells, located on the periphery of the muscle fiber "might be pertinent to the vexing problem of skeletal muscle regeneration". With these findings, observed through the lens of an electron microscope, the foundation for the field had been set. Their identification in humans by Dr. Ruben Laguens in 1963 opened new horizons in human muscle physiology. For the last nearly 60 years, the study of satellite cell biology has progressed, inextricably linked with muscle regeneration and repair.

Here, we turn to another legend. The fountain of youth, as suggested by Herodotus in *The Histories*, needs no introduction. Considerable evidence exists of an age-associated decline in satellite cell content and capacity to regenerate muscle. In 2005, pivotal work would be published suggesting that satellite cell activity of aged muscle could be rejuvenated, if the circulation of the aged animal was linked to one more youthful. Determining the humoural factors that alter the function of aged satellite cells, and how they are delivered through circulation, adds another page in the history of human physiology.

Hvar sem fjandinn er þar hefur hann sína.– ' a wise man changes his mind, a fool never will' – Icelandic proverb.

CHAPTER 1:

INTRODUCTION

1.1 OVERVIEW of the Aspects of Skeletal Muscle

In humans, skeletal muscle comprises ~40% of total body weight, therefore constituting one of the largest and most metabolically active tissues. From a mechanical perspective, skeletal muscle maintains posture, facilitates breathing, generates locomotion and is associated with independence. From a metabolic perspective, skeletal muscle provides storage for amino acids and carbohydrates, as well as the cellular machinery to provide energy for physical activity and the maintenance of homeostasis.

Muscle fibres are arranged into bundles, separated by connective tissue, forming skeletal muscle. Muscle fibres are highly vascularized, in order to provide sufficient oxygen and nutrient delivery, and are innervated by type I (slow contracting, fatigue resistant, highly oxidative) and type II (fast contracting, low fatigue resistant, highly glycolytic) (205) motor neurons. The relative proportion of these distinct myofibre isoforms ultimately determines the contractile property of a muscle. Irrespective of fibre-type, the processes for muscle contraction are the same, with action potentials triggering actin filaments to slide over the myosin filaments, resulting in contraction (179). Taken together, skeletal muscle exists as a functional unit, comprised not only of myofibres but also motor neurons, muscle capillaries and extracellular matrix as structural support.

Skeletal muscle is one of the most dynamic tissues in the human body, capable of remarkable plasticity and adaptation. Whereas endurance-type exercise generally leads to adaptations within skeletal muscle that leads to increased oxidative capacity, resistance training is characterized by increases in muscle mass and fibre size leading to increased force generation. Myofibres themselves are post-mitotic and multi-nucleated, and thus

derive their extensive plasticity primarily from existing myonuclei and the presence of muscle resident stem cells (known as satellite cells). However, recently discovered cell populations such as progenitor interstitial cells (112, 133), side population cells (150, 185) and even circulating hematopoetic stem cells (56, 106) have been suggested to contribute to the remodelling of skeletal muscle tissue.

1.1.1 **Origins of Skeletal Muscle**

During vertebrate embryonic development, the generation of muscle, termed 'myogenesis', is completed in distinct phases (184). In the early embryonic phase, three divergent germ layers; the ectoderm, mesoderm and endoderm, are formed (4). The mesoderm is then further separated, with the paraxial aspect condensing into somites, developing from the anterior aspect of the embryo down toward the tail (6). The growing structure of the embryo is altered by fluctuating gene expression (47) and various signalling molecules, with the spatiotemporal nature of these morphogens causing different cellular responses in different regions of the embryo (75). These somites, following signalling from the Notch, noggin and Wnt pathways, subsequently develop both dorsal and ventral compartments (71). The most dorsal aspect of the somite becomes the 'dermo-myotome' from which the vast majority of human skeletal muscles, minus some muscles of the head, are derived (145). The expression of the paired box transcription factor (Pax) 7 can be observed in cells within the dermomyotome structure (93) and this cell population continues to mature into the myotome comprised primarily of progenitor cells. These progenitor cells exhibit heightened expression of MyoD and Myf5 (142), both basic helix-loop-helix transcriptional activators belonging to the

myogenic regulatory factor (MRF) family that are considered indicators of terminal differentiation into the myogenic lineage (151). From the epaxial aspect of the myotome, the dorsal muscles develop (145), whereas the hypaxial aspect develops into the torso (36) and limb muscles (145). Muscles at the extremities, respiratory muscles, muscles of the tongue and some facial muscles are derived from cells at the junction of the ventral and lateral sides of the myotome from myogenic cells capable of extensive migration (194). Proliferating myogenic cells, originally exhibiting upregulated MyoD and Myf5, begin to express the later stage MRFs myogenin and MRF4, terminally differentiating and subsequently increasing myofibrillar protein specific genes such as myosin heavy chain (MHC), actin and muscle creatine kinase (31). Thus, the initial 'template' of multinucleated myofibres are generated during this period, as mononuclear myogenic cells fuse together. Extensive proliferation occurs until the myonuclear content reaches homeostasis, and myofibrillar protein synthesis reaches its peak (48). In this way, subsequent waves of proliferation and differentiation continue to develop perinatal muscle arranged upon the structural template.

During the course of embryonic development of skeletal muscle, a subpopulation of myogenic cells do not terminally differentiate and withdraw from the cell cycle, giving rise to a population resident satellite cell (SC) that remains mitotically quiescent (97, 157). Skeletal muscle, from the perinatal phase through maturation, is dependent on the contribution of SC in order to maintain tissue homeostasis (167) by contributing their nuclei to existing myofibres throughout the lifespan. Indeed, SC are unequivocally important for the maintenance, repair, and regeneration of myofibres (78, 120, 166) and

potentially skeletal muscle remodelling and adaptation in response to hypertrophic (95) and non-hypertrophic stimuli (89).

1.1.2 **Muscle stem cells**

Satellite cells were first identified via electron microscopy as mononucleated cells residing beneath the basal lamina and in close association with the sarcolemma (118). At the time of their discovery, it was hypothesized they may be involved in skeletal muscle regeneration (118).

Early radioactive nucleotide labeling experiments revealed that SC were capable of mitosis and contributed nuclei to the associated fibre (136, 158). Work by Snow et al. (178) suggested that while SC are normally mitotically quiescent in adult skeletal muscle, they can enter into the cell cycle following muscle injury. Observations from this study also suggested that this SC population could yield proliferative myogenic progenitor cells, termed myoblasts (178). Myoblasts had previously been shown to fuse together, creating multinucleated myotubes *in vitro* (100, 207). Consistent with this, there was a proliferation and expansion of the SC pool on isolated damaged myofibres, which was subsequently followed by fusion to form functional myotubes (13, 100). Taken together, these early observations support the notion that SC contribute to muscle regeneration and repair, via the donation of nuclei to damaged fibres.

Stem cells are defined by their ability to differentiate into distinct tissues and by their ability to self-renew, referred to as ‘stem-ness’. SC ‘stem-ness’ was identified through an elegant transplantation experiment, whereby ~7-22 SC, along with their intact myofibres, were transplanted from healthy mice into the muscles of an immune-deficient

muscular dystrophic mouse that had undergone muscle irradiation (40). Following the graft, it was observed that a single myofibre could give rise to ~100 *de novo* fibres, containing ~30,000 myonuclei, and observed a nearly 10-fold increase in the size of the SC pool (40). Similarly, even when single labelled SC were transplanted into the same irradiated dystrophic mouse muscle, new myofibres were generated and the progeny of the labelled SC remained in the muscle (164). Taken together, these findings support the notion that SC are a population of monopotent stem cells capable of terminal differentiation and self-renewal.

It is well established that SC are the primary source of progenitor cells in adult skeletal muscle. However, there is some evidence to suggest that muscle, albeit with a reduced functionality, is capable of being maintained without the presence of SC in adult skeletal muscle. The notion that non-SC progenitors, such as circulating bone marrow derived stem cells and hematopoietic stem cells (56, 106), as well as muscle resident $CD45^+/Sca1^+$ (50, 150, 185), $PW1^+$ interstitial cells (133), and $Twist2^+$ cells (112), could contribute in some capacity to muscle repair and/or maintenance has been suggested. However, it is important to note that while these various populations of atypical progenitor cells do exist, there is limited evidence to suggest that they play a meaningful role in the maintenance and/or repair of skeletal muscle. More importantly, few of them have been observed in human skeletal muscle and their precise function and/or purpose remain poorly understood.

While many aspects of muscle progenitor function remain nebulous, it is has been established that commitment to the myogenic lineage requires the paired box transcription

factor, Pax7 (133, 170). Together, Pax7 and the myogenic regulatory factors play a critical role in orchestrating the progression of SC from the quiescent state, through proliferation and into terminal differentiation.

1.1.3 **Pax7 and the myogenic regulatory factors**

The Pax7 gene is a member of the paired box (Pax) containing gene family of transcription factors and is specifically expressed in quiescent and activated SC (84, 170). Pax7 expression also appears to be necessary to maintain stem cell quality of the SC by facilitating self-renewal, as well as maintaining quiescence (140). While the precise cellular role of Pax7 has not been elucidated, it also appears that Pax7 plays a critical role in the initiation and progression of the myogenic program. Evidence by McKinnel et al. (126) suggests that Pax7 interacts with a specific histone methyltransferase complex that in turn directs the methylation of histone H3K4 to induce DNA modifications. The change to the chromatin stemming from this interaction facilitates the transcription of Myf5, thus initiating entry into the myogenic program. The interplay between Pax7 and the myogenic regulatory factors also appears to influence the progression of SC through the myogenic program. Indeed, down-regulation of Pax7 appears to be necessary for the initiation of myogenic differentiation (140, 141, 170). Pax7 co-expression with MyoD in SC during the proliferative phase appears to end with the down-regulation of Pax7 (212). Consistent with this, work by Olguin and colleagues (141) suggests that Pax7 and myogenin are reciprocal inhibitors of each other, and thus the down-regulation of Pax7 must occur in order to achieve terminal differentiation. Interestingly, during SC proliferation, a subpopulation of SC down-regulate Pax7 and thus initiate terminal

differentiation while other subpopulations down-regulate MyoD, retracting back to cellular quiescence and thus renewing the SC pool (49, 212). These observations suggest that MyoD expression does not necessarily warrant commitment to the myogenic program, and thus highlights the importance of Pax7 expression. The importance of Pax7 was demonstrated by the development of Pax7 null mice ($Pax7^{-/-}$). $Pax7^{-/-}$ mice survive embryonic development due to an increased activity of a Pax7 orthologue, Pax3 (23). However, these animals possess a marginal SC pool that diminishes rapidly during post-natal development (157) suggesting that Pax7 is critical to the existence and self-renewal of the SC pool. Together, these data would indicate that Pax7 is important for maintaining a viable SC population and temporal up- and down-regulation of the gene guides the SC through the myogenic program through interactions with the myogenic regulatory factors. The molecular regulation of SC proliferation and differentiation is driven by the expression of Pax7 and the myogenic regulatory factor (MRF) family, including MyoD, Myf5, myogenin and MRF4 (Figure 1). Upon exposure to a physiological stimuli, SC exit quiescence and become active. Satellite cell activation can be initiated by a number of growth factors and/or signaling pathways (discussed in Section 1.2.2). Activated SC are characterized by the upregulation of MyoD and Myf5 (42, 66, 206). In animal models, MyoD appears prior to any other indicator of cell proliferation (174) as early as ~12h following injury. Interestingly, MyoD upregulation appears following non-damaging and/or hypertrophic stimulation without the expansion of the total SC pool (90) in humans.

Figure 1. The myogenic program.

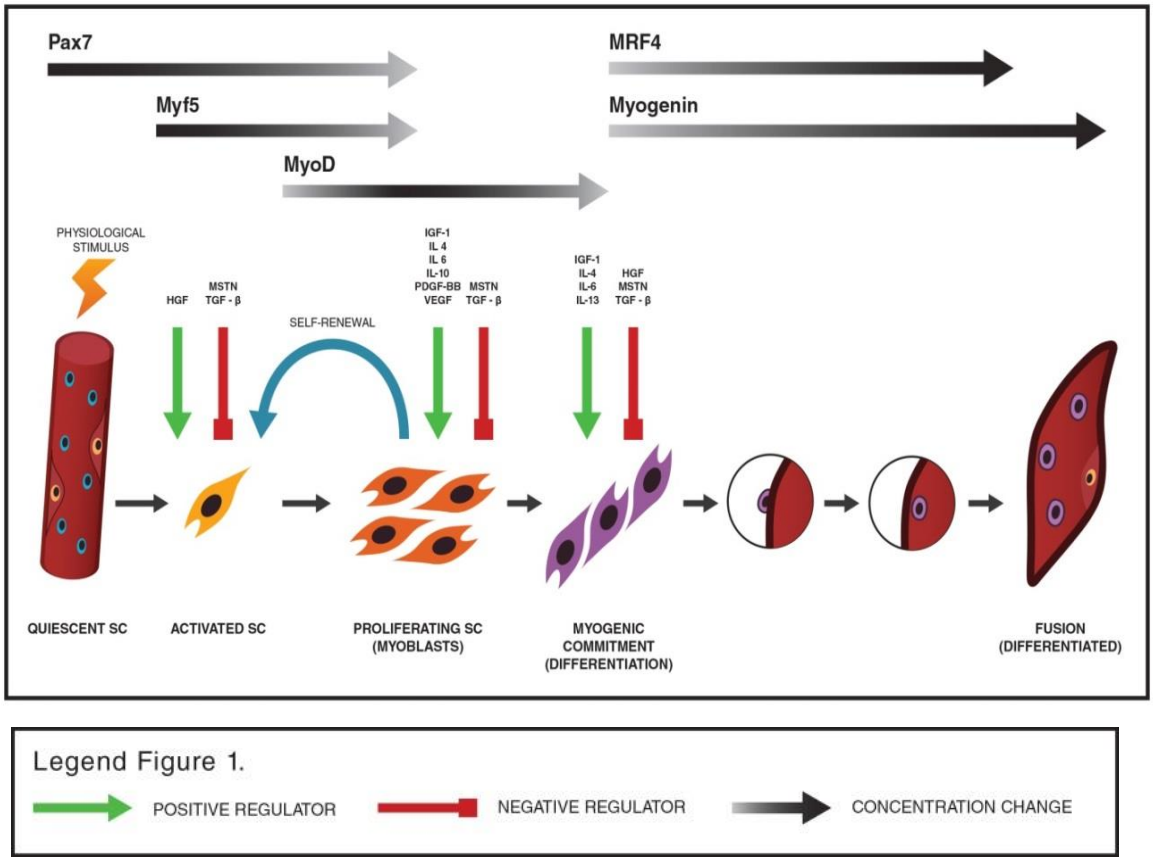

Figure 1. Schematic representation of the normal myogenic program in response to physiological stimuli. Satellite cells are typically mitotically quiescent and reside within a specialized niche situated beneath the basal lamina and the sarcolemma of the associated myofibre. Following stimuli, satellite cells begin the myogenic program by becoming activated and then begin to proliferate, expressing MyoD and Myf5. Following proliferation, the satellite cells begin differentiation, down-regulating the paired box transcription factor Pax7 and expressing MRF4 and Myogenin. During differentiation, the satellite cells fuse to themselves forming new myofibres, fuse to existing myofibres to donate their nucleus, or return to their quiescent state, thus renewing the satellite cell pool. Various growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), myostatin, platelet derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF) and a number of the interleukin family (IL-4, -6, -10, -13) have been shown to be regulators of the myogenic program.

This evidence suggests that the presence of MyoD may indicate that a SC has entered into the proliferative phase of the myogenic program, but may not continue all the

way into terminal differentiation. Furthermore, it appears that the early expression of MyoD is associated with a set of 'pre-committed' satellite cells, capable of differentiating without first undergoing proliferation (155), suggesting that there may be subpopulations of SC within the overall pool. Regardless of the implication that there may be heterogeneity amongst the SC pool (155), the vast majority of SC express either MyoD or Myf5 within 24h and typically co-express these factors within 48 hrs following injury (42, 43). Given that it appears that MyoD and Myf5 are upregulated concomitantly suggests that they may have differing functions in adult skeletal muscle. Work with $MyoD^{-/-}$ mice suggests that SC were more driven toward self-renewal as opposed to myogenic differentiation, whereas $Myf5^{-/-}$ mice demonstrate a reduced capacity for SC proliferation. Taken together, it appears that the entrance of the SC into various phases of the myogenic program is influenced by the expression of MyoD and/or Myf5 (163, 210).

Following proliferation, the majority of SC progress into differentiation and fuse with each other to create *de novo* myofibres or fuse with existing myofibres. Differentiation is driven by the upregulation of transcription factors myogenin and MRF4 (43, 206). The downstream targets of myogenin and MRF4 are genes that code for structural and/or contractile proteins essential for myofibrillar formation and functionality (12). Considerable work has established the intrinsic influence of Pax7 and the MRFs in orchestrating myogenesis, SC self-renewal and the progression through the myogenic program. However, there are also a number of extrinsic regulators and signaling molecules that initiate and influence myogenisis during the lifespan as well.

1.1.4 **The muscle stem cell niche**

The microenvironment provides important structural and signalling cues to stem cells, regardless of tissue type (139). Satellite cells are situated in a specialized location, termed the 'SC niche'. This niche is surrounded by microvasculature, is influenced by innervation, and is associated with and secured within the extracellular matrix (ECM). Within the SC niche, the SC is influenced by cell-to-cell interaction (92), as well as autocrine and paracrine signalling. The upper boundary of the satellite cell niche, the basal lamina, is comprised of two primary constituents, collagen type IV and laminin-2 (α2, β1 and γ1 chains), which assemble into cross-linked networks and are further linked by the glycoprotein nidogen (189). Amongst the basal lamina are a series of bindings sites for α7/β1-integrins that anchor the actin skeleton of the SC to the ECM (119) and allow for the transduction of mechanical force into biochemical signalling (24), thus being crucial for SC regulation. Resident fibroblasts secrete a number of growth factors and facilitate organization of the ECM by the deposition of collagen and other proteins (189). Proliferating cell nuclear antigen (PCNA) was no longer detected in SC following muscle denervation, suggesting that loss of motor neuron activity may limit the ability of SC to proliferate (105). Taken together, these findings suggest that cells and structures (e.g., fibroblasts, macrophages, motor neurons) associated with the SC niche play an indispensable role in regulating SC. However, the proximity of SC to capillaries, residing just outside the niche, and the demonstrated importance of cellular cross talk between endothelial cells and SC demands considerable attention.

Vasculature associated with SC niche

To maintain skeletal muscle homeostasis and to respond to increased metabolic demands, the delivery of cytokines, nutrients and oxygen via the microvasculature is an absolute necessity. Indeed, work by Christov and colleagues (35) suggests that muscle SC may be in close spatial proximity to capillaries and consequently endothelial cells. After determining the close physical juxtaposition of endothelial cells and SC across species via electron microscopy, this group observed that SC were more frequently associated with capillaries, as compared to myonuclei (35).

Furthermore, these endothelial cells are capable of stimulating myoblast proliferation by the secretion of a number of growth factors (35) such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), platelet derived growth factor (PDGF-BB), vascular endothelial growth factor (VEGF) and fibroblast derived growth factor (FGF) (further discussed in Section 1.2.2). By co-culturing endothelial cells and myogenic cells, there was a marked increase in proliferation in the myogenic population. In contrast, when growth factors IGF-1, HGF, bFGF, PDGF-BB, and VEGF were specifically inhibited individually, there was a decrease in SC proliferation ~50%, however, global inhibition of growth factors resulting in ~90% reduction in proliferation. The cross-talk between muscle SC and endothelial cells may be reciprocal for endothelial cell proliferation as well, as following incubation of myogenic cell-derived growth factors, pro-angiogenic effects and capillary structure formation were observed (35). This relationship between SC and the microvasculature is particularly evident during muscle regeneration, with the process of myogenic repair and angiogenesis occurring simultaneously (113). During muscle regeneration, the tissue undergoes extensive re-

vascularization (72, 77, 192). The primary driver of angiogenesis is VEGF (20), and the overexpression of VEGF can stimulate SC proliferation (5) *in vivo*, while the inhibition of VEGF can result in diminished myoblast differentiation *in vitro* (22). While also being secreted from endothelial cells, it also appears that VEGF can be produced and secreted (34), by primary myoblasts (70), and is upregulated during terminal differentiation (22). Furthermore, VEGF can also act on myogenic cells in an autocrine fashion, stimulating cellular movement and/or migration and promoting differentiation (33, 34, 70). Work has previously shown that VEGF is capable of stimulating myogenic cell migration (70), and can promote the establishment of myofibres with centrally located nuclei (5). Previous work has shown that VEGF receptor mRNA expression (VEGF receptor-1; VEGF receptor-2) is also present in myogenic cells, and appears to be upregulated in satellite cells following damage (5). Taken together, these data suggest that there may be critical interactions between muscle SC and the microvasculature in skeletal muscle. This suggestion is supported by observations in patients with amyopathic dermatomyositis, in which individuals have a reduction in muscle capillaries without myofibre damage (57). In this clinical population, a proportionate reduction in muscle SC and capillarization in the same muscle has been observed (35). Importantly, in areas of the muscle cross-section where capillarization is preserved, there is maintenance of SC quantity (35). Taken together, patients with amyopathic dermatomyositis undergo specific SC loss, occurring selectively in muscle fibres with a reduced number of supporting capillaries. While these limited observations are important, there remains a marked paucity in the literature regarding the relationship between SC and the microvasculature in healthy

human models, particularly during muscle repair or in the context of aging. In part, this may be due to challenges in accurately identifying the activation status of a relatively rare muscle stem cell *in vivo*.

1.1.5 **The identification of quiescent and active satellite cells in vivo**

Using electron microscopy, Mauro and colleagues (118) first identified SC, characterizing their location as beneath the basal lamina and above the sarcolemma of a myofibre. However, the progression of technology and immunohistochemistry has made the identification of SC possible via immuoflourescent microscopy. Schubert and colleagues (168) used the glycoprotein Leu-19, and determined that SC were not only morphologically different from myonuclei, but confirmed that they resided beneath the basal lamina. Further study of Leu-19 identified that it may be involved in cell adhesion, analogous to the function on neural cell adhesion molecule (NCAM)/CD56 on neural cells (108), and that both proteins had identical staining patterns. Thus, the antigen NCAM, expressed on the surface of SC, has been used frequently in the identification of human SC by various groups (32, 94, 95, 114, 138, 196). However, there are some limitations to the use of NCAM as a SC identifier, as the protein is expressed in other cell types including Schwann cells, intramuscular nerves and motor unit end terminals (86, 128). In light of this, alternative markers have been utilized to properly quantify SC such as c-Met (110, 125), cell adhesion protein M-cadherin (M-Cad), (156, 165), and Pax7 (117, 125), though each with their own challenges. C-met is a receptor for hepatocyte growth factor and is present in both quiescent and activated SC (44) but also appears in capillaries and some interstitial cells in human skeletal muscle (110). C-met expression is

low in human SC, and may potentially only identify a subpopulation of the overall pool and therefore is not considered a good measure of human SC (125). M-cad is a less frequently used marker of SC (175), due to the difficulty in producing a quality antibody for use in human tissue, but is remains an identifier of SC that closely mirrors results using NCAM (87).

In contrast to cell surface proteins, Pax7 is a nuclear marker of SC that is expressed in mature muscle (102, 170). Pax7 has been extensively used to identify SC in human tissue (175) and has also been shown to be reliable in mice (211). Studies demonstrate that SC quantification using Pax7 or NCAM/CD56 yield results that are within 5% of each other (111, 117, 125, 130, 196). Some of the variation that exists may be due to the differences in the expression of Pax7 or NCAM/CD56 during the early (183) phases of terminal differentiation (25).

Irrespective of what antibody is used to identify SC, it is the combination of the SC marker and its location between the sarcolemma and the basal lamina that is critical in accurately determining SC content (110, 111). Furthermore, accurate enumeration of the SC population also requires the analysis of a significant number of myofibres. Work by Mackey et al. (117) illustrated that at least 50 type I and 75 type II muscle fibres are required to make a reliable estimation of fibre type specific satellite cell content in healthy young men. However, whether enumerating the same number of fibres will provide an accurate estimation of SC content in other populations has not been well-established. In a population such as elderly individuals, in which SC content has been reported to be significantly lower than in healthy young men (124, 195, 196) the counting

of additional muscle fibres (and thus increasing the number of SC including in the analysis) may yield a more accurate assessment. Furthermore, a growing number of studies suggest that SC must be enumerated in a fibre type specific manner. In both healthy young and clinical populations, the SC pool at rest can be significantly different amongst fibre types (7, 181, 196), including hybrid fibres (89). The SC pool also appears to respond to exercise stimuli in a fibre type specific manner, depending on exercise modality (28), highlighting the relevance of fibre-type specific analysis of SC content.

While the quantification of the SC pool in a quiescent state is valuable, evaluating the responsiveness of the SC pool to a given stimuli (i.e., exercise, muscle damage, injury) is equally important. Therefore, various markers indicative of the cell cycle or of their stage of the myogenic program (i.e, quiescence through to proliferation and/or differentiation) have been utilized in combination with a SC marker. In humans, while proteins that indicate proliferation such as PCNA; (117), Ki67 (116), or MyoD (90), have been utilized, there does not appear to be a consensus regarding which marker best describes the proliferating SC pool. Ki-67 is typically expressed during the early G1 phase of the cell cycle, increasing through S and G2 and finally peaking in M phase (69). On the other hand, PCNA is expressed only in the S-phase and late G1, and is not visible in the nuclei in the G2 or M phases (103). There appears to be a discrepancy in the number of Ki-67$^+$ cells as compared to PCNA$^+$ cells following exercise in humans (28). This discrepancy may be due to the relatively short (~90 min) half-life of the protein Ki-67 (79) as compared to the longer (~20 hour) half-life of PCNA (16).

The protein MyoD is considered an excellent marker of SC proliferation given the

repeated observation that it is a primary driver of myoblast proliferation (99, 129). MyoD may also serve as an excellent marker for the early phase of differentiation, as the upregulation of MyoD occurs concomitantly with the downregulation of Pax7 during that phase of the myogenic program (210). When assessing MyoD co-localization with nuclear markers (i.e., 4',6-diamidino-2-phenylindole; DAPI) and anatomical location (i.e., the presence of laminin), in the absence of Pax7, it can be used as a marker to identify SC that may have moved entirely out of quiescence, through proliferation and is in the early phase of differentiation.

As immunohistochemistry, flow cytometry and related molecular biology techniques have improved over the last decade, the identification of SC cells in various stages of the myogenic program has become easier. However, we are still limited by the fundamental shortcomings of these techniques, often amounting to a limitation in the number of molecular markers that can be visualized at the same time.

