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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

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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.

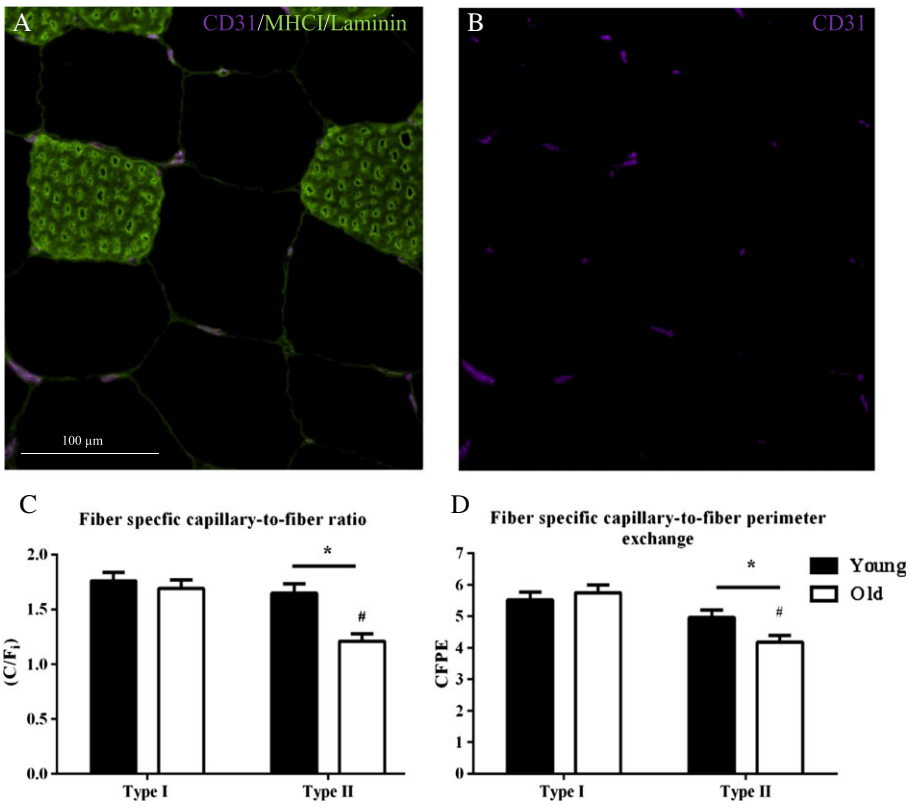