1.2 MUSCLE STEM CELLS AND EXERCISE

1.2.1 Acute satellite cell response in humans

In humans, SC function can be assessed by evaluating the SC pool following an acute bout of exercise. Unaccustomed, forced muscle lengthening contractions (i.e., eccentric muscle contractions) have been used to induce ultrastructural damage to muscle fibres and induce a repair response (8, 62, 153). A significant expansion of the SC pool has been detected as early as 6h following a single bout of eccentric exercise (45, 55, 60, 121, 122, 130, 138, 190). A list of findings regarding the expansion of the SC pool following eccentric contractions is shown in Table 1. The increase in satellite cell

number following eccentric contractions has been observed as early as 4h post-exercise recovery, with the peak typically occurring around 72h post-exercise (175). While a number of the studies in Table 1 analyzed SC content in 'whole muscle' (i.e., mixed muscle), eccentric exercise, known to preferentially recruit type II muscle fibres (137), results in a type II SC expansion in young men, suggesting that this model is also capable of inducing a fibre-type specific change in the SC pool (28).

Similarly, the SC pool response has also been evaluated following non-damaging exercise such as a single bout of resistance (RES), moderate intensity continuous (MICT) or high intensity interval training (HIIT) exercise. Following RES, there is a robust increase in the activation and expansion of the SC pool (11, 85, 124, 201). Interestingly, it appears that as few as three maximal isometric contractions are capable of upregulating myogenin mRNA expression, associated with SC differentiation (2). While inducing a less robust response from the SC pool than either eccentric- or RES exercise, HIIT and MICT also induce a SC response even under non-hypertrophic conditions across a range of subjects including healthy young and older individuals (90,114). Regardless of the modality, it appears that exercise can activate and/or expand the resident population of SC in human skeletal muscle. Considerably less understood are the growth factors and/or cytokines that are released during exercise that may play a role in the guidance of the myogenic program.

1.2.2 **Satellite cell regulators**

Eccentric contraction-induced muscle damage as well as other forms of exercise are sufficient to prompt a SC response. However, the precise factors that initiate SC activity are not well-understood. Within the niche, SC can be influenced by a variety of factors, including cytokines.

Table 1. Percentage change in satellite cell content in response to a single bout of eccentric exercise

Citation	Age	Exercise Protocol	Fibre Type	1h	3h	4h	24h	48h	72h	96h	120h	196h
Crameri et al., 2004	25 ± 3	50 one legged drop down jumps 8 x10 reps, 30 deg. s^{-1} 8 x10 reps, 120 deg. s^{-1}	Mixed					146		192		168
Dreyer et al., 2006	23–35	6 x16 reps at 60 deg. s^{-1}	Mixed				141					
O'Reilly et al., 2008	21 ± 2	10x 30 reps, 180 deg. s^{-1}	Mixed			5	138		148		119	
McKay et al., 2009	22 ± 1	10 x 30 reps, 180 deg. s^{-1}	Mixed			73	155		185		108	
Mikkelsen et al., 2009	23 ± 3	200 reps, 120 deg. s^{-1}	Mixed									96
McKay et al., 2010	21 ± 2	10 x30 reps, 180 deg. s^{-1}	Mixed				36					
Toth et al., 2011	21 ± 2	10 x30 reps, 180 deg. s^{-1}	Mixed	15	17		27					
Cermak et al., 2013	23 ± 1	10 x 30 reps, 180 deg. s^{-1}	Mixed I II				25 0 73					
Hyldahl et al., 2014	23 ± 2	196 reps, 180 deg. s^{-1}	Mixed I				25 30					

			II	25		
Buford et al., 2013	23 ± 4	110% 1 RM, one legged plantar flexors	Mixed	23		19

A compilation of studies that have assessed satellite cell content in response to a single bout of eccentric exercise in adults. Type I, type I muscle fibres; Type II, type II muscle fibers; Mixed, mixed muscle fibres. All values are expressed as a percentage increase from the baseline (i.e., prior to eccentric exercise) value.

Cytokines are small secreted proteins released by cells and have a specific effect on the interactions and communications between cells, and drive autocrine, endocrine and paracrine signaling that orchestrate progression of SC through the myogenic program. Regulatory factors such as HGF, IGF-1, myostatin, PDGF-BB, VEGF and IL-6 have been shown to be key regulators of the myogenic program (175, 210). HGF, a stromal mesenchymal-derived growth factor is a potent activator of quiescent SC as demonstrated in rodents (131, 186, 187). Consistent with this finding, O'Reilly and colleagues (138) reported that 24h following eccentric contraction-induced damage in humans there was a concomitant increase in SC number and HGF activator (HGFA) protein content. Another cytokine that has received considerable attention due to its hypothesized involvement in SC activation in both rodents (29) and humans (74, 122) is IGF-1, which is produced by the liver and muscle alike. IGF-1co-localizes with $Pax7^+$ SC in human muscle (74), and there is an increase in $IGF\text{-}1^+/Pax7^+$ cells 24h after eccentric contraction-induced damage (122). Furthermore, three spliced variants of IGF-1 have been described in skeletal muscle (i.e., IGF-1Ea, IGF-1Eb, and IGF-1Ec) and are all thought to make contributions to skeletal muscle regeneration (29, 162, 208, 209). IGF-1Ec (i.e., mechano growth

factor; MGF) and Myf5 appear temporally linked following eccentric damage suggesting that IGF-1Ec may be involved in SC activation/proliferation (122). Conversely, IGF-1Ea and Eb are temporally linked with myogenin expression suggesting that IGF-1Ea and Eb may be associated with differentiation or the transition from proliferation to differentiation. Myostatin (Mstn), a member of the transforming growth factor-β (TGF-β) family, has long been thought to regulate SC function due to its involvement in muscle fibre hypertrophy and/or hyperplasia during muscle growth (127). In Mstn knock-out mice, SC proliferation and pool size are significantly higher as compared to wild-type counterparts. It has also been shown that Mstn is expressed in human skeletal muscle SC, and in resting conditions approximately 80% of the SC pool is $Mstn^{+}$, suggesting that Mstn may play a role maintaining SC in the quiescent state (124, 176). Following a bout of resistance exercise, there is a significant decrease in the proportion of SC co-localized with Mstn (124, 176). The decrease in the proportion of SC co-localized with Mstn occurs at the same time that MyoD is up-regulated and a greater proportion of SC are in the S-phase of the cell cycle (124), which may suggest Mstn downregulation is required for SC proliferation. The interleukin family of cytokines, many of which are known to increase during/following exercise (51, 61, 146), can be secreted from skeletal muscle, making them potential candidates as SC regulators. Traditionally known as an inflammatory cytokine, IL-6 is the most well characterized interleukin in the context of exercise, is known to respond to various forms of exercise (9, 54, 61, 123, 146, 190) and importantly is known to play a role in SC function (123, 190). Signal transducer and activator of transcription 3 (STAT3) is a downstream target of IL-6 (191), and following

IL-6 binding to the IL-6Rα receptor, JAK2 is phosphorylated and initiates the phosphorylation of STAT3 leading to dimerization and translocation of STAT3 to the nucleus initiating transcription of downstream targets. Both IL-6 (121) and STAT3 (190) co-localize with SC following an acute bout of eccentric contractions. The genetic knockout of IL-6 has been shown to result in diminished SC proliferation by impairing STAT3 activation and expression of its target gene cyclin D1 (171). Likewise, IL-6 treatment of rat SC led to increased proliferation, phosphorylation of STAT3 and increased cyclin D1 expression (104). In humans, the temporal response of IL-6 following exercise appears to differ depending on intensity and duration of exercise (147). In humans, serum IL-6 has been shown to increase rapidly following eccentric exercise and remains elevated similar to the time course for SC expansion (121) with a peak in IL-6 occurring a few hours post-exercise. While serum IL-6 concentration may not perfectly reflect the cytokine concentration in the SC niche, these data suggest that the IL-6 response may play an important role in regulating SC during post-exercise recovery.

1.2.3 **Satellite cells and hypertrophy**

In rodent models, it has been well-established that SC are necessary for successful skeletal muscle regeneration and/or repair (120, 166). However, there is still a healthy debate regarding the contributions of SC during muscle fibre hypertrophy. As a post-mitotic tissue, skeletal muscle relies on the addition of *de novo* nuclei from an exogenous source, predominately SC. The nuclei within the muscle (termed 'myonuclei') have been postulated to be responsible for the governance of a given volume of the myofibrillar cytoplasm (76), in what has been termed the 'myonuclear domain theory' (3).

Hypertrophy, therefore demands the addition of new myonuclei in order to support additional cytoplasm (3, 76). While it appears that minor increases in muscle fibre size do not require significant increases in myonuclei (148), sustained and considerable growth in fibre size require the accretion of myonuclear content (21), derived from SC activity. Early work suggested that mice that had undergone muscle-targeted irradiation (and thus presumably damaged the resident SC) were not capable of hypertrophy following 3 months of synergist ablation, whereas the control non-irradiated mice were able to mount a significant response (1). However, work by McCarthy and colleagues (2011) examined the importance of SC during muscle fibre hypertrophy using a $Pax7^{CreER}$ - diphtheria toxin A **(**DTA) strain of rodents, which allows for the controlled ablation of Pax7 cells through tamoxifen administration. This study demonstrated that there were no differences in the hypertrophic response to two weeks of synergist ablation-induced hypertrophy in animals that underwent SC ablation as compared to wild-type mice. In a follow up study, the same group reported that hypertrophy was not compromised following two weeks using synergist ablation, but was indeed affected following a period of 8 weeks (64) with impaired growth observed in the $Pax7^{CreER}$ –DTA mouse. Taken together, these studies represent the on-going debate regarding the role of SC during the hypertrophic response, as it appears that short term hypertrophy can occur without SC, but long term hypertrophy and/or sustained hypertrophy is reduced in the absence of SC. While elegantly designed, these studies represent 'supra-physiological' conditions, achievable only in the animal model. Therefore, descriptive human studies are required

in order to understand the associations between muscle fibre hypertrophy and changes in SC content and myonuclear number.

In humans, skeletal muscle hypertrophy is achieved through resistance exercise training. In a growing number of studies, it has been observed that an increase in muscle fibre size is associated with an increase in SC content and/or myonuclear accretion following resistance training (60, 96, 195). Interestingly, a strong positive correlation between the increase in SC content and the hypertrophic response to resistance exercise training (i.e., the change in fibre size) is conserved across men and women, regardless of age (11, 115, 148, 197). Furthermore, it appears that muscle hypertrophy is accompanied by an increase in myonuclear content (11, 96, 149), with larger myofibres containing more nuclei than smaller fibres (58, 96, 172).

1.3 AGING MUSCLE

The progressive loss of skeletal muscle mass and strength is a hallmark of aging and is referred to as 'sarcopenia'. The impact of sarcopenia is broad, including impaired functional capacity, loss of independence, increase in incidence of injury and an increase in morbidity (37, 88, 107). Considering that the world's population 60 years and older will triple within ~50 years, and octogenarians are the fastest growing subpopulation in the world (204), sarcopenia represents an immense economic burden in Canada and around the world (180).

The structural changes associated with human aging are extensive, including changes in the size and number of myofibres (109), which leads to a decrease in maximum force production (177, 200). An extensive shift in muscle fibre type

distribution toward a greater percentage of type I fibres as compared to type II fibres occurs with aging (32, 55, 63, 196). Furthermore, the prevalence of denervation in older human skeletal muscle has also been shown to be associated with fibre type grouping of denervated type II myofibres (161, 193). As a consequence of this fibre type grouping, older skeletal muscle may lose the mosaic-like distribution of fibres (161, 193), which has been proposed as a possible mechanism for the loss of functional strength observed in older individuals (98). Furthermore, aged muscle is more susceptible to injury (18, 19) and demonstrates a diminished capacity for muscle repair/regeneration (73, 91) as compared to young muscle. Due to these impairments in the ability to repair and/or remodel skeletal muscle with aging, satellite cells have been implicated as a potential factor contributing to sarcopenia.

1.3.1 **Satellite cells and aging**

In studies assessing muscle CSA via histochemical and/or immunohistochemical methods, there is a general consensus that while type I muscle fibre size remains undiminished with age, there is a loss of type II muscle size (30, 38, 55, 109, 148, 196). Furthermore, human studies have repeatedly observed that the loss of type II muscle fibre size in older muscle occurs concomitant with a reduction in type II muscle fibre SC content (116, 123, 124, 195-199). Work by Brack and colleagues (14) supports the notion that there is a loss of SC content prior to age-related muscle atrophy. Given that SC are the primary source for nuclear addition to existing myofibres, the authors proposed that the loss of SC content and thus the inability to contribute new nuclei leads to an increased domain size that triggers cytoplasmic atrophy (14) in mice. In humans, muscle CSA in

elderly men was positively correlated with both myonuclear content and SC content, supporting the notion that the decline in SC content may play a role in the loss of muscle CSA typically observed in aging (197). However, recent work by Fry and colleagues (65) challenges this notion as they used the $Pax7^{CreER}$ –DTA strain of mice to selectively ablate muscle SC, thus severely impairing regenerative function and/or remodeling ability as compared to wild type mice. Following a two-year aging period, similar muscle loss was observed in both the experimental and wild-type mice, suggesting that the loss of muscle SC typically observed with aging may not be a major contributor to the progression of sarcopenia (65). The translation of animal data can be challenging, as experimental animals are kept in a controlled environment, primarily sedentary. Given that a major contributor to sarcopenia appears to be adverse events (i.e., illness, musculoskeletal injuries, periods of bedrest or even inclement weather) (10, 143, 202), the interpretation of results from animal studies must be done with caution, and validated in humans when possible. While not sarcopenia *per se*, the atrophy or immobilization models have been used in humans in order to more fully understand changes to the muscle with disuse. In a study by Suetta and colleagues (182), single leg immobilization was applied for 2 weeks, followed immediately by 4 weeks of reloading in a cohort of healthy young and old men. Whereas the loss in muscle size and SC content observed with immobilization was recovered in the young men, this was not the case with their older counterparts (182). The authors postulated that a reduction in sensitivity to growth factors controlling muscle size and SC function may explain the impairment in recovery in older men. Collectively, these data suggest that not only are elderly individuals

undergoing a naturally occurring decline in muscle mass, but may also be at risk for singular events that accelerate the loss of muscle mass and function.

The age-related decline in muscle size appears to be related in some capacity to the loss of SC content. However, there also appears to be an age-related loss of muscle SC functionality and/or ability to progress through the myogenic program, which may also contribute to the progression of sarcopenia. The impact of aging on SC function in human skeletal muscle is best demonstrated using a single bout of damaging or non-damaging exercise to stimulate a SC response. Following a single bout of eccentric (55, 201) or resistance exercise (124, 176) the SC response appears to be both delayed and blunted in older as compared to younger men. This impaired response is mainly attributed to the lack of response in type II muscle SC (124, 176). Age related dysfunction in SC activation, driven primarily by the impaired response of type II muscle SC, is supported by experiments examining the cell cycle in both young and older individuals (124). Following a resistance bout of exercise, there was a delayed entry into S phase of the cell cycle in old as compared to young men, with young men exhibiting a significant increase 24h post-exercise while the older men did not realize a significant expansion until 48h post-exercise recovery. There also appears to be a diminished number of SC in S phase at 48h post-exercise in old as compared to the young group. Taken together, these data reinforce the notion that there is a blunted proliferative response in older adults, reflected by both cell cycle kinetics and a lack of MyoD up-regulation in the SC pool.

Furthermore, while older muscle can hypertrophy following prolonged resistance training (195, 197, 199), the response appears to be blunted in older men especially in

type II myofibres (101, 148). Taken together, these studies suggest that the activation and/or expansion of SC following exercise can be mainly attributed to the reduction in function of type II associated SC. The deficiency in function, paired with the loss of type II SC content with aging, may play a role the observed loss of muscle mass with aging. In order to combat sarcopenia, a mechanism that might explain the loss of SC must be elucidated.

1.3.2 **Age-related loss of muscle capillarization**

Muscle capillaries are responsible for the delivery of oxygen, nutrients and growth factors to skeletal muscle. Lined by endothelial cells, capillaries are suited for gas exchange/growth factor delivery due to their thin structure (67, 83, 132). Through arterial stiffening, wall thickening or a decrease in endothelial cells and thus a loss of muscle capillaries (i.e., microvasculature), the circulatory system is adversely affected by aging (169). Importantly, the loss and/or alteration of the peripheral microcirculation plays a role in age-associated organ damage (132). With aging there is reduced blood flow (52) and vascular conductance (53) to the lower limbs at rest. Consistent with this, blood flow and vascular conductance were attenuated in aged individuals as compared to young (53). Furthermore, observations of a blunted microvascular blood flow responses in post-absorptive and post-prandial conditions following protein ingestion have been reported (134, 173). Taken together, these findings suggest that there is a concomitant loss of function with aging, and many groups have identified morphological or content changes in capillarization with aging as well.

The morphological aspects of the capillaries and their relationship to the size and/or tortuosity of the myofibre has been shown to be relevant for the exchange and perfusion of circulating factors (80, 82). Indeed, the quantification of muscle capillarization must be performed accurately to assess changes related to diffusion or perfusion. Early work utilized the analysis of capillary contacts (CC), or the individual capillary-to-fibre ratio (C/F_i), the latter of which includes a sharing factor (SF) calculation in order to determine a fibre-type specific C/F_i (80,82). These measurements can be used to accurately detect the capillarization of a given area, but fibre area-based measurements, such as capillary density (CD) may be a more appropriate marker to estimate the delivery of substrates (59) and/or the removal of waste products (188) from the myofibres via primarily passive diffusion. However, indexes of the capillary-to-fibre surface, such as the capillary-to-fibre perimeter exchange (CFPE) index may provide the most substantial information regarding the capacity of either oxygen or growth factors reliant on receptor-mediated processes at the myofibre membrane. The CFPE is calculated by the equation $CFPE = C/F_i / P$. The inclusion of the myofibre perimeter (P) as related to individual capillary supply to the myofibre is crucial, as P is related to the 3D surface area of the fibre. In contrast, the measurement of fibre area (i.e., CSA) is proportional to the volume of the fibre. Therefore, by using P and not FA, the CFPE index takes into account the capillary-to-fibre surface, and thus can accurately assess microvascular supply to the most relevant area of oxygen flux and/or growth factor receptors (80, 81).

While the measurement of capillarization has not been consistent on a study-to-study basis, it appears that human aging has a deleterious impact on muscle

capillarization (144). Coggan and colleagues (38, 39) observed a ~25% decline in both number and density of microvascular capillaries in older as compared younger women. In a longitudinal study, Frontera and colleagues (63) observed that there was a ~20% decrease in CD in men from the age of 65 to 77. Under closer examination, there also appeared to be a fibre-type specific response in the loss of capillaries with age. Indeed, when analyzing both isoforms of type II myofibres (i.e., IIA, IIB), Proctor and colleagues (1995) observed a significant decline in capillarization in older adults as compared to young individuals. Consistent with this, a loss of type II myofibre capillarization with age have been repeatedly reported (38, 46, 152). The loss of endothelial function, exacerbated by a loss of type II fibre capillarization, may help to explain the 'anabolic resistance' observed in older adults (17, 135, 203) and may also be an underlying factor in the loss of type II CSA observed with aging. Furthermore, this loss of muscle capillarization and/or perfusion may limit the delivery of growth factors and nutrients to the muscle SC niche. While not a model of aging *per se*, there are other clinical populations that highlight the relationship between muscle capillaries and SC. Individuals suffering from amyopathic dermatomyositis show a reduction in muscle capillarization in the absence of myofibrillar damage (57). Interestingly, these individuals also exhibit a loss of SC content proportionate to the loss of muscle capillarization (35). Furthermore, it appears that in areas of the muscle with preserved capillary content, there is also a maintenance of SC content (35). Taken together, these data indicate that SC loss occurs selectively in muscle fibres with a diminished network of capillaries. The loss of capillary content leading to a reduced delivery of SC-regulating growth factors or some

combination of both factors may lead to an altered niche and subsequent loss of functional SC in aging.

1.3.3 **Implications of an altered SC environment with aging**

An age-related reduction in muscle SC content and function has a considerable impact on the preservation of muscle size, as well as the ability for skeletal muscle to repair following injury. There is growing evidence to suggest that the dysregulation of SC during muscle repair may stem from extrinsic signaling within the SC niche. When aged muscle is grafted into a youthful environment, there is an improvement in regenerative capacity (27, 160) ; in contrast, when young muscle is transplanted into aged animals, there is a significant impairment in regeneration (26, 27). In an elegant experiment, Conboy and colleagues (41) demonstrated that when the circulation of an aged mouse is paired with a young counterpart (termed 'heterchonic parabiosis'), there is a rescuing of the regenerative potential in the muscle of the aged mice. These findings are reinforced by cell culture experiments observing that when SC from young mice are exposed to the serum from aged mice, there is a decrease in their myogenic potential (15, 27). The aged environment also appears to impair function in SC derived from young animals, therefore suggesting that the systemic environment may determine the cell regenerative capacity (15, 154). Taken together, these observations suggest that there are systemic factors that facilitate SC function, and that the absence of these factors may be pivotal to the impaired SC response in aged muscle.

While the parabiosis model is a powerful model to study the impact of the systemic environment with aging, it does not take into account the altered delivery of

these circulating cytokines through skeletal muscle that may occur normally with aging. This point becomes particularly relevant when considering that the impact of aging on skeletal muscle capillarization in rodent models is not entirely clear, with some studies reporting a reduction (159), others reporting no change and/or an increase (68). A loss of skeletal muscle capillarization with aging could limit the ability of growth factors and nutrients to reach the SC micro-environment. In the parabiosis model, the exposure of old muscle to circulating growth factors/cytokines of the young animal may be sufficient to improve SC function. However, a reduction of either conduction via the microvasculature or the concentration of the circulating growth factors likely both contribute to impaired SC function under normal aging conditions. Therefore, it is crucial more experimental work must be conducted in integrated human models.

The loss of myofibre capillarization with aging has therefore been seen as an important component for skeletal muscle maintenance and growth. Given that there are a number of cytokines and growth factors in circulation that have been observed to be regulators of SC function, the delivery of these signals to the muscle SC would therefore rely, in part, on sufficient perfusion of the myofibres. Cytokine delivery to the SC niche, paired with the cell-cell cross talk observed between endothelial cells and SC (34, 35) suggest the importance of muscle capillarization to SC function.

The functional relationship between the SC and the muscle microvasculature has not been well elucidated. Furthermore, how this relationship is altered in a population suffering from a reduction in SC content concomitant with a reduction in muscle capillarization, such as aged individuals, remains an important question.

1.4 Content of Thesis

1.4.1 **Purpose of Thesis**

The research experiments contained in the current thesis were designed to investigate the relationship between human skeletal muscle capillaries and resident muscle SC.

i) In Chapter 2, we examined whether the spatial relationship between SC and capillaries were relevant for the activation status of the SC pool following a single bout of resistance exercise. To gain insight whether there were any changes with this relationship with aging, we compared the relationship between SC and the nearest capillary between healthy young and older men.

ii) In Chapter 3, we examined the activation response of the SC pool following a single bout of resistance-type exercise prior to and following 16 weeks of progressive resistance training.

iii) In Chapter 4, we assessed the activation status and expansion of the SC pool following a bout of eccentric contraction-induced muscle damage in a group of healthy young men with a broad scaling of muscle fibre capillarization

1.4.2 **Hypotheses**

i) We hypothesized that following exercise, activated SC would be situated in a closer proximity to the nearest capillary as compared to their quiescent counterparts. We hypothesized that there would be a greater distance between type II associated SC in muscle capillaries in older as compared to younger men

ii) We hypothesized that following long term RT there would be an enhanced SC activation response. We hypothesized that following training, there would be increased markers of capillarization and muscle perfusion that may influence SC activation

iii) We hypothesized that individuals with higher indices of muscle capillarization would exhibit a greater activation and/or expansion of the SC pool in response to exercise-induced muscle injury.

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CHAPTER 2:

SKELETAL MUSCLE SATELLITE CELLS ARE LOCATED AT A CLOSER PROXIMITY TO CAPILLARIES IN HEALTHY YOUNG COMPARED WITH OLDER MEN.

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Skeletal muscle satellite cells are located at a closer proximity to capillaries in healthy young compared with older men

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Abstract

Background Skeletal muscle satellite cells (SC) are instrumental in maintenance of muscle fibres, the adaptive responses to exercise, and there is an age-related decline in SC. A spatial relationship exists between SC and muscle fibre capillaries. In the present study, we aimed to investigate whether chronologic age has an impact on the spatial relationship between SC and muscle fibre capillaries. Secondly, we determined whether this spatial relationship changes in response to a single session of resistance exercise.

Methods Muscle biopsies were obtained from the *vastus lateralis* of previously untrained young men (YM, 24 ± 3 years; $n = 23$) and older men (OM, 67 ± 4 years; $n = 22$) at rest. A subset of YM ($n = 9$) performed a single bout of resistance exercise, where additional muscle biopsies taken at 24 and 72 h post-exercise recovery. Skeletal muscle fibre capillarization, SC content, and activation status were assessed using immunofluorescent microscopy of muscle cross sections.

Results Type II muscle fibre SC and capillary content was significantly lower in the YM compared with OM ($P < 0.05$). Furthermore, type II muscle fibre SC were located at a greater distance from the nearest capillary in OM compared with YM (21.6 ± 1.3 vs. 17.0 ± 0.8 μm, respectively; $P < 0.05$). In response to a single bout of exercise, we observed a significant increase in SC number and activation status ($P < 0.05$). In addition, activated vs. quiescent SC were situated closer ($P < 0.05$) to capillaries.

Conclusions We demonstrate that there is a greater distance between capillaries and type II fibre-associated SC in OM as compared with YM. Furthermore, quiescent SC are located significantly further away from capillaries than active SC after single bout of exercise. Our data have implications for how muscle adapts to exercise and how aging may affect such adaptations.

Keywords Muscle stem cells; Pax7; MyoD; Capillaries; Perfusion

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Introduction

The gradual loss of skeletal muscle mass is a hallmark of aging. The loss of muscle can mainly be attributed to the reduction in type II muscle fibre size[1,2] and is accompanied by a decline in type II muscle fibre satellite cell (SC) number.[2–4] Skeletal muscle SC are known to play an obligatory role in regeneration following injury, and while typically residing in a quiescent state, they can become active in response to a stimulus like exercise and/or mechanical damage. Following activation, SC proliferate and differentiate to supply additional myonuclei or return to a quiescent state again to replenish the resident pool of SC.[5] The addition of new myonuclei to existing muscle fibres represents an essential step in the maintenance and remodelling process of skeletal muscle. Hence, a reduction in SC number and/or function has been hypothesized to play a critical role in the development or progression of muscle fibre atrophy with age.[6–8]

A host of circulating growth factors [e.g. insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), interleukin 6 (IL-6), myostatin] have been hypothesized to be regulators

of SC function.[7–12] The delivery of these systemic signals to the muscle SC would rely in part on their proximity to local microvascular flow. Delivery of these systemic growth factors to the muscle fibre could be a key event in SC recruitment thereby supporting muscle repair and or remodelling/adaptation. Interestingly, a study by Christov *et al.* reported that a spatial association exists between SC and capillaries in skeletal muscle.[13] This study reported that activated and differentiating SC were located closer to a capillary as compared with quiescent SC.[13] Hence, the distance of a SC to its nearest capillary may be an important factor in whether SC become activated in response to stimulation. This observation may be particularly relevant in senescent muscle, as muscle fibre capillarization declines with advanced age,[14] with a specific reduction in type II fibres.[15–18] However, whether the distance between SC and capillaries changes with age remains unknown. Therefore, we assessed the spatial proximity of SC to capillaries in the resting state in both type I and type II muscle fibres in a group of healthy young and older men. To gain insight into the relevance of the SC–capillary distance, we examined whether the spatial relationship between SC and capillaries may be of importance in SC function in response to a single resistance exercise session. We hypothesized that there would be a greater distance between type II-associated SC and capillaries in older compared with younger men. In addition, we hypothesized that following exercise, activated SC would be closer to capillaries than their quiescent counterparts suggesting that SC proximity to circulating factors is important in their mobilization.

Methods

Participants

Twenty-three healthy young men [YM: 24 ± 3 years; mean ± standard error of the mean (SEM)] and 22 older men (OM: 67 ± 4 years) were recruited to participate in this study. All participants were recreationally active with no formal weight training experience in the previous 6 months. Exclusion criteria included smoking, diabetes, the use of non-steroidal anti-inflammatory drugs or statins, and history of respiratory disease and/or any major orthopaedic disability. Participants were informed about the nature and risks of the experimental procedures before their written consent was obtained.

Muscle biopsy sampling

Percutaneous needle biopsies were taken, after an (~10 h) overnight fast, from the mid-portion of the *vastus lateralis* under local anaesthetic using a 5 mm Bergstrom needle adapted for manual suction. Subjects had not participated in any physical activity at least 96 h before the collection of the baseline biopsy (Pre). In a subgroup of YM ($n = 9$), two additional muscle biopsies were taken from the same leg, 24 and 72 h after a single session of resistance exercise. Incisions for the repeated muscle biopsy sampling were spaced by approximately 3 cm to minimize any effect of the previous biopsy. Upon excision, muscle samples were immediately mounted in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C until further analyses.

Exercise protocol

To determine the impact of exercise on SC content and activation status in relation to skeletal muscle fibre capillarization, a subset of YM ($n = 9$) performed a single session of exercise. In short, the YM performed a single session of exercise that consisted of four sets of eight repetitions each at 80% of 1 RM on leg press (Maxam, Hamilton, Ontario), leg extension (Atlantis, Laval, Quebec), calf press, and leg curl (Hur, Kokkola Finland). The final set of each exercise was performed to volitional failure.[19] A resting period of 2 min between sets was allowed. All participants were verbally encouraged during the exercise session to complete the entire protocol. Prior to and following the resistance exercise, a 5 min warm up/cool down was performed on a cycle ergometer. We selected a resistance-type exercise protocol based on previous work that suggests that either concentric and/or eccentric muscle contractions are sufficient to cause an expansion of the SC pool and are well tolerated by participants[20]

Immunofluorescence

Muscle cross sections (7 μm) were prepared from unfixed OCT embedded samples, allowed to air dry for 15–45 min, and stored at −80°C. Samples were stained with antibodies against Pax7, myosin heavy chain type I, laminin, MyoD1, and CD31. For immunofluorescent detection, appropriate secondary antibodies were used. Detailed antibody information is found in Table 1. Nuclei were labelled with 4′,6-diamidino-2-phenylindole (DAPI) (1:20 000, Sigma-Aldrich, Oakville, ON, Canada), prior to cover slipping slides with fluorescent mounting media (DAKO, Burlington, ON, Canada). 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% bovina serum albumin, 5% foetal bovine serum, 0.2% Triton X-100, 0.1% sodium azide, and 5% goat serum (GS). Following blocking, sections were incubated in the primary antibody Pax7 at 4° C overnight. Following washes in PBS (3 × 5 min), sections were then incubated in the appropriate secondary antibodies. Sections were then re-fixed in 4% PFA and re-blocked in

Table 1. Antibody information

Antibody	Species	Source	Clone	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	1:1	Alexa 594, 488 goat anti-mouse 1:500
Anti-laminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488, 647 goat anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 slow isoform	1:1	Alexa Fluor 488 goat anti-mouse, 1:500
Anti-CD31	Rabbit	Abcam	ab28364	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Mouse	Dako	5.8A	1:50	Goat anti-mouse biotinylated secondary antibody, 1:200; streptavidin-594 fluorochrome, 1:250

Detailed information on primary and secondary antibodies and dilutions used for immunofluorescent staining of the frozen muscle cross sections.

a blocking solution containing 0.01% Triton X-100 and 5% GS in PBS. Following blocking, sections were incubated in the primary antibody CD31 at 4°C overnight. Following washes (3 × 5 min in PBS), sections were then incubated in the appropriate secondary antibodies. Sections were then again re-blocked in 10% GS in PBS. Sections were then incubated sequentially in the third primary antibodies, either a primary antibody cocktail (i.e. MHC and laminin) for the fibre-specific SC quantification or MyoD1 for the quantification of activated SC. This was followed by incubation in the appropriate secondary antibody (Table 1). The staining procedures were verified using negative controls, in order to ensure appropriate specificity of 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 Instruments, Melville, NY, USA). Images were captured and analysed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments, Inc., USA). All images were obtained with the 20× objective, and at least ≥200 muscle fibres/subject/time point were included in the analyses for SC content/activation status, fibre cross-sectional area (CSA), and fibre perimeter. Slides were blinded for both group and time point. The quantification of muscle fibre capillaries was performed on 50 muscle fibres/subject/time point.[21] Based on the work of Hepple *et al.*,[22] quantification of (i) capillary contacts (CC, the number of capillaries around a fibre), (ii) the capillary-to-fibre ratio on an individual fibre basis (C/F*i*), (iii) the number of fibres sharing each capillary (i.e. the sharing factor), and (iv) the capillary density (CD). The CD was calculated by using the cross-sectional area (μm^2) as the reference space. The capillary-to-fibre perimeter exchange index (CFPE) was calculated as an estimate of the capillary-to-fibre surface area.[22] The SC-to-capillary distance measurements were performed on all SC that were enclosed by other muscle fibres. The measurement was taken by identifying a SC (i.e. $Pax7^+$ co-localized with DAPI, beneath the basal lamina) and tracing the perimeter of the muscle fibre of which it was associated to, down to the nearest capillary (*Figure* 2A). If two capillaries were situated within visually similar distances, both distances were traced and the lesser of the two was recorded. All areas selected for analysis were free of 'freeze fracture' artefact, and care was taken such that longitudinal fibres were not used in the analysis. Muscle fibres on the periphery of muscle cross sections were not used in the analysis. SC-to-capillary distances were verified by two independent researchers, showing an inter-observer reliability correlation of 0.98.

Statistical analysis

Statistical analysis was performed using Sigma Stat 3.1.0 analysis software (Systat Software, Chicago, IL, USA). A two-way ANOVA was conducted for baseline comparisons between the young and old group in a fibre type-specific manner. For the subset of participants who performed the single session of resistance-type exercise, a two-way repeated measures ANOVA for time (Pre, 24 and 72 h) and SC population ($Pax7^+/MyoD^-$ and $Pax7^+/MyoD^+$ cells) as within-subjects factors. Significant main effects or time × SC population interaction were analysed using Tukey's *post-hoc* test. Statistical significance was accepted at $P < 0.05$. All results were presented as means ± SEM.

Results

Muscle fibre CSA and fibre-type distribution

There was no difference in type I muscle fibre CSA and perimeter observed between YM and OM (Table 2). In type II fibres, muscle fibre CSA and muscle fibre perimeter were significantly lower in OM compared with YM (both $P < 0.05$, Table 2). Muscle fibre-type distribution was not significantly different between YM and OM. The percentage of type II muscle fibres was significantly greater than type I fibres in both groups ($P < 0.05$, Table 2).

Muscle fibre capillarization

No differences in type I muscle fibre CC, C/Fi, CFPE, or CD were observed in resting muscle biopsy samples between YM and OM. In type II muscle fibres, CC, CFPE, and C/F*i* ratio

Table 2. Skeletal muscle fibre characteristics in young and old men

	Fibre type	YM ($n = 22$)	OM ($n = 23$)
Fibre area (μm^2)			
	I	5716 ± 393	5899 ± 265
	II	6677 ± 465*	4940 ± 216*#
Fibre perimeter (μm^2)			
	I	299 ± 10	296 ± 8
	II	334 ± 11*	286 ± 8**
Fibre-type distribution >(fibre %)			
	I	33 ± 3	38 ± 2
	II	67 ± 3*	62 ± 2*

Mean ± SEM.
YM, young men; OM, old men.
*Significant effect of fibre type ($P < 0.05$).
**Significant effect for age ($P < 0.05$).

Table 3. Skeletal muscle capillarization in young and old men

	Fibre type	YM ($n = 23$)	OM ($n = 22$)
Capillary contacts			
	I	3.30 ± 0.24	3.16 ± 0.20
	II	2.27 ± 0.18*	1.90 ± 0.09*
Individual capillary-to-fibre ratio (C/Fi)			
	I	1.76 ± 0.08	1.69 ± 0.08
	II	1.65 ± 0.07*	1.21 ± 0.07***
Capillary density (capillaries × mm^{-2})			
	I	573 ± 28	542 ± 32
	II	352 ± 30*	392 ± 21*
CFPE (capillaries × 1000 μm^{-1})			
	I	5.95 ± 0.24	5.75 ± 0.25
	II	4.96 ± 0.24*	4.16 ± 0.21***

Mean ± SEM.
YM, young men; OM, old men; CFPE, capillary-to-fibre perimeter exchange index.
*Significant effect of fibre type ($P < 0.05$).
**Significant effect for age ($P < 0.05$).

were significantly greater in YM compared with OM (all $P < 0.05$, *Figure* 1C–D, Table 3). In both groups, CC, CD, and CFPE were greater in type I compared with type II muscle fibres (all $P < 0.05$, *Figure* 1C–D, Table 3).

Satellite cell content and distance to nearest capillary

At rest, the number of type I-associated SC was not different in YM (10.6 ± 0.7 $Pax7^+$ cells/100 type I myofibre) compared with OM (10.9 ± 0.9 $Pax7^+$ cells/100 type I myofibre; *Figure* 2F). However, type II muscle fibre SC content was significantly lower in OM compared with YM (7.9 ± 0.7 vs. 11.9 ± 0.7 $Pax7^+$ cells/100 type II myofibres, respectively; $P < 0.05$; *Figure* 2F).

Figure 1 Fibre type-specific staining with muscle capillaries. (A) Representative image of a MHCI/laminin/CD31 stain of a muscle cross section. Single channel views of (B) CD31. (C) Fibre-specific capillary-to-fibre ratio (C/F_i). (D) Fibre-specific capillary-to-fibre perimeter exchange index (CFPE). Values represent means ± SEM; *$P < 0.05$, significant vs. type I; **$P < 0.05$, significant vs. young.

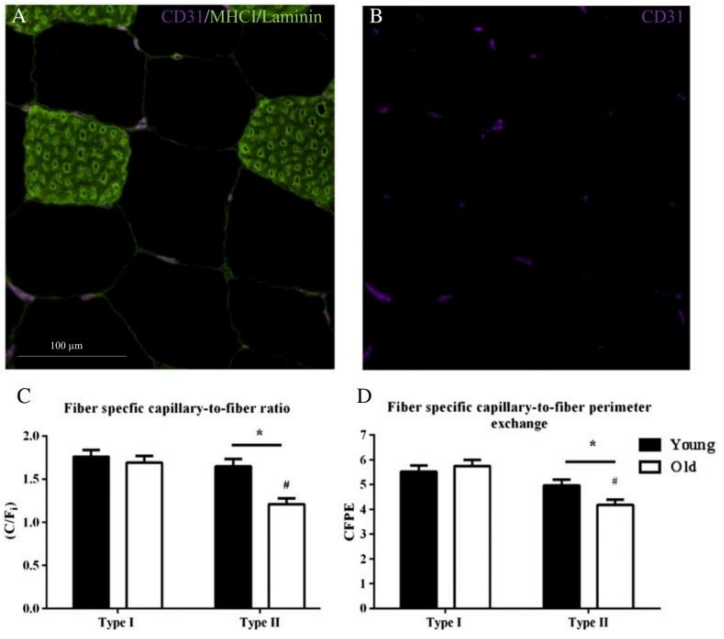
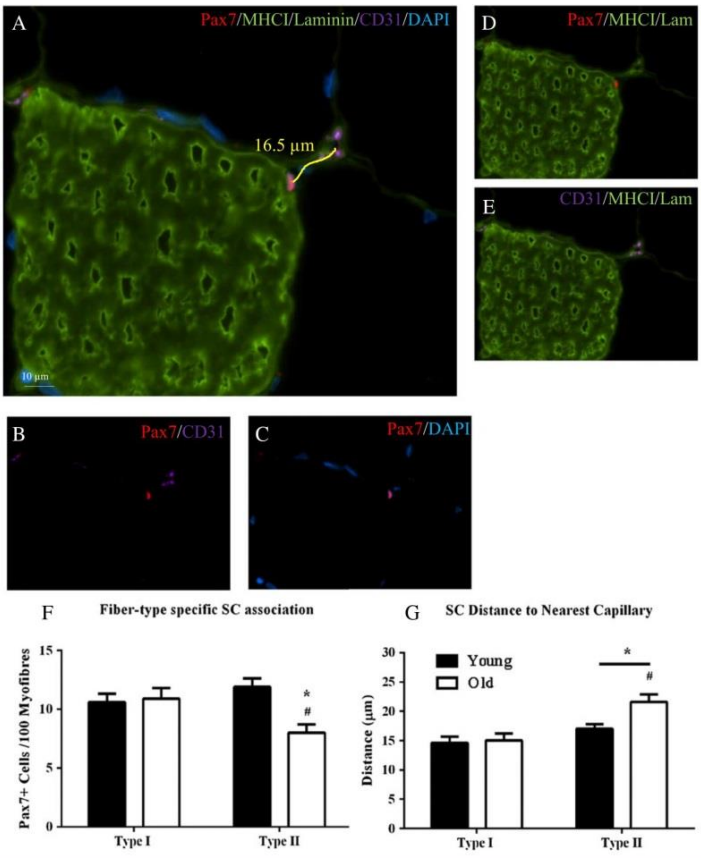


Figure 2 The number of type I and type II satellite cells per muscle fibre. (A) Representative image of a MHCI/laminin/CD31/Pax7 stain of a muscle cross section. Single channel views of (B) Pax7/CD31, (C) Pax7/DAPI, (D) Pax7/MHCI/laminin, and (E) CD31/MHCI/laminin. (F) The number of type I and type II satellite cells per muscle fibre and (G) distance to the nearest capillary. Values represent means ± SEM, $*P < 0.05$, significant vs. type I; $**P < 0.05$, significant vs. young.



In relation to type I-associated SC, there was no difference in distance to nearest capillary between YM and OM (14.6 ± 1.1 vs. 15.0 ± 1.2 µm, respectively; *Figure* 2G). There was a greater distance between type II-associated SC and the nearest capillary in OM as compared with YM (21.6 ± 1.3 vs. 17.0 ± 0.8 µm, respectively, $P < 0.05$; *Figure* 2G).

Satellite cell content, activation status, and distance to nearest capillary in response to exercise

In response to the single session of resistance exercise, total $Pax7^+$ cells/100 fibres were higher at 24 h but did not reach significance until 72 h compared with Pre in YM ($P < 0.05$, Table 4). $Pax7^+/MyoD^-$ cells/100 fibres did not change from Pre to 24 and 72 h after exercise (Table 4). $Pax7^+/MyoD^+$ cells/100 fibres were significantly increased at 24 and 72 h as compared with Pre ($P < 0.05$, Table 4).

$Pax7^+/MyoD^+$ cells were closer to the nearest capillaries compared with $Pax7^+/MyoD^-$ cells both prior to exercise (Pre) and at 24 h post-recovery ($P < 0.05$, Table 4). This difference was abolished at 72 h post-exercise, as there were no differences in measured distance between SC and capillaries that were $Pax7^+/MyoD^-$ or $Pax7^+/MyoD^+$ ($P < 0.05$). $Pax7^+/MyoD^-$ cells were located closer to the nearest capillary at 72 h as compared with 24 h ($P < 0.05$, Table 4).

Table 4. SC activation status after a single bout of exercise in young men

	Cell population	Pre	24 h	72 h
SC (per 100 myofibre)				
	Total Pax7$^+$	9.6 ± 1.0	12.0 ± 0.9	12.6 ± 1.0*
	Pax7$^+$/MyoD$^-$	3.1 ± 0.9	7.7 ± 0.9*	6.3 ± 0.6*
	Pax7$^+$/MyoD$^+$	0.1 ± 0.1	2.4 ± 0.5*	5.3 ± 1.1***
SC distance to capillary (μm)				
	Total Pax7$^+$	16.0 ± 1.6	18.5 ± 2.0	15.6 ± 1.6
	Pax7$^+$/MyoD$^-$	18.3 ± 1.5	21.4 ± 1.6	15.1 ± 1.6**
	Pax7$^+$/MyoD$^+$	13.7 ± 1.5***	15.3 ± 2.0***	16.0 ± 1.3

Mean ± SEM.
SC, satellite cell.
*Significantly different compared with Pre ($P < 0.05$).
**Significantly different compared with 24 h ($P < 0.05$).
***Significantly different compared with Pax7$^+$/MyoD$^-$ within time point ($P < 0.05$).

Discussion

The present study observes a spatial relationship between SC and capillaries in both type I and type II muscle fibres in human skeletal muscle. We report a greater distance between capillaries and type II muscle fibre-associated SC in OM compared with YM. In addition, we report that active SC are situated in closer proximity to capillaries in response to a single session of resistance exercise as compared with quiescent SC.

It has been well documented that the loss of muscle mass with age can mainly be attributed to the reduction in type II muscle fibre size,[1,23] directly impacting physical function in older adults. Likewise, we report significantly smaller type II muscle fibre size and perimeter in OM compared with YM (Table 2). Skeletal muscle SC have been suggested to play an important role in muscle fibre maintenance and remodelling.[24] As such, it is hypothesized that a reduction in SC number and/or function may be a critical factor in the development of type II muscle fibre atrophy with aging.[2,4,6,8] Consistent with previous literature from our own laboratory[8] as well others,[3,25] we report a lower number of type II-associated SC in OM compared with YM. Understanding the sources of impaired SC regulation in aging muscle is of importance in developing intervention strategies to more effectively combat the loss of muscle mass with age.

Adequate muscle fibre perfusion and the consequent delivery of nutrients and growth factors are indispensable for muscle mass maintenance.[26] However, CD has been reported to decline with increasing age, with a reduction specific to the type II muscle fibres.[14,16,18,27] In agreement, we report a ~25% lower C/F*i* ratio for type II fibres in OM compared with YM. Furthermore, type II muscle fibre CFPE index was also markedly lower in OM compared with YM. CFPE is indicative of capillary supply relative to the fibre surface.[28] Therefore, a decrease in the capillary-to-fibre surface area (e.g. a decrease in CFPE) would result in a reduction in diffusional conductance from the capillary lumen to the muscle cell membrane, potentially limiting the delivery of systemic nutrients and/or signalling factors to the muscle fibre. There are numerous circulating growth factors that control activation and expansion of the SC pool (e.g. IGF-1, FGF, MGF, myostatin, IL-6 and HGF).[12] Therefore, a greater distance between the point of delivery and the actual SC may therefore impact SC function.[13,29] Interestingly, Christov *et al.*[13] have shown that a spatial relationship exists between SC and muscle fibre capillaries, with active SC located at a closer proximity compared with quiescent SC. We extend on these findings by showing that the distance between a quiescent SC (Pax7$^+$/MyoD$^-$) and its closest capillary was greater in type II compared with type I muscle fibres in both YM and OM. Interestingly, the distance between type II-associated SC and the nearest capillary was significantly greater in OM as compared with YM. The fibre-type specificity of this observation is in line with previous studies[15–17] showing that aging mainly has an impact on type II muscle fibres. Therefore, the grouping of type II fibres that has been observed in aging[30] may result in considerably less muscle capillarization and/or perfusion and may play a role in the observed greater distance between type II SC and capillaries in OM.

The importance of the spatial relationship between SC and capillaries is also highlighted by observations made in clinical populations. Patients suffering from amyopathic dermatomyositis have a reduction in muscle capillaries without myofibre damage.[31] In these individuals, a proportionate reduction in muscle SC and capillarization in the same muscle has been observed.[13] Importantly, in areas of the muscle cross section where capillarization is preserved, there is maintenance of SC quantity.[13] Taken together, the observations that individuals presenting with amyopathic dermatomyositis undergo specific SC loss, occurring selectively in muscle fibres with a reduced number of supporting capillaries, are important. Therefore, we propose that the greater distance between type II muscle fibre SC and capillaries in older adults is an important factor underlying the impaired SC response to acute exercise[8] and loss in type II muscle fibre size observed in aging muscle.

In YM, a robust increase in SC number and activation status is typically observed in response to single session of resistance-type exercise.[6,19,32] MyoD is a primary myogenic

regulatory factor known to be expressed during SC proliferation and during the transition between SC proliferation and differentiation.[33] We observed that activated SC ($MyoD^+$/$Pax7^+$) were more closely situated to capillaries compared with quiescent SC prior to (13.7 ± 1.5 vs. 18.3 ± 1.5 μm, respectively) and 24 h (15.3 ± 2.0 vs. 21.4 ± 1.6 μm, respectively) following the single session of resistance exercise. However, the distance between quiescent SC and the nearest capillary was markedly reduced between 24 and 72 h of post-exercise recovery. We speculate that this may be because of a greater exposure to circulating growth factors (as delivered by capillaries), which may cause a more rapid activation of muscle SC closer to capillaries while those with reduced exposure to growth factors remain quiescent. Alternatively, SC have been reported to have extensive migratory behaviour.[34,35] As such, SC that are located near capillaries and situated close to the site requiring repair or remodelling may be activated quickly, and the reduction in SC-to-capillary distance observed 72 h following exercise may be reflective of muscle SC migration.

The results from the present study clearly indicate that the spatial relationship between SC and muscle fibre capillaries may be important in overall SC function. Previously, we have shown that the increase in type II muscle fibre SC content is delayed in response to a single session of exercise in older adults and is accompanied by a blunted SC activation response.[6,8] This attenuated response may play an important role in the reduced capacity of senescent muscle to increase muscle fibre size and/or mass with prolonged exercise training.[36,37] Whether an increase in muscle fibre capillarization may optimize SC function during post-exercise recovery, and thus augment the muscle adaptive response to prolonged exercise training in older adults, remains to be established.

We conclude that a spatial relationship exists between SC activation status and capillaries at rest as well as in response to a single session of resistance exercise. The greater distance between type II muscle fibre-associated SC and capillaries observed in OM may be a critical factor in the impaired regulation of the SC pool in senescent muscle.

Acknowledgements

The study was approved by the Hamilton Health Sciences Integrated Research Ethics Board and conformed to the guidelines outlined in the Declaration of Helsinki. Participants gave their informed written consent prior to their inclusion to the study. The authors certify that they comply with the ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2015.[38]

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Conflict of interest

None declared

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CHAPTER 3:

ALTERED MUSCLE SATELLITE CELL ACTIVATION FOLLOWING 16 WK OF RESISTANCE TRAINING IN YOUNG MEN

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RESEARCH ARTICLE | *Physical Activity and Inactivity*

Altered muscle satellite cell activation following 16 wk of resistance training in young men

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Nederveen JP, Snijders T, Joanisse S, Wavell CG, Mitchell CJ, Johnston LM, Baker SK, Phillips SM, Parise G. Altered muscle satellite cell activation following 16 wk of resistance training in young men. *Am J Physiol Regul Integr Comp Physiol* 312: R85–R92, 2017. First published November 9, 2016; doi: 10.1152/ajpregu.00221.2016.—Skeletal muscle satellite cells (SC) play an important role in muscle adaptation. In untrained individuals, SC content and activation status have been observed to increase in response to a single bout of exercise. Muscle fiber characteristics change considerably when resistance exercise is performed chronically, but whether training status affects the activity of SC in response to a single bout of exercise remains unknown. We examined the changes in SC content and activation status following a single bout of resistance exercise, before and following a 16-wk progressive resistance training (RT) program in 14 young (25 ± 3 yr) men. Before and after RT, percutaneous biopsies from the vastus lateralis muscle were taken before a single bout of resistance exercise and after 24 and 72 h of postexercise recovery. Muscle fiber size, capillarization, and SC response were determined by immunohistochemistry. Following RT, there was a greater activation of SC after 24 h in response to a single bout of resistance exercise (Pre, 1.4 ± 0.3; 24 h, 3.1 ± 0.3 $Pax7^+$/$MyoD^+$ cells per 100 fibers) compared with before RT (Pre, 1.4 ± 0.3; 24 h, 2.2 ± 0.3 $Pax7^+$/$MyoD^+$ cells per 100 fibers, $P < 0.05$); no difference was observed 72 h postexercise. Following 16 wk of RT, MyoD mRNA expression increased from basal to 24 h after the single bout of exercise ($P < 0.05$); this change was not observed before training. Individual capillary-to-fiber ratio (C/F*i*) increased in both type I (1.8 ± 0.3 to 2.0 ± 0.3 C/F*i*, $P < 0.05$) and type II (1.7 ± 0.3 to 2.2 ± 0.3 C/F*i*, $P < 0.05$) fibers in response to RT. After RT, enhanced activation of SC in response to resistance exercise is accompanied by increases in muscle fiber capillarization.

muscle stem cells; Pax7; MyoD; capillaries; perfusion

THE ACTIVATION, proliferation, and/or differentiation of satellite cells (SC) are important events in postexercise recovery leading to muscle fiber adaptation, remodeling, and repair. After a single bout of damage (21, 22) or resistance exercise (37) in humans, expansion of the SC pool is observed by 24 h, peaking at 72 h postexercise (36). Irrespective of the model employed, these aforementioned studies (21, 22, 37) were primarily performed on exercise-naive participants. Presumably then, the typically observed increase in SC content may be a result of general stress rather than a refined adaptive response to an exercise bout. It is well established that repeated bouts of exercise result in markedly reduced indexes of muscle damage and stress following subsequent bouts (20). Similarly, exercise-trained individuals typically demonstrate an attenuated damage or stress response to a habitual exercise challenge (28, 29, 44), suggesting that adaptation has occurred. However, whether the acute SC response following a single bout of exercise is altered in exercise-trained individuals (i.e., individuals who are accustomed to the exercise stimulus) compared with exercise-naive individuals following a single exercise session remains unknown. Consequently, comparing the change in SC content in the untrained and trained state following a single bout of exercise can provide insight to the nature of adaptation.

The progression of SC through the myogenic program is orchestrated by a transcriptional network collectively known as the myogenic regulatory factors (i.e., MyoD, Myf5, Myogenin and MRF4). There is relatively little known regarding adaptation in the myogenic program following exercise-training. In addition, various regulatory factors such as hepatocyte growth factor (HGF), interleukin 6 (IL-6), myostatin, insulin-like growth factor-1 (IGF-1) have been shown to be key regulators in the process of activation, proliferation and/or differentiation (21–23, 26). Some of these factors are produced locally by skeletal muscle (27, 39). As an 'endocrine organ', skeletal muscle tissue produces and releases various cytokines that act in a paracrine, autocrine, or endocrine fashion (27). Consistent with this notion, it has been shown that the systemic environment plays a critical role in SC function (3, 9). Although regulatory signals may originate locally, they may also be derived from other organs and the broader circulatory system (42). Therefore, it has been hypothesized that muscle fiber capillarization may play an important role in the regulation of SC (5).

In healthy young men, RT is sufficient to promote capillarization (11). The increase in capillary number, induced by training, likely reflects the necessity to match the demand for oxygen (15) and nutrients (6, 7) to support growing/adapting muscle fibers. Furthermore, the increase in capillary number is larger as compared with the increase in muscle fiber size, leading to a greater number of capillaries per area muscle, which suggests a more efficient perfusion of the muscle fiber following prolonged resistance exercise training (14). Whether

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increased muscle fiber capillarization influences SC regulation in healthy young adults remains unknown.

We assessed the activation of the SC pool in response to a single bout of resistance exercise in a group of healthy young men before (untrained state response; UTSR) and following (trained state response; TSR) 16 wk of resistance training (RT). We hypothesized that, following RT there would be an augmented activation of muscle SC in response to a single bout of resistance exercise and that this would be associated with enhanced muscle fiber perfusion.

METHODS

Participants

Fourteen healthy young men (YM: 25 ± 3 yr; mean ± SE) were recruited to participate in this study. All participants were recreationally active with no formal weight training experience in the previous 6 mo. The participants in this study were a subset of a larger project investigating the adaptation of skeletal muscle tissue to prolonged resistance exercise training in healthy young men and included data relating to fiber cross-sectional area, strength changes with training, and expansion of the quiescent satellite cell pool (1, 24). The participant selection for the present study was based upon the availability of tissue for all time points for which to perform immunohistochemical analysis. Exclusion criteria included smoking, diabetes, the use of nonsteroidal anti-inflammatory drugs (NSAIDs), and/or statins, and history of respiratory disease and/or any major orthopedic disability. The study was approved by the Hamilton Health Sciences Integrated Research Ethics Board and conformed to the guidelines outlined in the Declaration of Helsinki. Participants gave their informed written consent before their inclusion to the study.

Muscle Biopsy Sampling

Percutaneous needle biopsies were taken after an (~10 h) overnight fast, from the midportion of the vastus lateralis under local anesthetic using a 5-mm Bergstrom needle adapted for manual suction (2). Subjects had not participated in any physical activity for at least 96 h before the biopsy collection before the bout of resistance exercise in the untrained condition (i.e., before resistance training) and the trained condition (i.e., following resistance training). The muscle biopsy procedure was repeated under the same fasted condition (~10 h) 24 h and 72 h following the single bout of resistance exercise detailed below. Incisions for the repeated muscle biopsy sampling were spaced ~3 cm apart to minimize any effect of the previous biopsy. Upon excision, muscle samples were immediately mounted in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C until further analyses.

Exercise Training

Exercise training was performed four times per week, divided into two upper and two lower body sessions under strict supervision as described previously (24). The lower body session consisted of five exercises: leg press, leg extension, leg curl, calf press, and plank exercise. The upper body session consisted of six exercises: chest press, shoulder press, lat pull down, row, biceps curl and triceps extension. Training progressed from two sets performed at 70% of 1 repetition maximum (RM) to four sets performed at 85% of 1RM, with the final set performed to the point of momentary muscle exhaustion. At the conclusion of each workout, and on the mornings of non-training days, participants consumed a beverage containing 30 g of whey protein, 25.9 g of carbohydrates and 3.4 g of fat (Musashi p30, Notting Hill Victoria, Australia).

Single Bout of Resistance Exercise

To determine the impact of resistance exercise on SC content and activation status in relation to RT, participants performed a single bout of resistance exercise both before and following 16 wk of RT. In short, the participants completed four sets of eight repetitions each at 80% of 1RM on leg press (Maxam, Hamilton, Ontario), leg extension (Atlantis, Laval, Quebec), calf press and leg curl (Hur, Kokkola Finland). The single bout of exercise was performed at the same relative intensity both before and following RT. The final set of each exercise was performed to volitional failure (1). A resting period of 2 min between sets was allowed. All participants were verbally encouraged during the exercise session to complete the entire protocol. Prior to and following the resistance exercise, a 5 min warm up was performed on a cycle ergometer.

Immunofluorescence

Muscle cross sections (7 μm) were prepared from unfixed OCT-embedded samples, allowed to air dry for 30 min and stored at −80°C. Samples were stained with antibodies against appropriate primary and secondary antibodies, found in Table 1, as previously described (25). Nuclei were labeled with DAPI (4′,6-diamidino-2-phenylindole) (1:20,000, Sigma-Aldrich, Oakville, ON, Canada), before being coverslipped with fluorescent mounting media (DAKO, Burlington, ON, Canada). The staining procedures were verified using negative controls, to ensure appropriate specificity of staining. Slides were viewed with the Nikon Eclipse *Ti* Microscope (Nikon Instruments), equipped with a high-resolution Photometrics CoolSNAP HQ2 fluorescent camera (Nikon Instruments, Melville, NY). Images were captured and analyzed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments). All images were obtained with the ×20 objective, and ≥200 muscle fibers per subject per time point were included in the analyses for SC content/activation status (i.e., $Pax7^+$/$MyoD^+$ or $Pax7^+$/$MyoD^-$), and fiber cross-sectional area (CSA), and perimeter. The activation status of SCs was determined via the colocalization of $Pax7^+$ and DAPI ($Pax7^+$/$MyoD^-$) and/or the colocalization of Pax7, MyoD and DAPI (i.e., $Pax7^+$/$MyoD^+$). Slides were blinded for both group and time point. The quantification of muscle fiber capillaries was performed on 50 muscle fibers per subject per time point (30). Based on the work of Hepple et al. (15), quantification of *1*) capillary contacts (CC; the number of capillaries around a fiber), *2*) the capillary-to-fiber ratio on an individual fiber basis (C/F*i*), *3*) the number of fibers sharing each capillary (i.e., the sharing factor), and *4*) the capillary density (CD) was performed. The CD was calculated by using the cross-sectional area (μm^2) as the reference space. The capillary-to-fiber perimeter exchange index (CFPE) was calculated as an estimate of the capillary-to-fiber surface

Table 1. *Antibody information*

Antibody	Species	Source	Clone	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	1:1	Alexa 594, 488 goat-anti mouse 1:500
Anti-laminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488, 647 goat anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 Slow isoform	1:1	Alexa Fluor 488 goat anti-mouse, 1:500
Anti-CD31	Rabbit	Abcam	ab28364	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Mouse	Dako	5.8A	1:50	goat anti-mouse biotinylated secondary antibody, 1:200; streptavidin-594 fluorochrome, 1:250

Detailed information on primary and secondary antibodies and dilutions used for immunofluorescent staining of the frozen muscle cross sections.

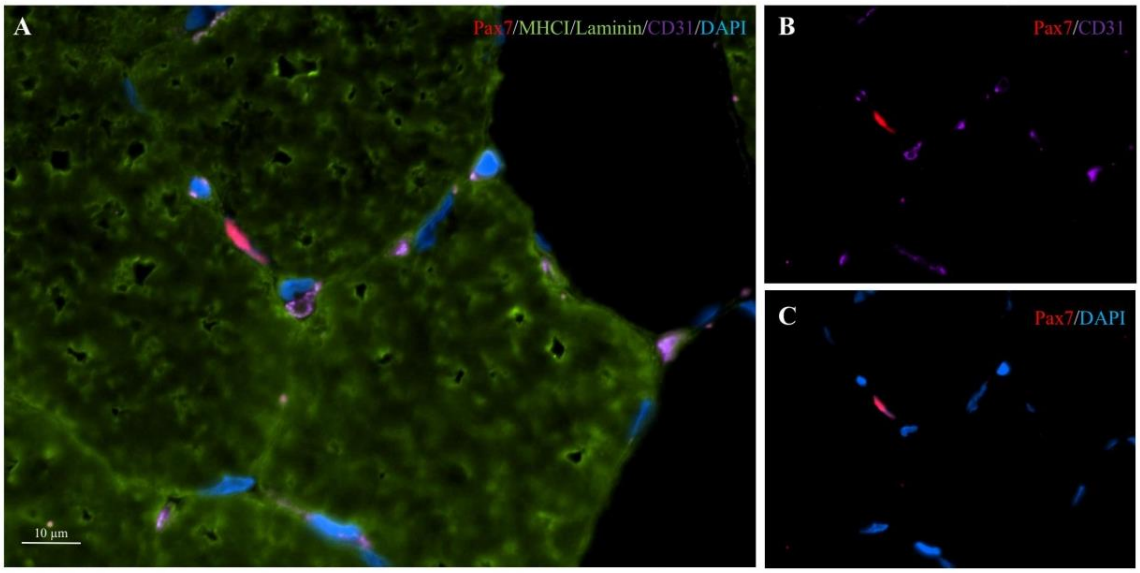

Fig. 1. Fiber type-specific staining with muscle capillaries. *A*: representative image of a MHCI/laminin/CD31/Pax7/DAPI stain of a muscle cross section. Channel views of CD31/Pax7 (*B*) and Pax7/DAPI (*C*).

area (15). The SC-to-capillary distance measurements were performed on all SC that were enclosed by other muscle fibers, and has been described previously as well as in Fig. 1 (25). All immunofluorescent analysis were completed in a blinded fashion.

RNA Isolation

RNA was isolated from 15 to 25 mg of muscle using the TRIzol/RNeasy method. All samples were homogenized with 1 ml of TRIzol Reagent (Life Technologies, Burlington, ON, Canada), in Lysing Maxtrix D tubes (MP Biomedicals, Solon, OH), with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) for a duration of 40 s at a setting of 6 m/s. After a 5-min room temperature incubation, homogenized samples were stored at −80°C for 1 mo until further processing. After thawing on ice was completed, 200 ml of chloroform (Sigma-Aldrich, Oakville, ON, Canada) were added to each sample, mixed vigorously for 15 s, incubated at RT for 5 min, and spun at 12,000 *g* for 10 min at 4°C. The RNA (aqueous) phase was purified using the E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA) as per manufacturer's instructions. RNA concentration (ng/ml) and purity (260/280) were determined with the Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD). RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Toronto, ON, Canada). Samples were reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in 20-μl reaction volumes, as per manufacturer's instructions, using an Eppendorf Mastercycler epGradient Thermal Cycler (Eppendorf, Mississauga, ON, Canada) to obtain cDNA for gene expression analysis.

Table 2. *Primer sequences for quantitative real-time PCR*

Gene Name	Forward Sequence (5′→3′)	Reverse Sequence (5′→3′)
Myf5	ATGGACGTGATGGATGGCTG	GCGGCACAAACTCGTCCCCAA
MyoD	GGTCCCTCGCGCCCAAAAGAT	CAGTTCTCCCGCCTCTCCTAC
MRF4	CCCCTTCAGCTACAGACCCAA	CCCCCTGGAATGATCGGAAAC
β-2-*m*	ATGAG TATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG

MyoD, myogenic determination factor; *Myf5*, myogenic factor-5; *MRF4*, myogenic regulatory factor-4; *β-2-m*, β-2-microglobulin.

Quantitative Real-Time RT-PCR

All QPCR reactions were run in duplicate in 25-μl volumes containing RT Sybr Green qPCR Master Mix (Qiagen Sciences, Valencia, CA), prepared with the epMotion 5075 Eppendorf automated pipetting system (Eppendorf), and carried out using an Eppendorf Realplex2 Master Cycler epgradient (Eppendorf). Primers are listed in Table 2 and were resuspended in 1× TE buffer (10 mM Tris·HCl and 0.11 mM EDTA) and stored at −20°C before use. mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, and fold changes from baseline were calculated using the $\Delta\Delta C_t$ method (18). Gene expression was normalized to the housekeeping gene β-2-microglobulin (β2M). Expression of β2M did not differ between time points.

Statistical Analysis

Statistical analysis was performed using Sigma Stat 3.1.0 analysis software (Systat Software, Chicago, IL). To assess the long-term changes in muscle fiber characteristics in response to 16 wk of RT, two way ANOVA was performed with time (pre- and postexercise training) and fiber type (type I and II) as within-subject factors, and

Table 3. *Skeletal muscle fiber characteristics before and after 16 wk of resistance exercise training in young men*

	Fiber Type	Pre	Post
Fiber area, μm^2	I	5,621 ± 409	6,263 ± 413#
	II	5,771 ± 381*	7,725 ± 519*#
Fiber perimeter, μm^2	I	294 ± 9	309 ± 11#
	II	319 ± 10*	359 ± 18*#
Fiber type distribution, fiber %	I	33 ± 3	38 ± 2
	II	67 ± 3*	62 ± 2*

Values are means ± SE. *Significant difference between fiber types ($P < 0.05$); #significant effect of exercise training ($P < 0.05$).

Table 4. *Skeletal muscle fiber capillarization characteristics before and following 16 wk of resistance exercise training in young men*

	Fiber Type	Pre	Post
Capillary contacts	I	3.18 ± 0.17	3.78 ± 0.22
	II	2.12 ± 0.16*	2.95 ± 0.21*
Individual capillary-to-fiber ratio (C/F*i*)	I	1.71 ± 0.08	1.94 ± 0.03#
	II	1.64 ± 0.09	2.07 ± 0.09#
Capillary density, capillaries/mm^2	I	586 ± 32	640 ± 54
	II	383 ± 34*	400 ± 33*
CFPE, capillaries/1,000 μm	I	5.89 ± 0.21	6.45 ± 0.22#
	II	5.07 ± 0.19*	5.95 ± 0.18*#

Values are means ± SE. CFPE, capillary to fiber perimeter exchange index. *Significantly different compared with type I muscle fibers ($P < 0.05$); #significant effect for exercise training ($P < 0.05$).

appropriate post hoc analysis was performed if interactions were detected. Separate one-way repeated-measures ANOVA, with time (Pre, 24 h, and 72 h) as a within factor, were performed to assess the following: the acute change in satellite cell activity status (i.e., Pax7$^+$/MyoD$^-$ and/or Pax7$^+$/MyoD$^+$ cells); the acute change in distance of activated SC to nearest capillary following a single bout of resistance type exercise; the acute change in MRF mRNA expression, before and following 16 wk of RT. In the one-way repeated-measures ANOVA design for the acute SC response, postexercise time points were only compared with baseline, and Bonferonni corrections were applied to account for multiple comparisons. In addition, to assess the difference in the acute SC response before and following 16 wk of exercise training, a paired sample Student's *t*-test was utilized to compare the change in SC content and activation status (Pre vs. 24 h, and Pre vs. 72 h), before and following 16 wk of RT. Statistical significance was accepted at $P < 0.05$. All results were presented as means ± SE.

RESULTS

Muscle Fiber CSA and Fiber-Type Distribution

Muscle fiber CSA was significantly greater in type II compared with type I, both before and after RT ($P < 0.05$, Table 3). We previously reported a significant increase in muscle fiber CSA in a larger cohort (1). Analysis of this subset of subjects resulted in similar statistically significant changes to those observed in the larger cohort previously reported (1). The percentage of type II muscle fibers was significantly greater than type I fibers ($P < 0.05$, Table 3); muscle fiber type distribution did not change with RT. After 16 wk of RT, there was a significant increase in both type I and type II muscle fiber CSA and perimeter ($P < 0.05$, Table 3). Furthermore, after16 wk of RT, type II muscle fiber CSA was greater than type I ($P < 0.05$, Table 3).

Table 5. *Fiber type-associated SC content and distance to nearest capillary before and after 16 wk of resistance exercise training in young men*

	Fiber Type	Pre	Post
SC, Pax7$^+$ cells/100 myofibers	I	10.9 ± 0.8	13.4 ± 0.6#
	II	11.9 ± 0.8*	15.6 ± 0.9*#
SC distance to capillary, μm	I	15.2 ± 1.0	13.9 ± 0.7
	II	16.8 ± 0.7*	15.9 ± 0.9*

Values are means ± SE. SC: satellite cell. *Significant effect of fiber type ($P < 0.05$); #significant effect for exercise training ($P < 0.05$).

Muscle Fiber Capillarization

There was greater CC (the number of capillaries around a fiber), C/F*i* ratio (capillary-to-fiber ratio), CFPE (capillary-to-fiber perimeter exchange index), and CD (capillary density) in type I compared with type II muscle fibers ($P < 0.05$, Table 4). In both type I and type II muscle fibers, CFPE and C/F*i* was

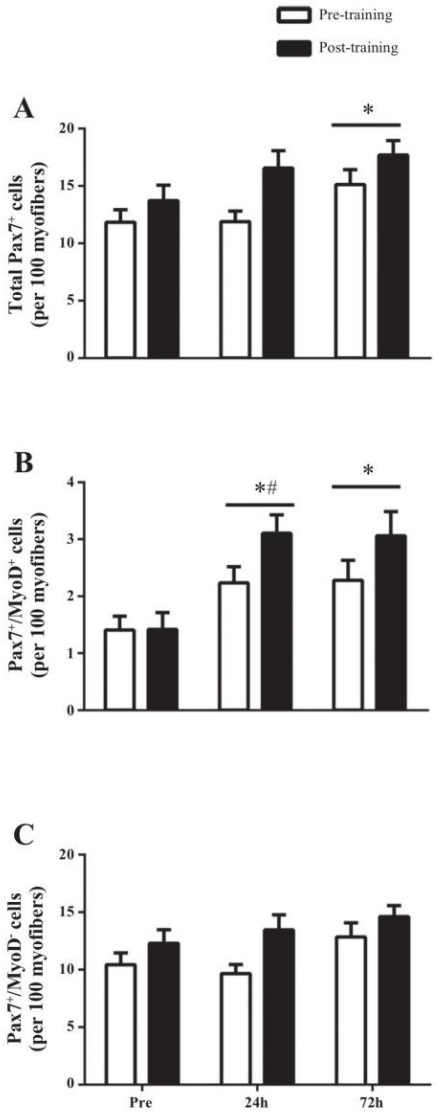

Fig. 2. Characterization of the activity status of satellite cell (SC) after a single bout of resistance exercise before (UTSR; open bars) and after 16 wk of RT (TSR; filled bars). Quantification of these cell populations as total number of Pax7$^+$ SC (*A*), number of MyoD$^+$/Pax7$^+$ (active SC; *B*), number of MyoD$^-$/Pax7$^+$ (quiescent SC; *C*) per 100 myofiber before, 24 h, and 72 h postexercise recovery. *Time effect vs. Pre ($P < 0.05$); bar indicates that effect of time is present for both before and after 16 wk of RT. #Significantly greater ($P < 0.05$) increase with time TSR vs. UTSR. Values are means ± SE.

significantly greater following RT (all $P < 0.05$, Table 4). In contrast, no differences in type I and type II muscle fiber CC and CD were observed with RT.

Fiber Type-Specific Satellite Cell Content and Distance to Nearest Capillary

In resting muscle, SC content was greater in type II than type I muscle fibers ($P < 0.05$, Table 5) both before and after RT, as previously reported (1). Type II-associated SC were located at a greater distance to their nearest capillary as compared with type I-associated SC ($P < 0.05$, Table 5) both before and after RT. Both the number of type I- and type II-associated SC increased after RT ($P < 0.05$, Table 5). There was no change in distance to the nearest capillary from either type I- or type II-associated SC after 16 wk RT (Table 5).

Satellite Cell Content and Activation Status in Response to an Acute Bout of Exercise

UTSR. Response to a single bout of exercise resulted in total $Pax7^+$ cells per 100 myofibers remaining unchanged at 24 h (11.9 ± 0.9 cells/100 myofibers) but increased significantly at 72 h (15.2 ± 1.3 cells/100 myofibers) compared with Pre (11.8 ± 1.1 cells/100 myofibers) ($P < 0.05$, Fig. 2*A*). $Pax7^+/MyoD^+$ cells per 100 myofibers were significantly higher at 24 h (2.2 ± 0.3 cells/100 myofibers) and 72 h (2.3 ± 0.4 cells/100 myofibers) after the single bout of exercise as compared with Pre (1.4 ± 0.3 cells/100 myofibers) ($P < 0.05$, Fig. 2*B*). $Pax7^+/MyoD^-$ cells per 100 myofibers did not change from Pre (10.4 ± 1.0 cells/100 myofibers) to 24 h (9.7 ± 0.8 cells/100 myofibers), but was trending toward significance at 72 h (12.9 ± 1.2 cells/100 myofibers) after the single bout of exercise ($P = 0.06$, Fig. 2*C*).

TSR. In response to a single bout of resistance exercise of the same relative intensity following 16 wk of RT, total $Pax7^+$ cells/100 myofibers was unchanged 24 h (16.6 ± 1.5 cells/100 myofibers) and increased significantly at 72 h (17.7 ± 1.3 cells/100 myofibers) compared with Pre (13.7 ± 1.4 cells/100 myofibers) ($P < 0.05$, Fig. 2*A*). $Pax7^+/MyoD^+$ cells per 100 myofibers were significantly increased at 24 h (3.1 ± 0.2 cells/100 myofibers) and 72 h (3.1 ± 0.4 cells/100 myofibers) after the single bout of exercise compared with Pre (1.4 ± 0.4 cells/100 myofibers) ($P < 0.05$, Fig. 2*B*). $Pax7^+/MyoD^-$ cells per 100 myofibers were unchanged from Pre (12.3 ± 1.2 cells/100 myofibers) to 24 h (13.5 ± 1.3 cells/100 myofibers), but was trending toward significance at 72 h (14.6 ± 1.0 cells/100 myofibers) after the single bout of exercise ($P = 0.08$, Fig. 2*C*).

UTSR vs. TSR. In comparing the UTSR and TSR responses we discovered that there was a greater change in the number of $Pax7^+/MyoD^+$ cells from Pre to 24 h postexercise recovery compared with UTSR (Fig. 2*B*).

Distance of SC to Nearest Capillary in Response to an Acute Bout of Resistance Exercise

UTSR. $Pax7^+/MyoD^+$ cells were closer to their nearest capillary compared with $Pax7^+/MyoD^-$ cells both before the single bout of exercise (Pre) and at 24 h postexercise recovery ($P < 0.05$, Fig. 3*A*). There were no difference in distance to the nearest capillary from SC that were $Pax7^+/MyoD^-$ or $Pax7^+/MyoD^+$ ($P > 0.05$, Fig. 3*A*) at 72 h postexercise. Before resistance training, there was no difference in the distance of $Pax7^+/MyoD^+$ or $Pax7^+/MyoD^-$ cells to the nearest capillary 24 h or 72 h following a single bout of exercise in comparison to the Pre distance.

TSR. $Pax7^+/MyoD^+$ cells were located closer to the nearest capillary compared with $Pax7^+/MyoD^-$ cells before the single bout of exercise ($P < 0.05$, Fig. 3*B*). However, at 24 h postexercise recovery, the difference in distance between SC and its nearest capillary was abolished, such that there was no difference between the two SC populations (Fig. 3*B*). At 72 h, there was a reestablishment of the relationship observed at the Pre time point, such that $Pax7^+/MyoD^+$ cells were again located closer to their nearest capillary compared with $Pax7^+/MyoD^-$ cells ($P < 0.05$, Fig. 3*B*). After 16 wk resistance training, there was no difference in the distance of $Pax7^+/MyoD^+$ or $Pax7^+/MyoD^-$ cells to the nearest capillary 24 or 72 h after a single bout of exercise compared with baseline measurements.

MRF Genes in Response to an Acute Bout of Resistance Exercise

UTSR. In response to a single bout of exercise, MyoD mRNA expression did not increase from basal levels at 24 h (1.1-fold change) or 72 h postexercise recovery (1.8-fold change), compared with Pre (Fig. 4*A*). MRF4 mRNA expression did not significantly increase from basal expression at 24 h (1.2-fold change) or at 72 h postexercise recovery (1.3-fold change) (Fig. 4*B*). Myf5 mRNA expression did not significantly increase from basal expression at 24 h (1.4-fold change) or at 72 h postexercise recovery (1.1-fold change) (Fig. 4*C*).

TSR. After 6 wk of RT, a single bout of exercise resulted in MyoD mRNA expression increased 1.4-fold from basal levels

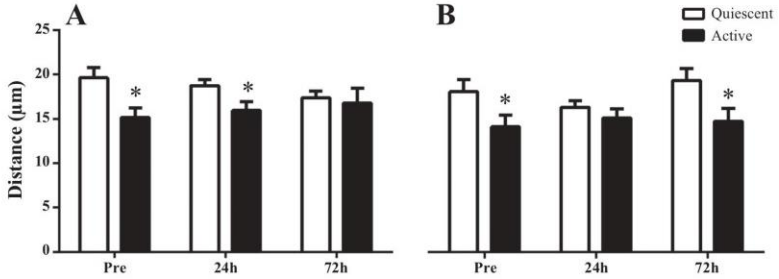

Fig. 3. Distance between activated ($MyoD^+/Pax7^+$) and quiescent ($MyoD^-/Pax7^+$) SC to nearest capillary following a single bout of exercise before compared with after 16 wk of RT. Response to resistance exercise before 16 wk RT exercise (UTSR; *A*) and after (TSR; *B*). *Significantly different compared with active SC within time point ($P < 0.05$). Values are means ± SE.

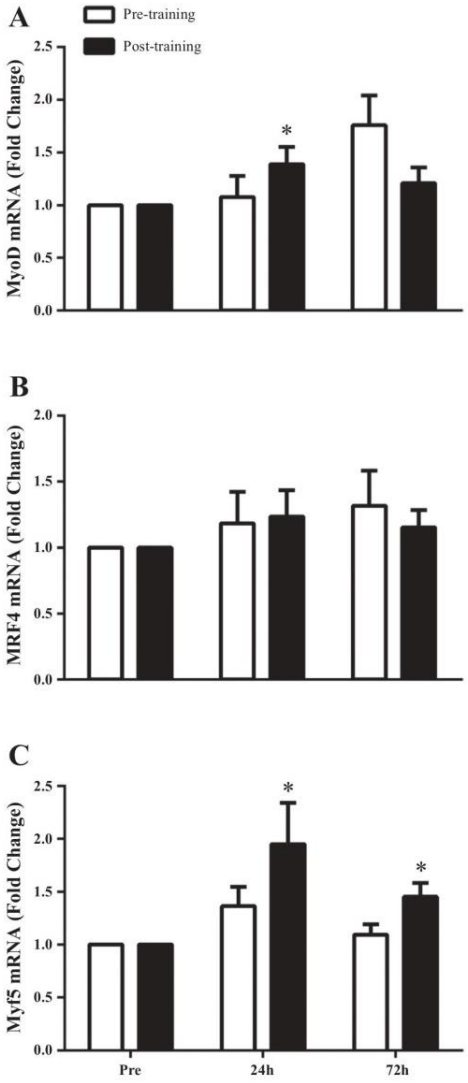

Fig. 4. Relative expression of MyoD mRNA (*A*), MRF4 mRNA (*B*), and Myf5 mRNA (*C*) expression in response to a single bout of exercise before (UTSR; open bars) compared with after 16 wk of RT (TSR; filled bars) expressed as fold change from Pre. Data are normalized to β-2-microglobulin. *Significantly different compared with Pre ($P < 0.05$). Values are means ± SE.

at 24 h postexercise recovery ($P < 0.05$, Fig. 4*A*). However, MyoD mRNA expression was no longer increased 72 h postexercise recovery compared with Pre (1.2-fold change) ($P > 0.05$, Fig. 4*A*). Myf5 mRNA expression was increased at both 24 h (2.0-fold) and 72 h (1.5-fold) postexercise compared with Pre ($P < 0.05$, Fig. 4*C*). MRF4 mRNA expression did not significantly increase from basal levels at 24 h (1.2-fold change) or at 72 h postexercise (1.2-fold change).

DISCUSSION

In the present study we observed an altered activation of the SC pool in response to a single bout of exercise after 16 wk of RT. We speculate that increased capillarization as a result of 16 wk of exercise training may be an important factor for enhancing SC activation in the postexercise period.

Activation, proliferation, and/or differentiation of SC are important events in the postexercise recovery period to support muscle fiber adaptation. Accordingly, SC number is increased substantially in the days following a single bout of resistance exercise (36). More importantly, a greater proportion of SC is in the active state following exercise, as defined by the colocalization of MyoD with Pax7 (23, 37). In the present study, before exercise training, there was an ~35% increase in active SC ($MyoD^+/Pax7^+$) 24 h after a single bout of resistance exercise. However, there was a significantly greater increase in active SC (~55%) at the same time point after 16 wk of RT. Consistent with this observation, we observed an increase in MyoD gene expression (~1.4-fold from Pre) 24 h postexercise after RT compared with no change in the untrained status response. These findings suggest an enhanced SC activation following 16 wk of RT. We suggest that this is an adaptive response to chronic exercise training that allows for an augmented postexercise response to acute exercise. To better understand the nature of this observation to an acute bout of exercise after training, we examined whether enhanced SC activation following RT in young men was accompanied by changes in muscle fiber capillarization.

Skeletal muscle fiber perfusion is essential for the delivery of oxygen, growth factors, and macronutrients to skeletal muscle fibers. Inadequate muscle fiber perfusion has been suggested to play a role in "anabolic resistance" and impaired nutritive flow in various populations (13, 32, 40). To meet increased metabolic demand and to support continuous muscle hypertrophy during resistance exercise, an increase in muscle capillarization may be required. Consistent with this notion, muscle fiber capillarization has been reported to increase significantly in response to RT in healthy young men (12, 14, 19). In agreement, we report a ~13% increase in C/Fi in type I and a ~26% increase in type II muscle fibers. Furthermore, we observed an increase in type I (~10%) and type II (~17%) CFPE index. As CFPE is regarded as a proxy measure of microvascular perfusion (16), an increase in CFPE suggests improved delivery of circulating nutrients and/or growth factors. Therefore, increases in muscle fiber vascularization and/or the reorganization of the microvascular bed after RT may result in enhanced supply of circulating growth factors during the postexercise period that could influence the SC response.

There are many growth factors that may play a role in regulating SC function (e.g., IL-6, IGF-1, myostatin, HGF) (17). Therefore, an increase in muscle fiber perfusion may result in enhanced exposure of SC to regulatory growth factors in circulation (4, 5). We and others have reported an anatomical relationship between muscle SC and capillaries (5) and have also noted that activated SC are closer to capillaries than quiescent SC (5, 25) suggesting that proximity of a SC to a capillary could be an important factor for SC function. Accordingly, it has been hypothesized that SC content (5, 10) and/or activation status (4, 5, 25) may be related to muscle fiber capillarization. In the present study, activated SC cells were

located in closer proximity to capillaries compared with quiescent SC at baseline (Pre; before the single bout of resistance exercise) in both the UTSR and the TSR condition. We were unable to observe any direct or significant correlation between the increase in muscle capillarization and the altered acute SC response in the TSR. However, we observed that the temporal-spatial relationship between both quiescent and active SC and the nearest capillary had been changed in response to a single bout of exercise at 24 h after 16 wk RT. These small changes may be indicative of an adaptive response of the spatial relationship between SC and capillaries after chronic training. Whether the small changes in the relationship between active and/or quiescent SC and the distance to the nearest capillary can explain the enhanced activation of SC in response to a single bout of exercise following 16 wk of RT remains unknown and requires further study. Furthermore, SC activation status was not determined in a fiber type-specific manner, and future studies should address this issue.

Although we observed an increase in capillarization following RT that accompanied an altered SC response to resistance exercise, there remains an incomplete understanding of how the SC response to a stimulus is initiated. Indeed, there is evidence to suggest that numerous cytokines and growth factors produced by skeletal muscle and/or the microvasculature may stimulate SC in an autocrine/paracrine fashion rather than through circulation. IL-6, previously reported to have a role in SC regulation (34, 41), is produced locally by contracting muscles (39). Interestingly, cell types such as endothelial cells within the muscle have also produce IL-6 under certain conditions (35, 45), as well as IGF-1 and HGF (5). Given the established spatial relationship between capillaries and SC, it would stand to reason that cellular cross-talk between endothelial cells and SC may influence angiogenesis (5, 33). Indeed, Chazaud et al. (4) reported that human muscle progenitor cells undergoing differentiation produce VEGF, a key factor for angiogenesis (4). Taken together, these findings indicate that the relationship between microvascular capillaries and SC may be predicated not only on the exposure to systemic factors, but also the immediate paracrine cross-talk between endothelial cells and SC. Future studies should address whether cytokines released from skeletal muscle or the microvasculature stimulate the SC response through autocrine/paracrine pathways, or exposure to endocrine-derived signals delivered through the microvasculature, or some combination of both.

Given the increased muscle perfusion after 16 wk of RT, we speculate that SC may have received enhanced input from circulating growth factors and more rapidly initiated the myogenic program and migratory function of SC leading to a loss in the observed anatomical relationship between SC and capillaries in the rested state and early activated state after exercise. Although we do not find a significant correlation between the altered (post-RT) response and the increase in capillarization, recent work might lead us to speculate that capillarization may play a role in resistance training adaptation. Indeed, Snijders et al. (38) recently observed that capillarization was linked to changes in muscle cross-sectional area following resistance training in older men. The study observed that individuals who started with a higher muscle fiber capillarization at baseline had a greater muscle hypertrophy following resistance training in older men. Taken together, the changes in SC activation that accompany the increases in muscle capillarization following long-term RT warrant further study into the relationship between capillaries and the SC pool. In compromised populations, such as older adults, who can have a relatively reduced muscle capillarization (8, 31) and reduced muscle mass (43), an impaired SC activation in response to exercise has been observed (23, 37). Furthermore, it would be interesting to investigate whether increasing muscle fiber capillarization would result in an augmented SC response during the postexercise period in older adults. In conclusion, we observed that an altered activation of the SC pool in response to a single bout of resistance exercise is accompanied by increased capillarization following 16 wk RT.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.P.N., T.S., S.J., C.G.W., C.J.M., L.M.J., S.K.B., and S.M.P. performed experiments; J.P.N., T.S., S.J., and C.G.W. analyzed data; J.P.N., T.S., S.J., and G.P. interpreted results of experiments; J.P.N. prepared figures; J.P.N. drafted manuscript; J.P.N., T.S., S.J., C.G.W., C.J.M., L.M.J., S.K.B., S.M.P., and G.P. edited and revised manuscript; J.P.N., T.S., S.J., C.G.W., C.J.M., L.M.J., S.K.B., S.M.P., and G.P. approved final version of manuscript.

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CHAPTER 4:

THE INFLUENCE OF CAPILLARIZATION ON SATELLITE CELL POOL EXPANSION AND ACTIVATION FOLLOWING EXERCISE-INDUCED MUSCLE DAMAGE IN HEALTHY YOUNG MEN

Preparation for submission to *Journal of Physiology*

ABSTRACT:

Factors that determine the skeletal muscle satellite cell (SC) response remain incompletely understood. It is known, however, that SC activation status is closely related to the anatomical relationship between SC and muscle capillaries. We investigated the impact of muscle fibre capillarization on the expansion and activation status of SC following a muscle damaging exercise protocol in healthy young men. Twenty-nine young men (21±0.5 yrs) performed 300 unilateral eccentric contractions (180 deg s^{-1}) of the knee extensors. Percutaneous muscle biopsies from the vastus lateralis and blood samples from the antecubital vein were taken prior to (Pre) and at 6h, 24h, 72h and 96h of post-exercise recovery. Type I and type II muscle fibre size, capillarization, and SC response were determined via immunohistochemistry. There was a significant correlation ($r= 0.39$; $p<0.05$) between the expansion of SC content (change in total $Pax7^+$ cells/100 myofibre) 24h following eccentric exercise and mixed muscle capillary-to-fibre perimeter exchange index. Subjects were retrospectively stratified based on their mixed muscle CFPE index. There was a greater increase in activated SC ($MyoD^+/Pax7^+$ cells/100 myofibre) in the High CFPE group as compared to the Low CFPE group 72h following eccentric exercise. The current study provides further evidence that muscle capillarization may play an important role in the activation and expansion of the SC pool during the muscle fibre repair process.

INTRODUCTION:

Skeletal muscle satellite cells (SC) are indispensable for muscle regeneration and repair following injury (Lepper *et al.*, 2011; McCarthy *et al.*, 2011; Sambasivan *et al.*, 2011). In response to a physiological cue (e.g. exercise), SC activate, proliferate and differentiate donating nuclei to existing muscle fibres to aid in repair/adaptation or return to a state of quiescence to replenish the basal SC pool (Bentzinger *et al.*, 2012; Yin *et al.*, 2013). The process of SC activation through terminal differentiation is orchestrated by a transcriptional network, known as the myogenic regulatory factors (MRFs), and is collectively referred to as the myogenic program. Expansion of the SC pool following a single bout of exercise or muscle fibre contraction-induced damage has been well characterized in humans (Bellamy *et al.*, 2014; McKay *et al.*, 2009; McKay *et al.*, 2008; McKay *et al.*, 2013; McKay *et al.*, 2012; Nederveen *et al.*, 2017) with appreciable expansion occurring by 24h and peaking 72h post-stimulus (Snijders *et al.*, 2015).

A number of cytokines and growth factors including, but not limited to, interleukin-6 (IL-6), insulin-like growth factor-1 (IGF-1), myostatin and hepatocyte growth factor (HGF) are known regulators of SC progression through the myogenic program (McKay *et al.*, 2009; McKay *et al.*, 2008; O'Reilly *et al.*, 2008). Many of these factors are produced by skeletal muscle in its function as an 'endocrine organ' (Pedersen & Febbraio, 2008; Steensberg *et al.*, 2000), or by other organs, tissues or cells (Velloso, 2008) and then delivered to the SC niche via the vasculature. Therefore, delivery of these factors to the SC niche may be a requirement of the myogenic response. Indeed, the

importance of extrinsic factors in regulating SC function has been demonstrated using parabiotic pairings of old and young rodents (Brack & Rando, 2007; Conboy *et al.*, 2005). Muscle capillaries function as the delivery mechanism for oxygen, fuel, cytokines and growth factors that may regulate SC, but may also act as an important modulator of the SC response. We and others have reported an anatomical relationship between muscle SC and the microvasculature, with activated SC situated geographically closer to capillaries than quiescent SC (Christov *et al.*, 2007; Nederveen *et al.*, 2016; Nederveen *et al.*, 2017). Consequently, it has been proposed that SC content (Christov *et al.*, 2007; Emslie-Smith & Engel, 1990) and/or SC activation status (Chazaud *et al.*, 2003; Christov *et al.*, 2007; Nederveen *et al.*, 2016) may be related to the extent of muscle fibre capillarization as a result of exposure of SC to circulating factors or direct communication between endothelial cells and SC during muscle repair (Chazaud *et al.*, 2003; Ochoa *et al.*, 2007). However, to what extent the muscle fibre microvascular bed may dictate the acute muscle SC response during muscle repair in humans remains unknown. Therefore, in the present study, we assessed the expansion and activation status of the SC pool following a single bout of exercise-induced muscle fibre damage in a group of healthy young men with varying degrees of muscle fibre capillarization. We hypothesized that individuals with a greater degree of muscle fibre capillarization would demonstrate a more rapid and pronounced SC response following a single bout of eccentric exercise.

METHODS

Participants. Twenty nine healthy young men (YM: 22 ± 0.5 yr; mean ± SEM) were recruited to participate in this study. Exclusion criteria included smoking, diabetes, the

use of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or statins, and a history of respiratory disease and/or any major orthopaedic disability. Subjects were told to refrain from exercising throughout the duration of the study, and refrain from the use of NSAIDs (Mackey *et al.*, 2016).The study was approved by the Hamilton Health Sciences Integrated Research Ethics Board, and conformed to the guidelines outlined in the Declaration of Helsinki. Participants gave their informed written consent prior to inclusion in the study. To assess the impact of muscle fibre capillarization on the muscle SC response following a single bout of eccentric exercise and subsequent muscle damage, participants were assigned into one of two groups (n = 10 per group) based on mixed muscle fibre capillarization (corrected for capillary sharing factor and muscle fibre perimeter, also known as capillary-to-fibre perimeter exchange (CFPE) index) for non-correlative statistical analysis. This resulted in a group with a relatively low (Low; CFPE: 5.2 ± 0.5 capillaries • 1000 μm^{-1}) and relatively high (High; CFPE: 7.6 ± 1.0 capillaries • 1000 μm^{-1}) mixed muscle fibre CFPE index. Stratification of participants resulted in a 'middle' group (n = 9) who were not used in non-correlative statistical analysis, with the intent to create a clear separation between the Low and High group.

VO_2peak test and anthropometric measurements. During an initial visit to the laboratory participants performed a VO_{2peak} test on a cycle ergometer (model: H-300-R Lode; Lode B.V., Groningen, The Netherlands) and had anthropometric measurements recorded. The VO_{2peak} test consisted of load-less pedaling for one minute, followed by a step-wise increase to 50 watts (W) for two minutes. After the increase to 50 watts, work rate was increased by 30 W/min until the participant reached volitional fatigue (determined by the

inability of the participant to maintain a minimum cadence of 60 RPM). Gas exchange was collected throughout the test using a metabolic cart (Moxus, AEI Technologies, Pittsburgh, PA) and VO_{2peak} was calculated using the highest 30 second average VO_2 during the final stage of the ramp protocol. Work rate (WR) was collected continuously throughout the test and peak aerobic power (WR_{peak}) was calculated using the average WR from the last 30 seconds of the test.

Muscle biopsy sampling. Percutaneous needle biopsies were taken, after an (~10h) overnight fast (Pre), from the mid-portion of the *vastus lateralis* under local anesthetic using a 5 mm Bergstrom needle adapted for manual suction. Subjects had not participated in any physical activity for at least 96 hours before muscle biopsy collection prior to the single bout of eccentric exercise. The muscle biopsy procedure was repeated at 6h, and in the fasted condition (~10h) 24h, 72h and 96h of post-exercise recovery. Incisions for the repeated muscle biopsy sampling were spaced approximately 3 cm apart to minimize any effect of the previous biopsy. Upon excision, muscle samples were immediately mounted in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen–cooled isopentane, while another part was directly frozen in liquid nitrogen, and stored at -80° C until further analyses.

Blood sampling. Blood samples were obtained from the antecubital vein immediately prior the muscle biopsy sampling procedure before and after 6h, 24h, 72h and 96h of the single bout of eccentric exercise. Blood (~10 mL) samples were collected in EDTA containing tubes and centrifuged at 1500 rpm for 10 min at 4 °C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. Plasma samples were analyzed for

IL-6 protein and creatine kinase activity using commercially available Enzyme-Linked ImmunoSorbant Assay (ELISA) (R & D Systems, Inc., USA) and activity assay kits (Abcam Inc., Canada), respectively, following the manufacturer's instructions. Statistics were performed on the raw values, and expressed as a percentage change from baseline.

Eccentric Muscle Damage Protocol. Maximal isokinetic unilateral muscle-lengthening contractions of the quadriceps were performed using the Biodex dynamometer (Biodex-System 3, Biodex Medical Systems, Inc., USA) at 180 deg s^{-1}. For each subject, one leg was selected randomly to perform the exercise protocol described below. Movement at the shoulders, hips and thigh were restrained with straps in order to isolate the knee extensors during the protocol. Immediately prior to the intervention, subjects underwent a brief familiarization with the equipment, involving 5–10 submaximal lengthening contractions of the leg to be exercised. Subjects were required to perform 30 sets of 10 maximal knee extensions with 1 min rest between sets, for a total of 300 lengthening contractions. During each set, investigators provided verbal encouragement for the subjects to complete and exert maximal force during each contraction. This protocol has been previously shown to induce a significant level of skeletal muscle damage (Beaton *et al.*, 2002).

Immunofluorescence. Muscle cross sections (7µm) were prepared from unfixed OCT embedded samples, allowed to air dry for 30 minutes and stored at -80˚C. Samples were stained with appropriate primary and secondary antibodies against specific antigens, found in Table 1, as previously described (Nederveen *et al.*, 2016). Nuclei were labelled with DAPI (4',6-diamidino-2-phenylindole) (1:20000, Sigma-Aldrich, Oakville, ON,

Canada), prior to cover slipping with fluorescent mounting media (DAKO, Burlington, ON, Canada). The staining procedures were verified using negative controls, in order to ensure appropriate specificity of 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 Instruments, Melville, NY, USA). Images were captured and analyzed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments, Inc., USA). All images were obtained with the 20x objective, and ≥ 200 muscle fibres/subject/time point were included in the analyses for SC content/activation status (i.e., $Pax7^+/MyoD^-$ or $Pax7^+/MyoD^-$), and fibre cross sectional area (CSA), and perimeter. The activation status of SCs was determined via the colocalization of $Pax7^+$ and DAPI ($Pax7^+/MyoD^-$) and/or the co-localization of Pax7, MyoD and DAPI (i.e., $Pax7^+/MyoD^+$). Cell membranes were labelled with Peroxidase conjugated Wheat Germ Agglutinin (WGA) (1 ug/mL, Vector PL-1026, Burlington, ON, Canada) and realized with a substrate kit (Vector, SK-4700, Burlington, ON, Canada) as per manufacturer's instructions. Slides were blinded for both group and time point. The quantification of muscle fibre capillaries was performed on 50 muscle fibres/subject/time point (Porter *et al.*, 2002). Based on the work of Hepple *et al.* (Hepple, 1997; Hepple & Mathieu-Costello, 2001), quantification of; i) capillary contacts (CC; the number of capillaries around a fibre), ii) the capillary-to-fibre ratio on an individual fibre basis (C/F*i*), iii) the number of fibres sharing each capillary (i.e., the sharing factor) and iv) the capillary density (CD) was performed. The CD was calculated by using the cross sectional area (μm^2) as the reference space. The capillary-to-fibre perimeter exchange

index (CFPE) was calculated as an estimate of the capillary-to-fibre surface area (Hepple, 1997). The SC-to-capillary distance measurements were performed on all SC that were enclosed by other muscle fibres, and has been described previously (Nederveen *et al.*, 2016). All immunofluorescent analysis were completed in a blinded fashion.

RNA Isolation. RNA was isolated from 15–25 mg of muscle tissue using the Trizol/RNeasy method. All samples were homogenized with 1 mL 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 6 m/sec. Following a five minute room temperature incubation, homogenized samples were stored at -80°C for one month until further processing. After thawing on ice, 200 ml of chloroform (Sigma-Aldrich, Oakville, ON, Canada) was added to each sample, mixed vigorously for 15 sec, incubated at RT for 5 min, and spun at 12000 g for 10 min at 4°C. The RNA (aqueous) phase was purified using the E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) as per manufacturer's instructions. RNA concentration (ng/ml) and purity (260/280) was determined with the Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA).

Reverse Transcription. Samples were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in 20 μl reaction volumes, as per manufacturer's instructions, using an Eppendorf Mastercycler epGradient Thermal Cycler (Eppendorf, Mississauga, ON, Canada) to obtain cDNA for gene expression analysis.

Quantitative real time RT-PCR. All QPCR reactions were run in duplicate in 25 µl volumes containing RT Sybr Green qPCR Master Mix (Qiagen Sciences, Valencia, CA, USA), prepared with the epMotion 5075 Eppendorf automated pipetting system (Eppendorf, Mississauga, ON, Canada), and carried out using an Eppendorf Realplex2 Master Cycler epgradient (Eppendorf, Mississauga, ON, Canada). Primers are listed in Supplementary Table 1 and were re-suspended in 1X TE buffer (10mM Tris–HCl and 0.11 mM EDTA) and stored at −20◦C prior to use. Messenger RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and fold changes from baseline were calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). Briefly, Ct values were first normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Supplementary Table 1). Ct values normalized to GAPDH were expressed as delta-Cts (ΔCt). ΔCt values were then normalized to Pre values, expressed as delta-delta Cts (ΔΔCt). Values were then transformed out of the logarithmic scale using the formula: fold change = $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Thus, mRNA values are expressed as a fold change from Pre (mean ±sem). GAPDH expression was not different from Pre at any of the post-intervention time-points.

Table 1. Antibody information

Antibody	Species	Source	Details	Primary	Secondary
Anti-Pax7	Mouse	DSHB	Pax7	1:1	Alexa 594, 488 goat-anti mouse 1:500
Anti-laminin	Rabbit	Abcam	ab11575	1:500	Alexa Fluor 488, 647 goat anti-rabbit, 1:500
Anti-MHCI	Mouse	DSHB	A4.951 Slow isoform	1:1	Alexa Fluor 488 goat anti-mouse, 1:500
Anti-CD31	Rabbit	Abcam	ab28364	1:30	Alexa Fluor 647 goat anti-rabbit, 1:500
Anti-MyoD	Mouse	Dako	5.8A	1:50	goat anti-mouse biotinylated secondary antibody, 1:200; streptavidin-594 fluorochrome, 1:250

Table 1. Detailed information on primary and secondary antibodies and dilutions used for immunofluorescent staining of the frozen muscle cross sections.

Statistical analysis. Statistical analysis was performed using Sigma Stat 3.1.0 analysis software (Systat Software, Chicago, IL, USA).

Baseline comparisons. Comparisons of participant demographics between the High CPFE and the Low CFPE groups are found in Table 2, and were performed via a Student's *t* test. Baseline comparisons of muscle fibre type specific characteristics between the High CFPE and the Low CFPE group were performed using a two-way ANOVA (group x fibre type).

Response to eccentric contractions. One-way repeated measures ANOVA were performed separately for each of the 'Overall' group, for the High CFPE group and for the Low CFPE group, with time (Pre, 6h, 24h and 72h and 96h) as a within group factor. These tests were performed to assess the following; the acute change in SC activity status (i.e., $Pax7^+/MyoD^+$ cells); the acute change in SC content (i.e., mixed muscle, type I and/or type II $Pax7^+$ cells separately); the acute change in plasma IL-6 content; the acute change in plasma creatine kinase activity; the acute change in quadriceps muscle force

production and the acute change in MRF mRNA expression, following the bout of eccentric exercise induced muscle damage. In these one-way repeated measures ANOVA design for the acute response, post-exercise time points were only compared with baseline (Pre) and Bonferonni corrections were applied to account for multiple comparisons.

Comparing the High CFPE vs. Low CFPE group. A Student's *t* test was used to determine the differences that existed between the High and Low CFPE at different time points. Specifically, a Student's *t* test was utilized to compare the change (i.e., Δ) from the Pre timepoint to the timepoint of interest (i.e, Pre vs 6h, Pre vs 24h, Pre vs 72h, and Pre vs 96h). For correlations, Pearson's correlation analyses were performed where appropriate between indices of muscle fibre capillarization and the SC response following eccentric exercise. Statistical significance was accepted at $p < 0.05$. All results were presented as means ± standard error of the mean (SEM).

RESULTS

Subject characteristics

Overall: Complete subject characteristics are reported in Table 2.

Low vs. High CFPE group: There were no differences in age or height between the groups (Table 2). There was a significant difference in bodyweight ($p<0.05$, Table 2) and a trend for BMI ($p = 0.06$, Table 2) between the groups. Both the VO_{2max} (mL/kg/min) and W_{peak} (W) was significantly greater in the High as compared to the Low group ($p<0.05$; Table 2). There was no significant difference in force production prior to single bout eccentric exercise in the High (272.6 ± 8.8 N·m) compared to the Low (314.4 ± 13.3 N·m) group (Table 2).

Table 2. Demographics and group characteristics

Variable	*Overall (n=29)*	*High CFPE*	*Low CFPE*
Age (yrs)	22 ± 1	22 ± 1	21 ± 0
Height (cm)	179.2 ± 1.3	176.8 ± 1.1	179.5 ± 0.9
Weight (kg)	80.9 ± 2.4	72.5 ± 1.6*	83.9 ± 2.3
BMI (kg/m^2)	25.1 ± 0.6	23.2 ± 0.5	26.0 ± 0.6
Force Production (N·m)	319.4 ± 13.8	272.6 ± 8.8	314.4 ± 13.3
VO_2 Peak (mL/kg/min)	49.9 ± 2.3	63.1 ± 1.5*	40.5 ± 1.4
Peak Wattage (W)	339.2 ± 11.8	385.3 ± 8.9*	290.6 ± 11.0

*; significant effect for group. Mean ± SEM.

Indices of muscle damage following repeated eccentric contractions.

Overall: Following eccentric contractions, force production (N·m) was significantly reduced at 6h (253 ± 15 N·m), 24h (233 ± 16 N·m), 72h (261± 18 N·m) and 96h (270 ± 17 N·m), as compared to Pre (319 ± 14 N·m) ($p<0.05$; Supplementary Fig 1A). Following the eccentric contraction protocol, plasma creatine kinase activity was significantly increased at 24h (103.7 ± 8.2 mU/mL, $p<0.05$; Supplementary Fig 1C) compared to Pre (75.4 ± 6.1 mU/mL), and returned back to baseline at 72h (76.3 ± 6.7 mU/mL) and 96h (79.1 ± 6.6 mU/mL).

High CFPE vs Low CFPE group: In the High group, force production was significantly reduced at 6h (211± 15 N·m), 24h (196 ± 17 N·m), 72h (230 ± 21 N·m) as compared to Pre (272 ± 15 N·m and was back at baseline levels again at 96h (241 ± 23 N·m) ($p<0.05$; Supplementary Fig 1B). In the Low group, force production was significantly reduced at 6h (248 ± 29 N·m), 24h (215 ± 34 N·m), 72h (242 ± 37 N·m) and 96h (257 ± 32 N·m) as compared to Pre (314 ± 23 N·m) ($p<0.05$; Supplementary Fig 1B). In the High group,

plasma creatine kinase activity was significantly increased 24h (93.3 ± 13.8 mU/mL, $p<0.05$) following eccentric exercise, but was not significantly different at 72h (56.0 ± 5.4 mU/mL) and 96h (66.4 ± 8.6 mU/mL) as compared to Pre (60.3 ± 8.4 mU/mL) ($p<0.05$; Supplementary Fig 1D). In the Low group, plasma creatine kinase activity was significantly increased 24h (107.9 ± 10.7 mU/mL, $p<0.05$) following eccentric exercise, but was not significantly different at 72h (92.9 ± 12.4 mU/mL) and 96h (91.1 ± 14.3 mU/mL) as compared to Pre (88.4 ± 11.2 mU/mL) ($p<0.05$; Supplementary Fig 1D). Prior to the intervention, there were no significant differences in creatine kinase activity in the High compared to the Low group; there were no differences in creatine kinase activity changes following eccentric exercise.

Skeletal muscle fibre characteristics

Overall: Muscle fibre CSA was significantly greater in type II (7500 ± 355 um^2) compared to type I fibres (6326 ± 205 um^2, $p<0.05$). Muscle fibre perimeter was significantly greater in type II (326 ± 6 um) compared to type I fibres (306 ± 5 um, $p<0.05$). The number of myonuclei per fibre was not different in type II as compared to type I (3.7 ± 0.2 vs 3.6 ± 0.2 myonuclei/fibre, respectively). Myonuclear domain size was significantly greater in type II as compared to type I muscle fibres (2019 ± 88 vs 1805 ± 53 um^2, $p<0.05$, respectively). Muscle C/Fi (2.08 ± 0.1 vs. 1.94 ± 0.1 capillaries per fibre), CFPE index (6.86 ± 0.2 vs. 6.03 ± 0.23 capillaries per fibre · 1000^{-1}, and CD (655 ± 26 vs. 478 ± 29 capillaries/ mm^2) was significantly greater in type I compared to type II muscle fibres, respectively ($p<0.05$). SC distance to nearest capillary was significantly greater in type II compared to type I muscle fibres (15.8 ± 0.7 vs 13.9 ± 0.7 um,

respectively, $p<0.05$) at baseline (Pre). Total $Pax7^+$ cell distance to nearest capillary at baseline was negatively correlated to mixed muscle CFPE index ($r = -0.49$, $p<0.05$) across all participants. Type II muscle fibre associated SC distance to nearest capillary at baseline was negatively correlated to type II fibre CFPE index ($r = -0.51$, $p<0.05$; Figure 4D).

High CFPE vs Low CFPE group: Type II muscle fibre CSA and perimeter were significantly greater compared with type I muscle fibre in both groups, with no difference between group ($p<0.05$; Table 3). Interestingly, the proportion of type I muscle fibres was significantly higher in the High group compared with the Low group ($p<0.05$, Table 3). Both type I and type II muscle fibre C/Fi, CFPE index, and CC were significantly higher in the High as compared to the Low group ($p<0.05$; Table 3). There were no significant differences ($p>0.05$) in type I or type II myonuclei per fibre and/or myonuclear domain size between groups.

SC distance to nearest capillary across both type I and type II was significantly lower in the High group as compared to the Low group ($p<0.05$, Table 3).

Table 3. Muscle fibre capillarization and muscle characteristics

	High CFPE Group	Low CFPE Group
CC		
Type I	4.19 ± 0.23$^{\#}$	3.97 ± 0.29
Type II	4.53 ± 0.28$^{\#}$	2.31 ± 0.11
C/Fi		
Type I	2.41 ± 0.10$^{\#}$	1.66 ± 0.07
Type II	2.32 ± 0.11$^{\#}$	1.50 ± 0.06
CFPE		
Type I	7.97 ± 0.34$^{*\#}$	5.75 ± 0.17*
Type II	7.24 ± 0.35$^{\#}$	4.91 ± 0.17
Fiber Type proportion (%)		
Type I	56.0 ± 3.1$^{\#}$	33.9 ± 2.1
Type II	42.2 ± 3.3	65.8 ± 2.1$^{\#}$
Muscle fiber size (μm^2)		
Type I	6185 ± 231	5937 ± 376
Type II	7121 ± 445*	6626 ± 568*
Myonuclear domain (μm^2)		
Type I	1817 ± 109	1736 ± 85
Type II	1896 ± 157	1969 ± 104
Myonuclei per fiber		
Type I	3.5 ± 0.2	3.5 ± 0.5
Type II	3.9 ± 0.2	3.3 ± 0.2
Muscle fiber perimeter		
Type I	306 ± 5	293 ± 11
Type II	327 ± 8*	308 ± 12*
Satellite cell distance to nearest capillary (μm)		
Type I	12.1 ± 0.8$^{\#}$	13.9 ± 0.9
Type II	13.3 ± 0.9$^{\#}$	17.3 ± 0.9
Satellite cell per 100 myofiber		
Type I	11.6 ± 1.6	10.5 ± 1.9
Type II	11.8 ± 1.3	10.9 ± 1.4

*; significant effect within group for fiber type, #; significant effect for group. Mean ± SEM.

Mixed muscle SC response

Overall: Following the eccentric contraction protocol, total mixed muscle $Pax7^+$ cells/100 myofibre tended to increase significantly at 6h (14.4 ± 1.1 cells/100 myofibre ($p = 0.056$), increased significantly at 24h (14.9 ± 1.1 cells/100 myofibre; $p<0.05$) and 72h (15.8 ± 1.0 cells/100 myofibre; $p<0.05$) compared to Pre (11.8 ± 0.7 cells/100 myofibre, Supplementary Table 2). The change in total mixed muscle $Pax7^+$ cells/100 myofibre between Pre and 24h ($r = 0.39$, $p<0.05$; Figure 1G) was positively correlated to mixed muscle CFPE index across all participants. The activation status of the SC pool was assessed by colocalizing SC with MyoD before and after the eccentric contraction protocol. Mixed muscle $Pax7^+/MyoD^+$ cells/100 myofibres were significantly elevated at 6h (1.8 ± 0.3 cells/100 myofibre, $p<0.05$), 24h (2.2 ± 0.2 cells/100 myofibre, $p<0.05$), 72h (1.9 ± 0.4 cells/100 myofibre, $p<0.05$) and 96h (1.1 ± 0.2 cells/100 myofibre, $p<0.05$) as compared to Pre (0.4 ± 0.1; Figure 2F). The change in total mixed muscle $MyoD^+/Pax7^+$ cells/100 myofibre between Pre and 6h ($r = 0.40$, $p<0.05$; Figure 1G) and Pre and 72 ($r = 0.37$, $p<0.05$; Figure 1H) was positively correlated to mixed muscle CFPE index across all participants.

High vs. Low CFPE Group: Prior to the intervention, there were no differences in mixed muscle total $Pax7^+$ cells/100 myofibre ($p>0.05$) between the High (11.0 ± 1.2 cells/100 myofibre) and the Low (11.9 ± 1.3 cells/100 myofibre) group. Compared to baseline, total mixed muscle $Pax7^+$ cells/100 fibre were significantly increased at 6h ($p<0.05$); 24h ($p<0.05$) and 72h ($p<0.05$) after the single bout of eccentric exercise in the High group

(Figure 1F, Supplementary Table 3). In contrast, total mixed muscle $Pax7^+$ cells/100 myofibre was only significantly increased at 72h ($p<0.05$) during post-exercise recovery in the Low group compared to baseline (Figure 1F, Supplementary Table 3). There was a significantly greater increase in mixed muscle total $Pax7^+$ cells/100 myofibres from Pre to 6h ($p<0.05$); Pre to 24h ($p<0.05$) and a trend for Pre to 72h ($p=0.052$) following eccentric contractions in the High compared with the Low Group.

Prior to the intervention, there were no differences in total MyoD+/$Pax7^+$ cells/100 myofibre in mixed muscle ($p>0.05$) between the High (0.3 ± 0.2 cells/100 myofibre) and the Low (0.2 ± 0.1 cells/100 myofibre) groups. Mixed muscle $Pax7^+$/$MyoD^+$ cells/100 myofibres were significantly higher in the High group at 6h ($p<0.05$), 24h ($p<0.05$), 72h ($p<0.05$), and 96h ($p<0.05$) as compared to Pre (Figure 2F, Supplementary Table 3). In the Low group, $Pax7^+$/$MyoD^+$ cells/100 myofibres in mixed muscle were only significantly elevated at 24h ($p<0.05$) as compared to Pre (Figure 2F, Supplementary Table 3) In comparing the Low and the High mixed muscle SC activation ($Pax7^+$/$MyoD^+$ cells) response to eccentric exercise, we observed that there was a significantly greater increase in the number of mixed muscle $Pax7^+$/$MyoD^+$ cells/100 myofibre from Pre to 6h, and from Pre to 72h post-exercise recovery in the High as compared to the Low group ($p<0.05$, Figure 2F).

FIGURE 1

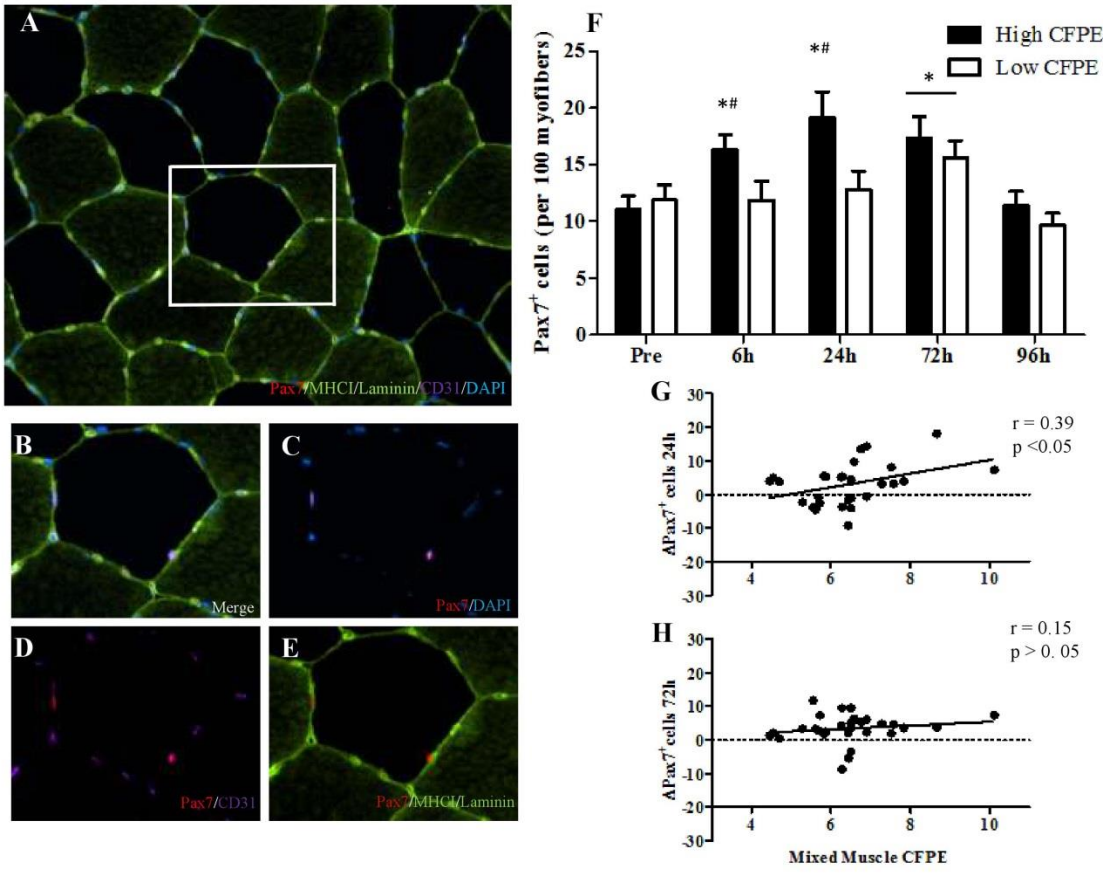

Fig. 1 Fibre type specific satellite cell staining with muscle capillaries. (***A***) Representative image of a MHCI/laminin/CD31/Pax7/DAPI stain of a muscle cross section. Channel views of ***(B***) Merge (***C***) Pax7/DAPI ***(D***) Pax7/CD31 (***E***) Pax7/MHCI/Laminin (***F***) Characterization of the expansion of the total mixed muscle satellite cell (SC) pool before and after 6h, 24h, 72h and 96h following eccentric contractions in the group with a High capillary to fibre exchange (CFPE) index and the group with Low CFPE index. *; Significantly different compared with Pre ($p<0.05$), bar indicates that effect of time is present for both groups. #; indicates a significantly greater increase with time High vs Low group ($p<0.05$). Data are expressed as mean ± sem. Relationship between the expansion of the total SC pool and mixed muscle CFPE following (***G***) Δ24h post-eccentric exercise ($r = 0.39$, $p<0.05$) and (***H***) Δ72h-post exercise ($r = 0.15$, $p>0.05$) across all participants.

FIGURE 2

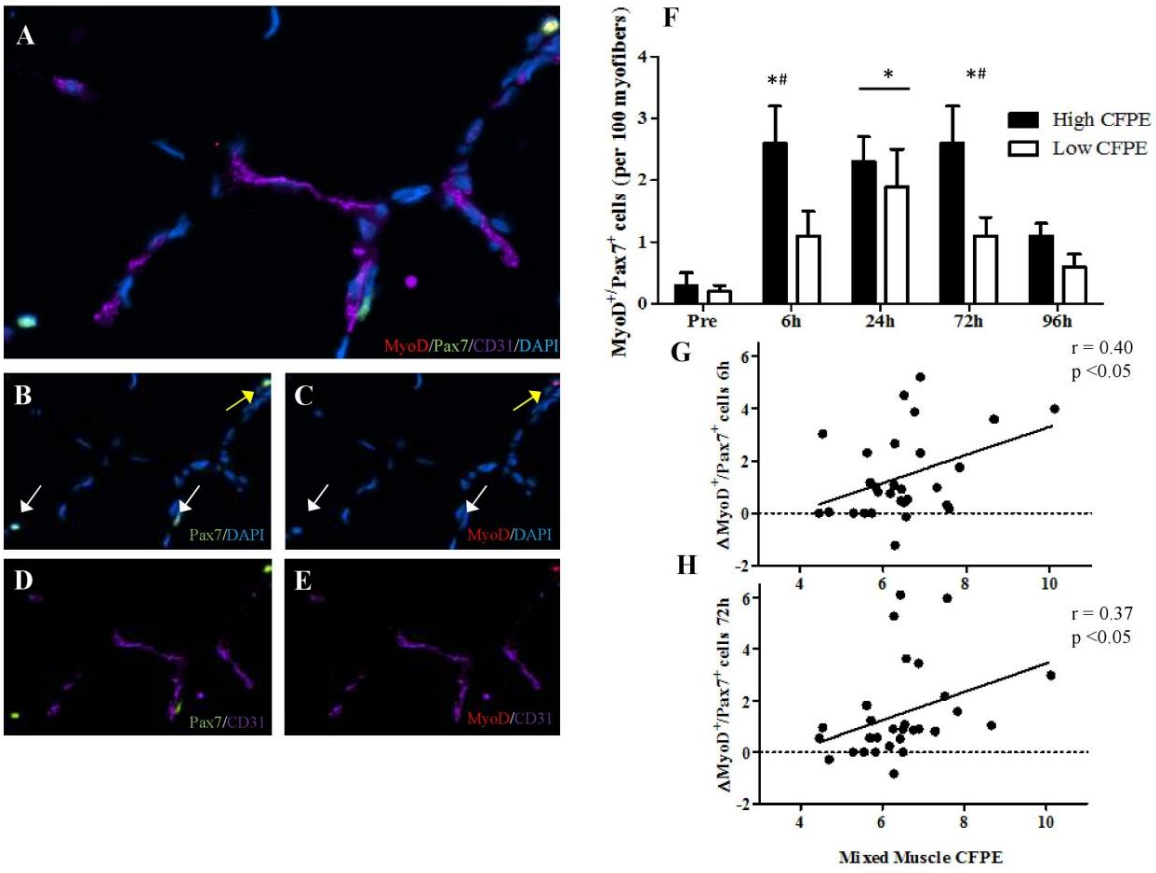

Fig. 2 Mixed muscle staining of satellite cell (SC) activation with muscle capillaries. (***A***) Representative image of a CD31/Pax7/MyoD/DAPI stain of a muscle cross section). Channel view of ***(B***) Pax7/DAPI (***C***) MyoD/DAPI ***(D***) Pax7/CD31 (***E***) MyoD/CD31 (***F***) Characterization of the activation status of the SC pool before and after 6h, 24h, 72h and 96h following eccentric contractions in the group with a High capillary to fibre exchange (CFPE) index and the group with Low CFPE index. *; Significantly different compared with Pre ($p<0.05$), bar indicates that effect of time is present for both High and Low group. #; indicates a significantly greater increase with time High vs Low group ($p<0.05$). Data are expressed as mean ± sem. Relationship between the activation of the SC pool ($\Delta MyoD^+/Pax7^+$ cells) and mixed muscle CFPE following (***G***) Δ6h post-eccentric exercise ($r = 0.40$, $p<0.05$) and (***H***) Δ72h-post exercise ($r = 0.37$, $p<0.05$) across all participants.

Type I and type II muscle fibre SC response

Overall: Prior to the intervention, there was no significant difference between type I-associated (11.5 ± 0.9 cells/100 myofibre) and type II-associated $Pax7^+$ cells/100 myofibres (11.8 ± 1.0 cells/100 myofibre) across all participants ($p>0.05$, Supplementary Table 2). Type I-associated $Pax7^+$ cells/100 myofibres remained unchanged at 6h, 24h, 96h and trended towards a significant increase at 72h ($p=0.09$), as compared to Pre (Supplementary Table 2).

Type II-associated $Pax7^+$ cells/100 myofibres remained unchanged at 6h and 24h but increased significantly at 72h ($p<0.05$), returning to basal levels at 96h as compared to Pre (Figure 3A).

The change in type II-associated $Pax7^+$ cells/100 myofibre between Pre and 6h ($r = 0.45$, $p<0.05$; Figure 3B) and Pre and 24h ($r = 0.42$, $p<0.05$; Figure 3C) following eccentric exercise was positively correlated with type II CFPE index across all participants.

The change in type II associated SC $Pax7^+$ cells/100 myofibre from Pre to 24h ($r = -0.37$, $p<0.05$; Figure 4B) following eccentric exercise was negatively correlated to type II SC distance to nearest capillary at baseline across all participants. There were no relationships between type I associated SC and type I SC distance to nearest capillary at baseline across all participants.

High vs. Low CFPE Group: Prior to the intervention, there were no differences in type I-associated $Pax7^+$ cells/100 myofibre or type II-associated $Pax7^+$ cells/100 myofibre

between Low and High groups. ($p>0.05$; Table 3). Type I $Pax7^+$ cells/100 myofibre was not significantly changed at 6h, 24h, 72h, or 96h as following eccentric contractions compared to Pre in the High ($p>0.05$) or Low group ($p>0.05$, Supplementary Table 3). In comparing the Low group to the High group following eccentric exercise, there were no differences between Type I $Pax7^+$/100 myofibres between Pre and any post-exercise time point. Type II $Pax7^+$ cells/100 myofibre were significantly increased at 6h ($p<0.05$) 24h ($p<0.05$) and 72h ($p<0.05$) following eccentric contractions in the High group, as compared to Pre (Figure 3A, Supplementary Table 3).

In the Low group, Type II $Pax7^+$ cells/100 fibre was only significantly elevated at 72h ($p<0.05$), as compared to Pre (Figure 3A, Supplementary Table 3). In comparing the Low and the High muscle fibre type specific SC response to eccentric exercise, we observed that there was a greater change in the number of Type II $Pax7^+$ cells/100 myofibres from Pre to 6h, and from Pre to 24h post-exercise in the High group as compared to the Low ($p<0.05$, Figure 3A, Supplementary Table 3).

SC distance to nearest capillary response following eccentric

Overall: SC distance to nearest capillary in mixed muscle fibres and/or type I/II-associated SC did not change in response to the single bout of eccentric exercise.

High vs. Low CFPE Group: Type I SC distance to nearest capillary did not change ($p>0.05$) following eccentric contractions in either the High (Pre: 12.2 ± 0.8 ; 6h: 13.0 ± 0.9; 24h: 12.4 ± 0.8; 72h: 12.6 ± 0.7; 96h: 11.9 ± 1.2 μm) or the Low group (Pre: 13.9 ± 0.9; 6h: 12.1 ± 0.7; 24h: 17.0 ± 1.1; 72h: 17.5 ± 1.8; 96h: 15.0 ± 0.9 μm) as compared to

FIGURE 3

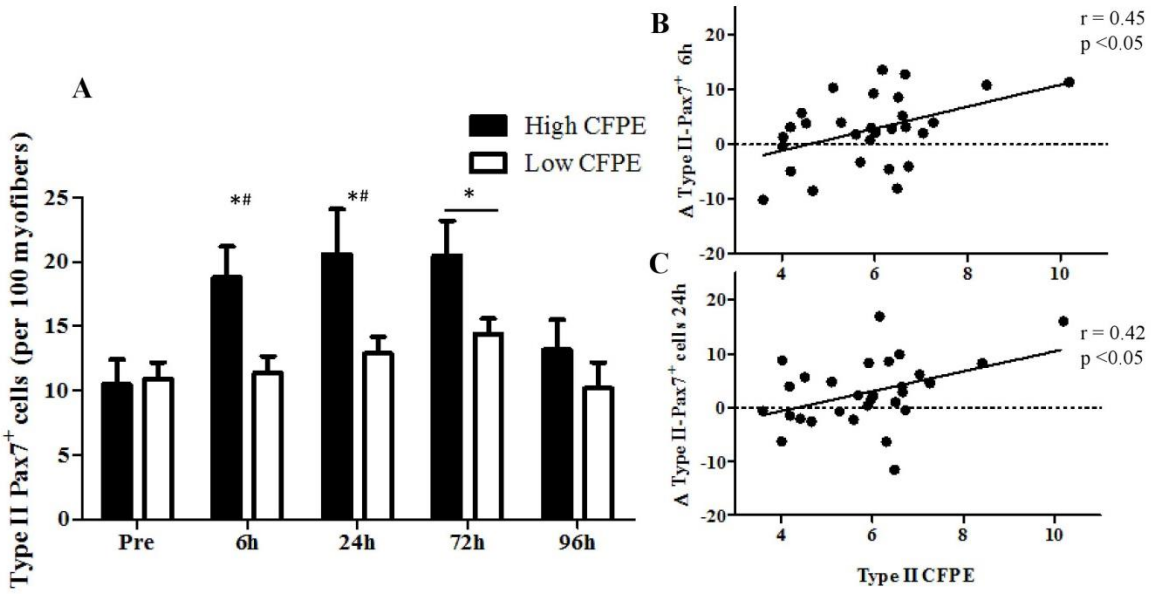

Fig. 3 Characterization of the expansion of type II fibre-associated satellite cell (SC) pool following eccentric contractions before and after 6h, 24h, 72h and 96h following eccentric contractions in the group with a High capillary to fibre exchange (CFPE) index and the group with Low CFPE index. (***A***) *; Significantly different compared with Pre ($p<0.05$), bar indicates that effect of time is present for both groups. #; indicates a significantly greater increase with time High vs Low group ($p<0.05$). Data are expressed as mean ± sem. Relationship between the expansion of the type II SC pool and type II CFPE following (***B***) Δ6h post-eccentric exercise ($r = 0.45$, $p<0.05$) and (***C***) Δ24h-post exercise ($r = 0.42$, $p <0.05$) across all participants.

baseline values. Type II SC distance to nearest capillary did not change ($p>0.05$) following eccentric contractions in either the High (Pre: 13.3 ± 0.8 ; 6h: 13.1 ± 1.3; 24h: 15.1 ± 1.2; 72h: 16.0 ± 1.0; 96h: 12.5 ± 1.3 μm) or the Low group (Pre: 17.3 ± 0.9; 6h: 17.0 ± 1.0; 24h: 16.9 ± 0.8; 72h: 20.0 ± 1.5; 96h: 16.8 ± 1.1 μm) as compared to baseline values.

FIGURE 4

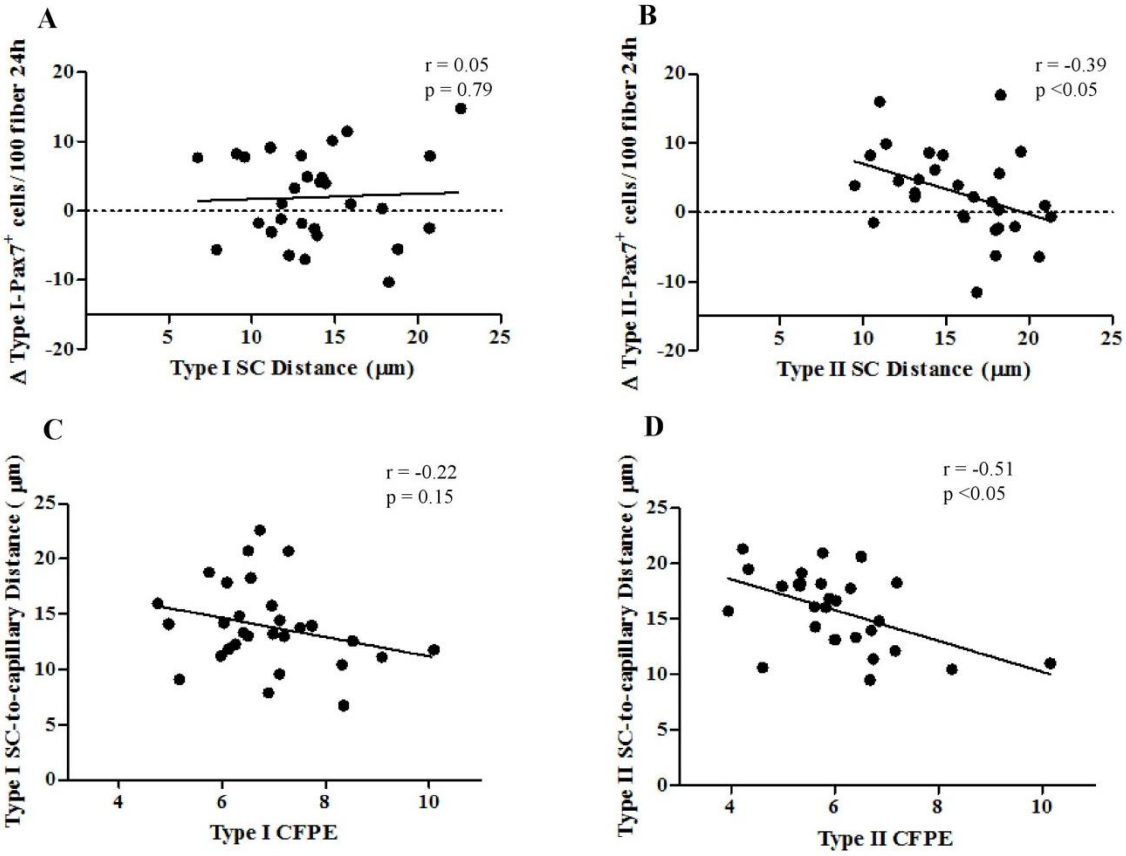

Fig. 4 Relationship between the expansion of the satellite cell (SC) pool from Pre to 24h post-eccentric exercise in a fibre type specific manner and fibre type specific CFPE for (***A***) type I-associated SC (r = 0.05, p = 0.79) and (***B***) type II-associated SC (r = -0.39, p<0.05) across all participants. Relationship between fibre type specific Capillary to fibre exchange (CFPE) index and distance of $Pax7^+$ SC to nearest capillary following eccentric exercise prior to eccentric damage for (***C***) type I-associated SC (r = -0.22, p = 0.15) and (***D***) type II-associated SC (r = -0.51, p<0.05) across all participants.

Myogenic regulatory factor response

Overall: MyoD, MRF4 and Myogenin mRNA expression were significantly increased at 6h (2.2-, 1.8- and 4.4-fold change, respectively), 24h (1.4-, 2.1- and 4.0-fold change,

respectively), 72h (1.6-, 1.6-, and 2.0-fold change, respectively) and 96h (1.4-, 2.3- and 1.9-fold change, respectively) after the single bout of eccentric exercise. Myf5 mRNA expression was only significantly increased at 24h (1.6-fold change), 72h (1.6-fold change) and 96h (1.9-fold change) following exercise.

High vs. Low CFPE Group: In comparing the Low and the High myogenic gene mRNA expression in response to eccentric exercise, we observed that there was a greater change in MyoD mRNA expression from Pre to 72h in the High group as compared to the Low group ($p<0.05$, Supplementary Table 4). We also observed that there was a trend for a smaller increase in Myogenin mRNA gene expression from Pre to 24h, in the High group as compared to the Low ($p=0.055$).

Cytokine response to repeated eccentric contractions

Overall: Plasma IL-6 concentrations were significantly increased at 6h (2.2 ± 0.2 pg/mL, $p<0.05$, Supplementary Figure 1E) and 24h (1.6 ± 0.1 pg/mL, $p<0.05$) but at 72h (1.2 ± 0.1 pg/mL) was not different anymore from Pre (1.1 ± 0.1 pg/mL). The change in plasma IL-6 between Pre and 6h ($r = 0.42$, $p<0.05$, Supplementary Figure 2A), as well as Pre and 72h ($r = -0.42$, $p<0.05$) following eccentric contractions was negatively correlated to mixed muscle CFPE index across all participants.

High CFPE vs Low CFPE group: Prior to the intervention, there were no significant differences in plasma IL-6 concentrations in the High compared to the Low group. In the High group, plasma IL-6 concentration was significantly increased at 6h (1.9 ± 0.3 pg/mL, $p<0.05$) and 24h (1.8 ± 0.2 pg/mL, $p<0.05$) but was not significantly different at 72h (1.2 ± 0.2 pg/mL) as compared to Pre (1.2 ± 0.2 pg/mL). In the Low group, plasma

IL-6 concentration was significantly increased at 6h (2.7 ± 0.2 pg/mL, $p<0.05$) and 24h (1.7 ± 0.1 pg/mL, $p<0.05$) but was not significantly different at 72h (1.3 ± 0.1 pg/mL) as compared to Pre (1.2 ± 0.2 pg/mL). In comparing the IL-6 to eccentric exercise in the High CFPE as compared to the Low CFPE group, we observed that there was a greater change in plasma IL-6 concentrations from Pre to 6h in the Low group as compared to the High group (Supplementary Figure 2B; $p<0.05$).

DISCUSSION:

In the present study, we observed that there was an enhanced expansion and activation of the SC pool in individuals with high as compared to low capacity for muscle perfusion following eccentric contractions. Therefore muscle fibre capillarization may be a critical factor for the activation and expansion of the SC pool in response to muscle damage in humans.

SC are indispensable for the repair and/or regeneration of damaged muscle in rodents (Lepper *et al.*, 2011; McCarthy *et al.*, 2011; Sambasivan *et al.*, 2011). In humans, a single bout of high-velocity eccentric contractions results in increased plasma creatine kinase, reduced force production and myofibrillar ultrastructual damage (Beaton *et al.*, 2002; Clarkson & Hubal, 2002; Paulsen *et al.*, 2012). Consequently, eccentric contractions are an effective tool for expansion of the muscle SC pool (Cermak *et al.*, 2013; Crameri *et al.*, 2004; Dreyer *et al.*, 2006; McKay *et al.*, 2009; McKay *et al.*, 2008) though the degree of expansion is dependent on many factors (Snijders *et al.*, 2015). However, factors that determine the degree of activation and expansion of the SC pool are not well understood. In agreement with previous literature, we report that there is an

expansion in the SC pool (as determined by total $Pax7^+$ cells/100 myofibre) and an increase in SC pool activation (as determined by $MyoD^+/Pax7^+$ cells/100 myofibre) in the days following a single bout of eccentric contractions. To better understand factors that determine the degree of activation and expansion of the SC pool we examined whether muscle fibre capillarization may be a determining factor following an acute bout of eccentric contractions in young men. Skeletal muscle capillarization and perfusion is necessary for the delivery of oxygen, growth factors and macronutrients to muscle fibres and resident cell populations alike. We and others have previously already reported an anatomical relationship between muscle SC and capillaries (Christov *et al.*, 2007; Nederveen *et al.*, 2016; Nederveen *et al.*, 2017), suggesting that the proximity of SC to their nearest capillary may be a determining factor in their activation status (Christov *et al.*, 2007; Nederveen *et al.*, 2016). In the present study, there was a positive correlation between the expansion of the total SC pool 24h post-eccentric exercise and mixed muscle CFPE, an index of muscle perfusion, suggesting that the greatest SC pool size expansion was experienced by subjects with the highest capacity for muscle fibre perfusion. When participants were retrospectively divided based on their mixed muscle CFPE index into a High CFPE and Low CFPE group, we observed that there was a greater expansion of the total $Pax7^+$ SC pool in the group with high CFPE (High; CFPE index 7.6 ± 1.0 capillaries $\cdot$ 1000 μm^{-1}) as compared to low CFPE (Low; CFPE index 5.2 ± 0.5 capillaries $\cdot$ 1000 μm^{-1}). This observation was made at 6h (~48% vs. ~1% $Pax7^+$ cells/100 myofibre, respectively) and 24h (~73% vs. ~10% $Pax7^+$ cells/100 myofibre, respectively) post-eccentric contractions. Work by Christov and colleagues (2007) supports these findings as

they observed a correlation between fibre capillarization and SC content in human deltoid muscle in the resting state, regardless of muscle fibre type. Furthermore, we observed that the High group had a greater activation of the SC pool at 6h (~750% vs. ~450% $MyoD^+/Pax7^+$ cells/100 myofibre, respectively) and 72h (~750% vs. ~300% ± $MyoD^+/Pax7^+$ cells/100 myofibre, respectively). Interestingly, although the degree of muscle damage was similar between groups (assessed by increases creatine kinase activity, reduction in force production), we observed that the force production returned to baseline again at 96h post-exercise recovery in the High group, whereas this was not the case in the Low group. Together with the greater activation and expansion of the SC pool size observed in the High group during post-exercise recovery these data indicate that individuals with high CFPE index have an accelerated muscle fibre recovery response following an acute bout of damaging exercise.

In the present study we observed that participants in the High group had a significantly greater percentage of type I muscle fibres as compared with the Low group (~56% vs ~34% Type I fibres, respectively). Type I muscle fibres are more oxidative, associated with more capillaries and/or are perfused to a greater degree than their type II counterparts. Considering that muscle fibre capillaries are shared between the mosaic of fibre types in humans, a greater percentage of type I muscle fibres may result in enhanced perfusion of neighboring type II muscle fibres. A greater association with shared muscle fibre capillaries amongst the muscle fibre type mosaic may contribute to not only a greater type II muscle fibre perfusion, but also contribute to a closer proximity between type II $Pax7^+$ SC and the nearest capillary observed in the High group. Consistent with

this notion, we observed a negative correlation between type II muscle fibre CFPE index and type II $Pax7^+$ SC distance to their nearest capillary. Considering that SC distance to nearest capillary in type II muscle fibres was negatively correlated with a greater change in type II $Pax7^+$ cells/100 myofibre from Pre to 24h following eccentric exercise, we propose that the link between a greater SC activation/expansion in response to muscle fibre damage may be the reduced spatial proximity to microvascular capillaries. In line with this, we have previously observed that type II SC are located at a further distance from capillaries in older men as compared to their young counterparts (Nederveen JCSM). Older men typically exhibit an impaired expansion and/or activation response to exercise (McKay *et al.*, 2012; Snijders *et al.*, 2014), as well as lower basal SC content (Verdijk 2007) concomitant with a loss of muscle capillarization (Proctor 2005). Taken together, these data support a relationship between muscle capillarization and functional SC in humans.

We also observed that enhanced capacity for muscle perfusion (i.e., muscle CFPE) or a reduction in the distance of a SC to its nearest capillary was associated with an enhanced activation and expansion of the SC pool in response to eccentric contraction-induced muscle fibre damage in mixed, type I and type II muscle fibres. Previously, we have reported that following resistance training, there was an increase in muscle capillarization and also an enhanced activation of SC in response to an acute bout of resistance exercise (Nederveen *et al.*, 2017). Taken together, this suggests that muscle capillarization and the ability of the SC pool to activate and expand following exercise/damage are closely linked. Indeed, it is now well established that activated SC

are found at closer proximity to capillaries than their quiescent counterparts (Chazaud *et al.*, 2003; Christov *et al.*, 2007; Nederveen *et al.*, 2016). However, the specific cues for induction of the myogenic program in response to muscle fibre damage remain to be elucidated. The process of SC activation, proliferation and/or differentiation is regulated by a multitude of cytokines and growth factors (e.g., IL-6, IGF-1, myostatin, HGF) (Kadi *et al.*, 2005) considering that CFPE index is regarded as a proxy measure of microvascular perfusion (Hepple & Mathieu-Costello, 2001; Weber *et al.*, 2006), variations in CPFE index could modify delivery of circulating nutrients and/or growth factors and presumably change the local environment of a SC post exercise/damage(Brack & Rando, 2007; Conboy *et al.*, 2005). In this capacity, few growth factors have been as extensively investigated as the cytokine IL-6, a well characterized member of the interleukin family. IL-6 is known to respond to various forms of exercise (Pedersen & Febbraio, 2008), but importantly known to play a role in SC function (McKay *et al.*, 2013; Toth *et al.*, 2011). Furthermore, elevations of IL-6 concentration has been shown to be associated specifically with SC proliferation in response to muscle fibre injury (McKay *et al.*, 2013; Pedersen & Febbraio, 2008; Toth *et al.*, 2011). In the current study, we observed that individuals with a lower mixed muscle CFPE index had a greater increase in plasma concentration of IL-6 from Pre to 6h following eccentric exercise. In line with this, we observe that the Low as compared to the High group had a greater change in circulating IL-6 from the Pre to 6h (increase of ~163% vs. ~66%, respectively). Interestingly, the greater plasma concentration of IL-6 observed in the Low group occurred simultaneously with a lesser activation of muscle SC

(i.e., $MyoD^+/Pax7^+$ cells/100 myofibre) over this same time period. Diminished activation of SC in the Low group despite an elevated systemic plasma IL-6 response in comparison to the High group suggests that there may be other mechanisms that regulate the impact of systemic IL-6 concentration upon SC activation and/or proliferation. Previous work has established that the presence of IL-6 can reduce endothelial signalling in some physiological situations (Yuen *et al.*, 2009). Increased plasma IL-6 concentrations may have implications for an increased local SC niche concentration, and may therefore interfere with the observed cellular cross-talk between SC and endothelial cells that has been observed (Chazaud *et al.*, 2003; Ochoa *et al.*, 2007). Previous work has suggested that IL-6 is produced by various resident cell types such as macrophages (Zhang *et al.*, 2013), fibroblasts (Joe *et al.*, 2010) or endothelial cells (Sironi *et al.*, 1989; Yan *et al.*, 1995), the exercising muscle (Pedersen *et al.*, 2008, Steensberg *et al.*, 2000) as well as SC themselves (Kami & Senba, 2002). Future work should continue to address the paracrine and autocrine functions of increased IL-6 within the local SC niche.

Given the positive relationship between muscle capillarization and the activation and expansion of the SC pool we conclude that the SC response is modulated by cross-talk with endothelial cells within the microvasculature, exposure to circulating signals, or a combination of both. In the future, attention should be focussed on study populations who are compromised in terms of relatively reduced muscle capillarization (Coggan *et al.*, 1992; Proctor *et al.*, 1995), an impaired SC content at rest and in response to exercise (McKay *et al.*, 2012; Snijders *et al.*, 2014) such as those factors found commonly in elderly adults. These future studies may provide insight into whether the blunted post-

exercise SC response in elderly individuals can be improved with increases in muscle fibre capillarization. In conclusion, the present study shows that skeletal muscle fibre capillarization is a major contributing factor to muscle SC activation and pool size expansion, thereby accelerating the muscle repair response, following eccentric contraction induced muscle damage in healthy young men.

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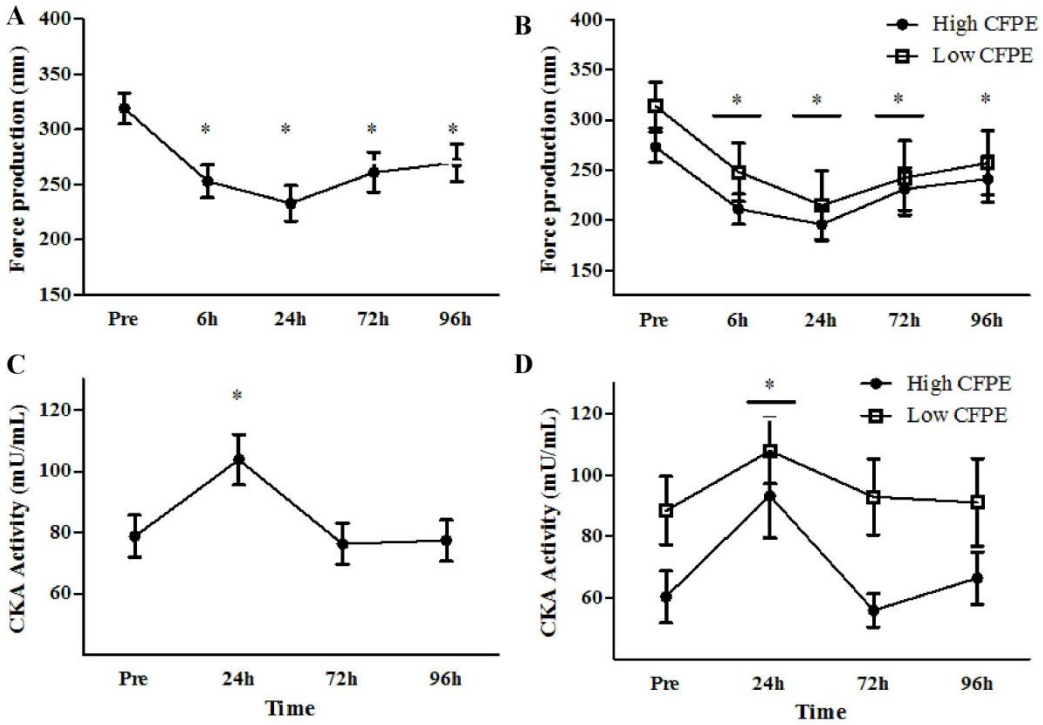
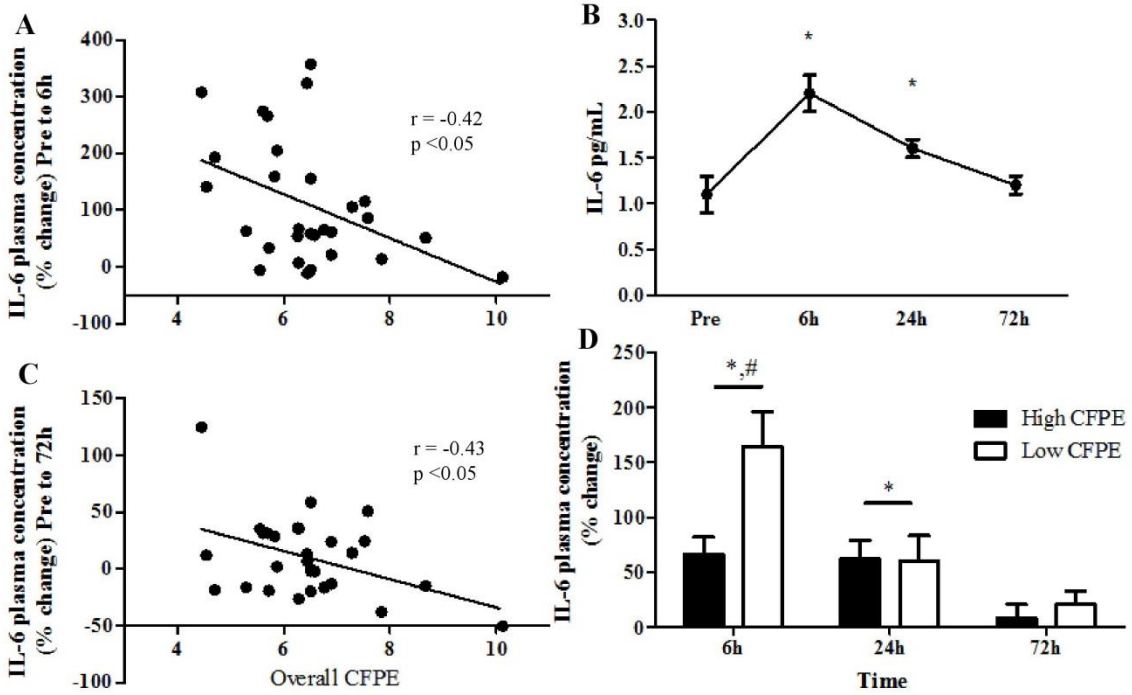
SUPPLEMENTARY FIGURES

Supplementary Figure 1



Sup.1 (**A**) Characterization of the force production (nm) response following eccentric contractions in the overall group. *; Significantly different compared with Pre ($p<0.05$) (**B**) in the group with a high capillary to fibre exchange (CFPE) index and the group with low CFPE index. *; Significantly different compared with Pre in the Low CFPE group ($p<0.05$), bar indicates that effect of time is present for both groups. (**C**) Characterization of the creatine kinase activity (CKA; mU/mL) response following eccentric contractions in the overall group *; Significantly different compared with Pre ($p<0.05$) (**D**) in the group with a high capillary to fibre exchange (CFPE) index and the group with low CFPE index. . Bar indicates significantly different compared with Pre ($p<0.05$), present for both group.

Supplementary Figure 2



Sup.2 (**A**) Relationship between the change IL-6 concentrations (expressed as a percentage) from Pre to 6h post-eccentric exercise and mixed muscle CFPE (r = -0.42, p<0.05) across all participants. (**B**) Characterization of the IL-6 (pg/mL) response following eccentric contractions in the overall group *; significantly different compared with Pre (p<0.05) **(C**) Relationship between the change IL-6 concentrations (expressed as a percentage) from Pre to 72h post-eccentric exercise and mixed muscle CFPE (r = -0.43, p<0.05) across all participants. (**D**) Characterization of the IL-6 response (expressed as a percentage) in the group with a high capillary to fibre exchange (CFPE) index and the group with Low CFPE index. Bar indicates significantly different compared with Pre (p<0.05), present for both group. #; indicates a significantly greater change with time High as compared to the Low CFPE group (p<0.05).

SUPPLEMENTARY TABLES

Supplementary Table 1. Primer sequences for quantitative real-time PCR

Gene Name	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)
Myf5	5′ - ATGGACGTGATGGATGGCTG -3′	GCGGCACAAACTCGTCCCCAA
MyoD	5′- GGTCCCTCGCGCCCAAAAGAT-3′	CAGTTCTCCCGCCTCTCCTAC
MRF4	5′ - CCCCTTCAGCTACAGACCCAA-3′	CCCCCTGGAATGATCGGAAAC
Myogenin	5′ -CAGTGCACTGGAGTTCAGCG-3′	TTCATCTGGGAAGGCCACAGA
GAPDH	5′ -CCTCCTGCACCACCAACTGCTT-3′	GAGGGGCCATCCACAGTCTTCT

Supplementary Table 1. *MyoD*, myogenic determination factor; *Myf5*, myogenic factor-5; *MRF4*, myogenic regulatory factor-4; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase

Supplementary Table 2. Satellite cell response following repeated eccentric contractions across all participants (n=29)

Time	Total Muscle $Pax7^+$ SC (per 100 fibre)	Total Muscle Activated SC ($MyoD^+/Pax7^+$) (per 100 fibre)	Type I $Pax7^+$ SC (per 100 fibre)	Type II $Pax7^+$ SC (per 100 fibre)
Pre	11.8 ± 0.7	0.4 ± 0.1	11.5 ± 0.9	12.1 ± 1.1
6h	14.4 ± 1.0	1.8 ± 0.3*	14.0 ± 1.4	14.7 ± 1.2
24h	14.9 ± 1.1*	2.2 ± 0.2*	14.2 ± 1.1	15.6 ± 1.6
72h	15.8 ± 1.0*	1.9 ± 0.4*	14.2 ± 0.9	17.4 ± 1.5*
96h	11.4 ± 0.8	1.1 ± 0.2*	10.4 ± 0.9	12.4 ± 1.3

*; significant as compared to Pre. Mean ± SEM.

Supplementary Table 3. Satellite cell response following repeated eccentric contractions in the High CFPE (n=10) and Low CFPE (n=10) groups

Time	Group	Total Muscle $Pax7^+$ SC (per 100 fibre)	Total Muscle Activated SC ($MyoD+/Pax7^+$) (per 100 fibre)	Type I $Pax7^+$ SC (per 100 fibre)	Type II $Pax7^+$ SC (per 100 fibre)
Pre					
	High CFPE	11.0 ± 1.2	0.3 ± 0.2	11.6 ± 1.6	10.5 ± 1.9
	Low CFPE	11.9 ± 1.3	0.2 ± 0.1	11.8 ± 1.3	10.9 ± 1.3
6h					
	High CFPE	16.3 ± 1.3*#	2.6 ± 0.6*#	13.9 ± 1.7	18.8 ± 2.4*#
	Low CFPE	11.9 ± 1.7	1.1 ± 0.4	12.3 ± 2.2	11.4 ± 1.3
24h					
	High CFPE	19.1 ± 2.3*#	2.3 ± 0.4*	17.7 ± 2.1	20.6 ± 3.5*#
	Low CFPE	12.8 ± 1.6	1.9 ± 0.5*	12.7 ± 1.5	12.9 ± 1.3
72h					
	High CFPE	17.3 ± 2.0*	2.6 ± 0.6*#	14.0 ± 1.3	20.5 ± 2.7*
	Low CFPE	15.6 ± 1.5*	0.8 ± 0.2	15.6 ± 1.9	14.4 ± 1.2*
96h					
	High CFPE	11.4 ± 0.8	1.1 ± 0.2*	9.5 ± 1.5	13.2 ± 2.3
	Low CFPE	9.6 ± 1.2	0.6 ± 0.2	9.1 ± 1.3	10.2 ± 2.0

Abbreviations : CFPE; capillary-to-fibre perimeter exchange (CFPE) index . *; significant as compared to Pre. #; significantly greater increase from Pre compared to Low. Mean ± SEM.

Supplementary Table 4. Myogenic regulatory factor mRNA expression following eccentric contractions in the High CFPE (n=10) and Low CFPE (n=10) Groups

		Time			
Gene	Group	6h	24h	72h	96h
MyoD					
	High CFPE	2.4*	1.5*	2.1*	1.6*
	Low CFPE	2.4*	1.2	1.4*	1.9*
Myf5					
	High CFPE	1.1	1.4	1.4*	1.8*
	Low CFPE	1.1	2.1*	1.9*	2.7*
MRF4					
	High CFPE	2.1*	1.9*	1.8*	1.9*
	Low CFPE	2.4*	3.1*	1.8*	3.2*
Myogenin					
	High CFPE	4.5*	2.0*	2.1*	2.2*
	Low CFPE	4.5*	7.9*	2.1*	2.5*

Values are expressed as fold change versus *Pre.* Abbreviations: MyoD, myogenic determination factor; Myf5, myogenic factor-5; MRF4, myogenic regulatory factor-4. *; significant versus *Pre,* within Group.

CHAPTER 5:

INTEGRATED DISCUSSION

5 INTRODUCTION

This thesis provides evidence of a relationship between skeletal muscle capillarization and the SC response to stimuli. We first sought to determine the spatial relationship between muscle SC and muscle capillaries in young and older men. We demonstrated, for the first time, that SC are located at a closer proximity to capillaries in younger men as compared to their older counterparts. This finding appeared primarily driven by a greater distance between capillaries and type II fibre-associated SC in older men. Furthermore, quiescent SC are located significantly further away from capillaries than active SC following a single bout of exercise (Chapter 2). We then demonstrated that a 16 wk progressive RT program resulted in enhanced activation of SC following a single bout of resistance exercise. This enhanced activation of SC with training was accompanied by increases in muscle fibre capillarization (Chapter 3). Furthermore, we report that muscle capillarization in healthy young men appeared to be related to a greater activation and/or expansion of the SC pool in response to eccentric exercise-induced muscle damage (Chapter 4). Taken together, this thesis demonstrates that muscle capillarization may be related to the SC response following acute resistance or exercise-induced injury, and may be implicated in adaptation to RT. Furthermore, the spatial relationship between muscle capillaries and SC appears to be altered with aging.

5.1 SIGNIFICANCE OF THE STUDIES

The activation, proliferation and/or expansion of the SC pool are central events in post-exercise recovery, the maintenance of muscle mass and the acute repair process in skeletal muscle.

Collectively, the studies highlight three novel contributions to the literature:

i) There is a greater spatial distance between SC and capillaries in OM as compared to YM in type II fibres, and this represents a potential mechanism underlying age-related SC dysfunction and loss of muscle mass in aging.

ii) Following long term RT, there is enhanced SC activation in response to a single bout of resistance exercise.

iii) Muscle capillarization appears to be positively related to SC pool activation and expansion, concomitant with an accelerated strength recovery, following muscle damage.

SC-capillary distance as a potential mechanism of age-related SC dysfunction

A loss of skeletal muscle SC content and/or function has been implicated in the development of sarcopenia, characterized by loss of muscle size and strength with aging, predominately in type II muscle fibres. SC play a central role in muscle fibre maintenance and plasticity (65) as they are the only established source of new myonuclei (40). Therefore, the loss of SC content and function observed with aging may be responsible, at least in part, for the loss of type II muscle fibre size (46, 50, 72, 73). We observed a lower number of type II associated SC in OM as compared to YM, which is validated by previous work (37, 46, 73). Given that skeletal muscle tissue perfusion is critical for sustaining muscle mass, as it is the means of delivering oxygen, growth factors and nutrients to the muscle, the loss of muscle perfusion may represent a mechanism for impaired SC function and subsequent loss of muscle size with aging. Age-related alterations in blood flow delivery have been attributed to chronic vasoconstriction (12),

loss of endothelial wall functionality (74) muscle capillarization (10, 57) and perfusion (59). Capillarization appears to decline progressively with age, with a reduction primarily observed in type II muscle fibres (10, 22, 25, 54). Therefore, it has been hypothesized that the reduction in type II muscle fibre size may be related to the reduction in type II associated SC and the reduction in type II fibre capillary content observed with advancing age. In Chapter 2, we reported that there is both a loss of capillarization (C/F*i*) and muscle perfusion (CPFE; (31)) with aging. We observed that type II muscle fibre SC are located at a greater distance to their nearest capillary in older as compared to young adults. The increased distance between muscle capillaries and SC, paired with the loss of muscle perfusion, may translate to an increased diffusion distance of circulating factors and/or factors secreted directly from the endothelial cells associated with the capillaries. Ultimately, these changes may be an important factor in age-related impaired recovery of skeletal muscle following exercise (13, 45, 46, 66). In Chapter 2, we also reported that activated SC are in closer proximity to capillaries as compared to their quiescent counterparts, supporting previous work (9). Older individuals have demonstrated a blunted SC activation following acute exercise (46, 66). The greater distance between SC and capillaries observed in OM may serve as a mechanism for this blunted response.

Recently, Snijders and colleagues (64) reported that elderly men with higher baseline capacity for muscle perfusion in type II fibres demonstrated greater muscle hypertrophy and a greater increase in the basal SC pool following resistance training. In light of these findings, it stands to reason that a diminished muscle capillarization, perfusion and/or greater SC-to-capillary distance may be linked to not only a fibre-type

specific loss of muscle mass but also SC content. Interestingly, SC loss has been observed to be proportionate to a reduction in microvasculature in humans (9, 14). The data presented in this thesis represents an addition to a growing body of evidence to suggest that a spatial relationship between muscle SC and capillaries exists, and we hypothesize that capillarization is a critical factor for SC function.

Enhanced SC activation following long term RT in young men

It has been well-established that chronic resistance exercise induces muscle remodeling (i.e., hypertrophy) and a concomitant increase in the basal SC pool (65). Previous work has shown that a single bout of resistance exercise (65) leads to expansion of the SC pool by ~24h, with a peak typically occurring 72h post-exercise (2, 66). Over time, these discrete responses to each training bout contribute to the change in phenotype observed in skeletal muscle with training. However, prior to this thesis, little work had attempted to compare the acute SC response to an exercise stimulus in an untrained versus trained state. In Chapter 3, we observed an enhanced activation of SC in response to a single bout of resistance exercise following chronic training, with a greater number of activated (i.e. $MyoD^{+}/Pax7^{+}$ SC) SC observed 24h post-exercise following 16 wk of RT as compared to the untrained state. Consistent with this observation, we observed an increase in MyoD gene expression (~1.4 fold from Pre) 24h post exercise following RT as compared to no change in the untrained state. The relationship between skeletal muscle capillarization and SC may play an important role in the activation of SC during post-exercise recovery and subsequent muscle fibre adaptation, and therefore we examined changes in muscle fibre capillarization. While RT has been shown to lead to

increases in muscle fibre capillarization in healthy young men (24, 29, 41), our findings that altered activation of the SC pool was accompanied by increased capillarization is novel. We also observed that the temporal-spatial relationship of both quiescent and activated SC in relation to the nearest capillary was changed in response to a single bout of exercise 24h following 16 wk RT. Prior to exercise in both the pre-trained and post-trained state, active SC were located in closer proximity than quiescent SC. In the pre-trained condition, this relationship appeared to be conserved until 72h post-exercise, during which time the distance between capillaries and both quiescent and active SC were not different. In the post-trained condition, the distance between active and quiescent SC was no longer different at the 24h post-exercise period. We speculate that this may be due, in part, to circulating growth factors either delivered via muscle capillaries or released from capillary associated endothelial cells. In turn, this may cause a more rapid activation of SC closer to capillaries, while those at a greater distance (and thus a diminished exposure to these factors) remain quiescent. These small changes may be indicative of an adaptive response of the spatial relationship between SC and capillaries.

Muscle capillarization influences expansion of the SC pool following damage

Using a model of muscle damage (i.e., eccentric contractions), in Chapter 4, we were able to expand our knowledge of the relationship between capillaries and SC activation during muscle repair. In order to induce ultra-structural damage, we used a single bout of muscle-lengthening contractions. A significant increase in serum CK levels and a dramatic reduction in force production indicated that we successfully induced myofibrillar damage in the vastus lateralis (1, 21). We recruited healthy young men with

a broad spectrum of muscle capillary content and perfusion, to examine the amplitude of the SC response to muscle damage in relation to muscle capillarization. It has been shown that SC pool size expands substantially following a single bout of eccentric exercise (13, 16, 38), particularly in SC associated with type II fibres (6, 21). In Chapter 4, we reported that skeletal muscle capillarization is a major factor contributing to muscle repair, as individuals with higher muscle perfusion had a greater SC pool activation and expansion following muscle damage. Furthermore, these individuals with a higher index of muscle perfusion as compared to those with a lower index also had an accelerated muscle force recovery following damage, suggesting that there is a functional improvement in muscle repair as well. We also observed that SC resided closer to capillaries in individuals with a higher capacity for muscle perfusion as compared to those with a lower capacity for muscle perfusion, suggesting that this relationship may be important for SC function. Taken together, the primary and novel finding of the current thesis is that there is an important relationship between muscle capillarization and the SC response to damaging exercise stimuli. Enhanced SC activation following stimuli was observed following increased capillarization (i.e., with training), whereas the age-related impaired SC activation was accompanied by a decrease in capillarization (i.e., with aging). Therefore, these data represent a growing understanding of the relationship between muscle capillaries and muscle SC in the context of skeletal muscle adaptation, repair, and aging.

5.2 POTENTIAL IMPACT OF MUSCLE CAPILLARIES ON SATELLITE CELL FUNCTION

Like other endocrine organs, skeletal muscle can produce and release various cytokines that may lead to paracrine-, autocrine-, or endocrine-mediated activation of SC (55, 67, 68). In Chapter 1, cytokines and/or growth factors that may play a role in regulating SC function (e.g., IL-6, IGF-1, Myostatin, HGF) were discussed. In Chapter 4, we examined changes in IL-6 concentration following exercise-induced muscle damage, as an elevation in IL-6 concentration has been shown to be associated with SC proliferation in response to muscle fibre injury (17, 43, 45, 55, 70). While these growth factors have been shown to mediate the myogenic process, muscle capillaries may be, in part, responsible for the production or delivery of these factors to the muscle and SC niche (outlined in Figure 1).

Cell-cell interaction

Evidenced by the data in this thesis, there appears to be a close spatial relationship between muscle SC and muscle capillaries. Previous work has suggested that there may be cellular cross-talk between SC and endothelial cells (8, 9). Work by Christov et al. (9) demonstrated that SC were supported through soluble growth factors stemming from a capillary homologue. Importantly, this study indicated that in co-culture, endothelial cells were able to promote proliferation in SC-derived myoblasts through the release of growth factors such as IGF-1, HGF, FGF and VEGF. Previous work in humans has shown the importance of IGF-1 (27, 44) and HGF (51) on the muscle SC response following exercise. Therefore, growth factors secreted from endothelial cells may be important for the function of SC. Consistent with this, it has also been suggested that endothelial cells are capable of producing cytokines, with IL-6 (63, 75) and detectable mRNA reported for

IL-3, IL-7, IL-8, IL-11, IL-14 and IL-15 in microvascular endothelial cells (49), suggesting that microvascular capillarization may contribute significantly to the network of regulatory pro- and anti- inflammatory cytokines that have been associated with muscle SC regulation. Factors being produced may also play a role in promoting migration of SC (3, 62). Indeed, SC located in closer proximity to capillaries and situated closer to the site requiring repair or remodeling may be activated quickly, and the reduction in SC to capillary distance following exercise may be reflective of muscle SC migration. Taking into account the close spatial proximity of SC to capillaries observed in Chapter 2, 3 and 4, it stands to reason that the production of cytokines from endothelial cells may play an important role in the activation and regulation of muscle SC.

Local niche environment

Cytokines and/or growth factors produced by various cell populations in the environment immediately surrounding the SC niche may also play an important role in the activation of SC. It is well documented that inflammatory cells and SC communicate with each other (7, 8). Macrophages promote SC proliferation (5, 8) and fibroblasts are known to produce a wide-range of cytokines (61), suggesting that muscle-resident cells may play a role in cytokine production following exercise. The cytokines derived from these cell populations may be sufficient to induce an appropriate physiological response. The distribution of locally produced cytokines may fall to the microvasculature within that muscle area. Therefore, the microvasculature may play a role in the distribution of locally produced cytokines in the area of muscle in which they are produced. Interestingly, both blood flow homogeneity and blood transit time through muscle is

extended significantly when there is a greater capillary content (33). By increasing the blood transit time through greater microvasculature, there may be a higher concentration of cytokines delivered to the local SC niche for extended time periods. Resident cell populations may therefore produce cytokines in concentrations sufficient to induce a local SC response, with more complete distribution through the muscle prior to clearance into general circulation.

Delivery of systemic factors

While the local production of cytokines may be important, these factors may also be derived from other muscles and/or organs and thus appear in general circulation following exercise. It has been consistently reported that IL-6 increases in concentration in blood plasma following muscle contractions (18, 55), depending on the modality, duration and intensity of exercise, up to 8,000-fold (39) representing a considerable systemic increase. The measurement of IL-6 concentrations and hemodynamics across the exercising leg via arteriovenous lines suggest that large amounts of IL-6 are cleared into general circulation (30, 68) from skeletal muscle. Evidence suggests that the systemic environment may play a significant role in regulating the SC population. Using heterochronic parabiosis, where an anastomosis between a young and an old animal is formed, effectively blending the circulation of an old with a young animal, Conboy and colleagues (2005) reported that the regenerative potential of aged muscle SC is improved, implicating key circulating factors in young animals that regulate SC function (11). While these data support the notion that circulating factors may be critical for SC function, the importance of timing of exposure and concentration is paramount. For

example, the role of IL-6 in the regulation of muscle SC is paradoxical, with IL-6 playing an important role in the regulation of SC (68) but elevated systemic levels may also be related to a diminished SC response following exercise in the elderly (45). Transient increases in IL-6 during post-exercise time periods may control SC function and cue myogenic processes, but chronic exposure to IL-6 can be detrimental to both muscle mass (47) and SC function (60). Consistent with this, chronic elevation of IL-6 impairs muscle protein synthesis, and is associated with a loss in skeletal muscle mass with age (28, 45) and physical inactivity (19, 53).

In Chapter 4, we observed that the group with Low CFPE had a greater change in circulating IL-6 from Pre to 6h, which occurred concomitantly with a lower content of activated (i.e., $MyoD^{+/}Pax7^{+}$) muscle SC during the same period. The larger systemic increase in IL-6 observed in the Low CFPE group may reflect a much greater IL-6 concentration at the level of the exercised muscle. For example, IL-6 concentrations that are measured directly at the level of the muscle are different compared to systemic levels (15). For example, the concentration of IL-6 is on the order of 5- to 100-fold higher in exercising muscle as compared to levels found in general circulation (58).

Evidence from Yuen et al. (76) suggests that excessive IL-6 can reduce insulin signaling and insulin-mediated increases in capillary recruitment. Elevated IL-6 concentrations that we observed in systemic circulation in the Low CFPE group may be indicative of a local niche concentration. Ultimately, this may lead to a reduction in the cross-talk between muscle SC and endothelial cells that has been previously observed (8, 52).

Figure 2. Satellite cells and muscle microvasculature.

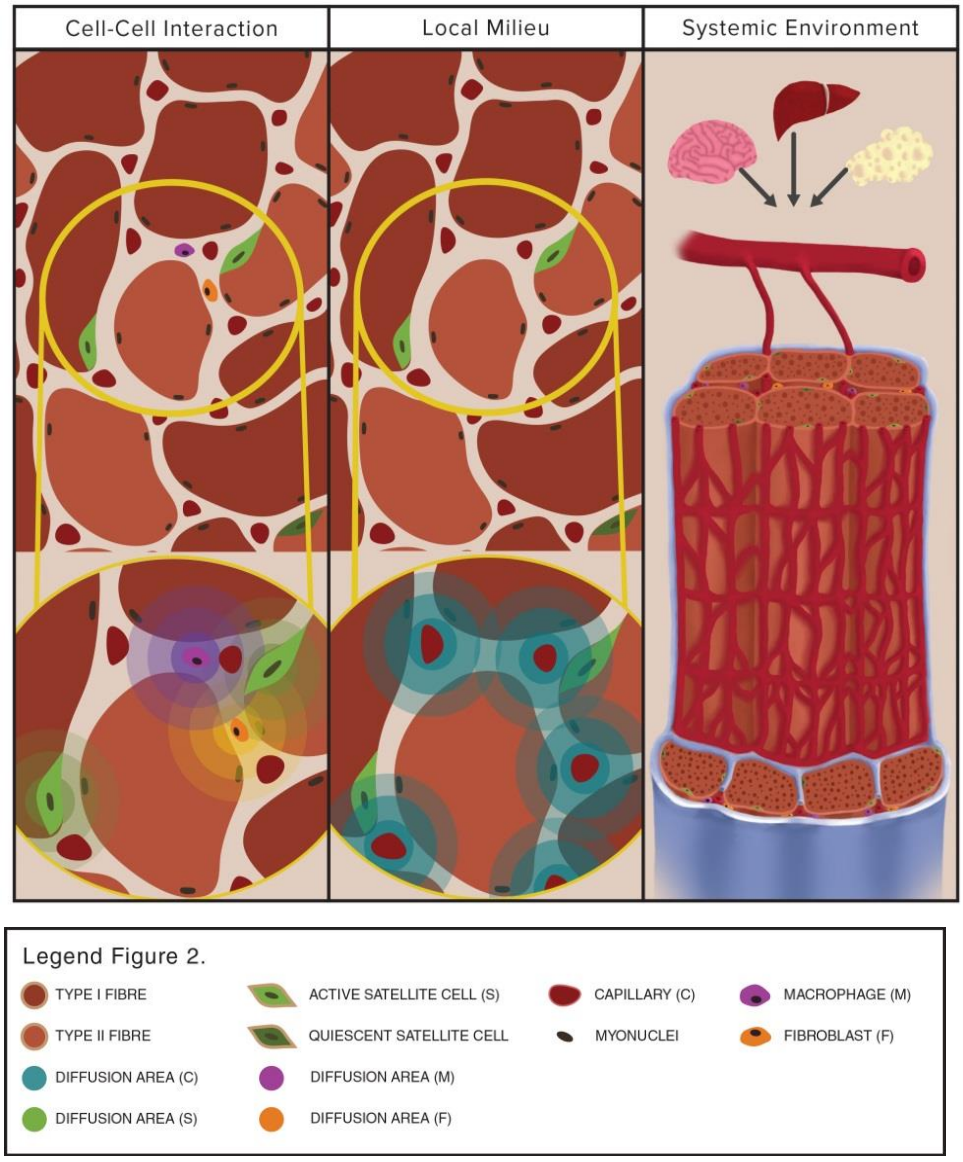


Figure 1. Schematic representation of skeletal muscle microvasculature and muscle fiber cross sections. (**A**) Cross-sections of muscle fibers illustrating the cell-cell interactions between muscle resident fibroblasts, macrophages and various other populations known to secrete growth factors capable of regulating satellite cell activity (**B**) Satellite cells reside in close spatial proximity to muscle capillaries. The cross-talk between endothelial cells, as well as the exposure to signaling molecules delivered from the local environment via capillaries, may be crucial for the regulation of satellite cell activation (**C**) The systemic environment, including blood-borne growth factors and cytokines, can be produced in various organs across the body, and while not necessarily reflective of the local environment, can influence satellite cell activity through prolonged exposure of systemic signals.

5.3 LIMITATIONS AND FUTURE DIRECTIONS

The data presented in the current thesis are novel in their description of the relationship between muscle capillaries and skeletal muscle resident SC. In Chapter 2, we observed that type II-associated SC are located at a further distance from the nearest capillary in older adults as compared to their younger counterparts. Extending the work of Chapter 2, Snijders et al. (64) reported that distance to the nearest capillary was greater in type II- as compared to type I-associated SC in older men. However, despite inducing hypertrophy via 24 weeks of RT in these older men, there was no increase in type II fibre capillarization, nor was there any change in type II SC distance to the nearest capillary. This may suggest that the distance to the nearest capillary may not be a limiting factor during muscle hypertrophy following RT in older men. However, this study did observe that the increase in type II muscle fibre size following 24 weeks of RT was mainly driven by individuals who had a relatively higher muscle capillarization and perfusion prior to training. A loss of muscle perfusion and thus an inadequate supply of nutrients has been suggested as a mechanism for sarcopenia-related loss of muscle mass and a blunted response to anabolic stimuli observed in the elderly (25). It would be interesting to examine whether aerobic training, in order to facilitate an increase in muscle capillarization, prior to the initiation of a long-term RT program would improve the increase in strength and/or muscle mass in older men above what is already observed following RT in older adults (34, 56). Furthermore, there appears to be an enhancement in SC activation following long-term RT in young men in response to a single bout of resistance-type exercise in healthy young men (Chapter 3). Future studies should address

whether long-term RT ameliorates the delayed SC response to exercise observed in elderly men (46, 48, 66). Previous work has demonstrated that the acute expansion of SC following a single bout of exercise translates into long-term changes in skeletal muscle mass following resistance exercise training in young men (2). Therefore, by improving the acute response to exercise in the elderly, larger muscle mass gains from training may be realized.

By isolating endothelial cells and SC from human skeletal muscle biopsies, and co-culturing the cells together in various cell ratios (i.e., high endothelial cells to SC, low endothelial cells to SC) or in various distances to each other, the alteration in influence of endothelial cells upon SC proliferation could be determined *in vitro*. Furthermore, by identifying the secretome of endothelial cells, further experiments could determine which of the growth factors and/or cytokines are influencing SC activity. Indeed, there are many factors (e.g., IL-6, IGF-1, HGF, myostatin, VEGF) that have been implicated in the regulation of SC activation and expansion of the SC pool that may stem from endothelial cells. In Chapter 4, we identified only the IL-6 response to eccentric contraction-induced muscle damage. Further studies should address the analysis of IL-6 in the muscle SC population, with markers of activation. Colocalization analysis could address this limitation by examining the influence of IL-6 upon SC activation, and could expand upon the observed difference in responses between individuals with high as compared to low muscle perfusion. Indeed, factors that have been well-established as co-localizing with SC such as IL-6 (43, 45), IGF-1 (27) and Mstn (46, 66) could be examined at both the systemic and cellular level. Future studies should also aim to examine growth factors that

may be secreted from the microvasculature. For example, VEGF and VEGF-receptors may play an important role in the content of both SC and muscle capillaries. During situations that require full regeneration of muscle (e.g., following myotrauma induced in rodents from methods such as the inducing injury via freeze-crush, ischemia and CTX injections), the site of injury undergoes extensive re-vascularization (32, 35). During muscle regeneration, extensive angiogenesis is required (26) and this process accompanies the restoration of muscle fibre CSA (36). The administration of VEGF, a primary regulator of capillary formation, following muscle injury improves skeletal muscle regeneration in mouse (4) and rabbit models (20). Considering that VEGF has been observed to function in an autocrine fashion via its receptors present on myogenic cells and is capable of stimulating migration, promoting differentiation and preventing apoptosis (8, 23), future work should address the influence of VEGF on the SC response following exercise in humans.

While the current thesis examined the spatial relationship between capillaries and SC, there are a multitude of other structures and cell types that comprise the SC niche and may contribute to SC function. Future work should address other age-related biophysical modifications to the SC niche including the ECM, synthesized by resident fibroblasts, a structure that is crucially important for integrity of the niche and provides regulatory cues to the SC (42, 69, 71). Recent evidence by Mackey et al. (38) points to extensive cross-talk between fibroblasts and muscle SC during muscle regeneration in humans, and thus the spatial relationship between those cell types may be crucial for SC function as well.

5.4 CONCLUSIONS

Skeletal muscle SC are critical for the maintenance of postnatal skeletal muscle throughout the lifespan, as they are indispensable for muscle repair following damage. Due to their anatomical location within the muscle, SC are exposed to a number of growth factors and cytokines that regulate activation and function. SC may be modulated in some capacity by cross-talk with endothelial cells within the microvasculature, exposure to circulating signals, or some combination of both. One of the major findings of this thesis is that there is a greater distance between type II muscle fibre associated SC and capillaries observed in older men as compared to their younger counterparts. We also observed that enhanced activation of the SC pool, and an altered spatial relationship between SC and capillaries occurs in response to a single bout of resistance exercise and is accompanied by increased capillarization following 16 wks RT in healthy young men. We also found that there is a positive correlation between muscle capillarization and the activation and/or expansion of the SC pool following eccentric-contraction induced muscle damage. Furthermore, individuals with higher muscle perfusion recover the loss of muscle strength following exercise-induced muscle injury. In conclusion, the current thesis has advanced our understanding of the relationship between human satellite cells and skeletal muscle capillaries in the context of aging, adaptation and muscle repair. These findings also provide groundwork for future experimentation that will continue to expand our understanding of the regulation of human skeletal muscle stem cells.

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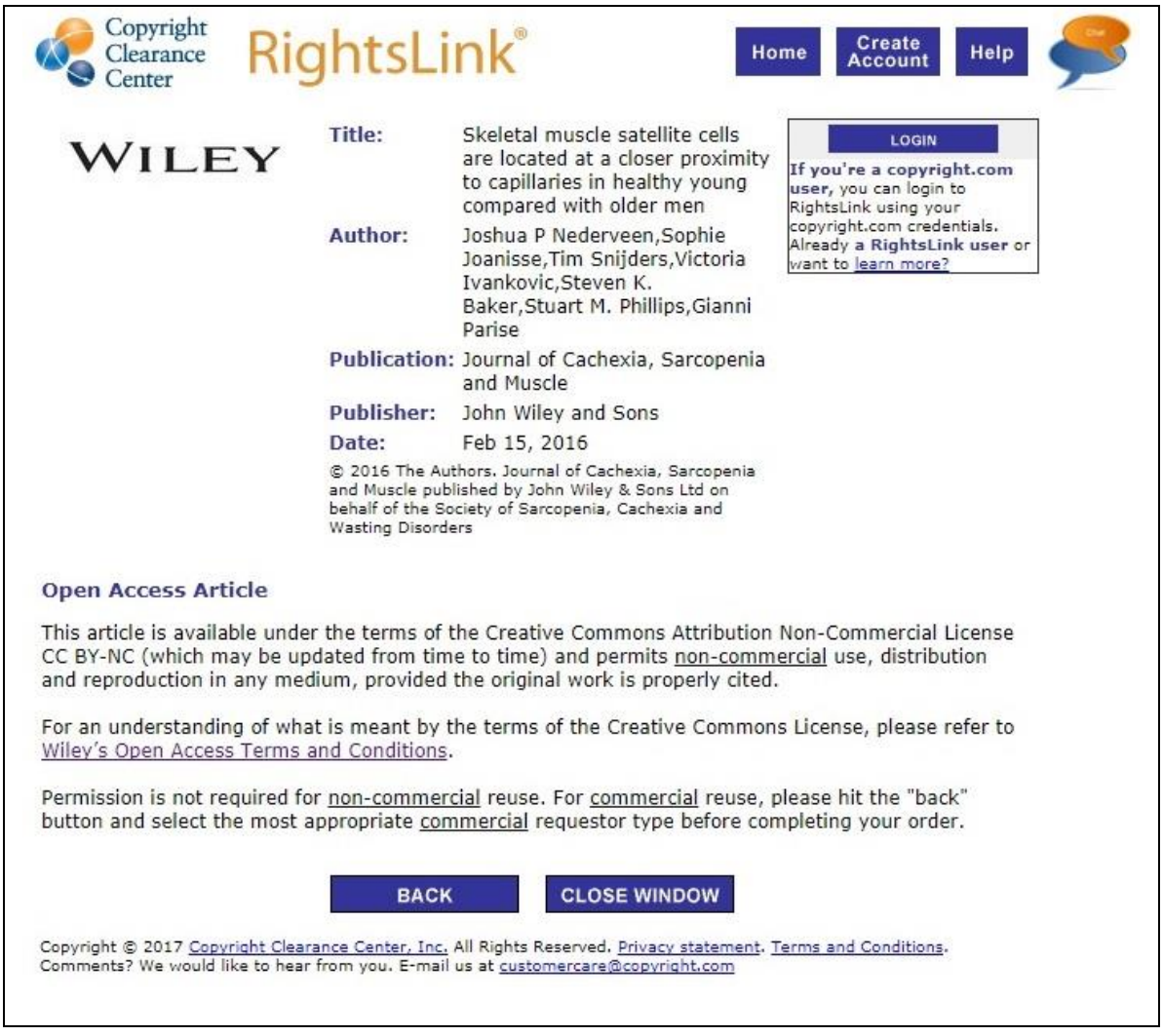

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Title: Skeletal muscle satellite cells are located at a closer proximity to capillaries in healthy young compared with older men

Author: Joshua P Nederveen,Sophie Joanisse,Tim Snijders,Victoria Ivankovic,Steven K. Baker,Stuart M. Phillips,Gianni Parise

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Peer-Reviewed Research Publications (22)

Nederveen JP, Joanisse S, Snijders T & Parise G (2017). The Influence and Delivery of Cytokines and their Mediating Effect on Muscle Satellite Cells. *Current Stem Cell Reports*, (**10**), 1-10.

Baker JM, **Nederveen JP**, Ibrahim G, Ivankovic V, Joanisse S & Parise G. Mechanical loading during exercise training, but not humoral factors, increases marrow cellularity in old mice. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme.* [*e-pub ahead of print*, 28.09.2017.apnm]

McLay KM, **Nederveen JP**, Koval JJ, Paterson DH & Murais JM. (2017). Allometric scaling of flow-mediated dilation: is it always helpful? *Clinical Physiology and Functional Imaging* , [*e-pub ahead of print*, 10.1111/cpf.12465]

Nederveen JP, Snijders T, Joanisse S, Wavell CG, Mitchell CJ, Johnston LM, Baker SK, Phillips SM & Parise G. (2017). Altered muscle satellite cell activation following 16 wk of resistance training in young men. *American journal of physiology Regulatory, integrative and comparative physiology* **312,** R85-R92.

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Nederveen JP, Joanisse S, Snijders T, Thomas ACQ, Kumbhare D, Parise G. *(in submission; JP-RP-2017-275155).* The influence of capillarization on satellite cell pool expansion and activation following exercise-induced muscle damage in healthy young men. *Journal of Physiology*

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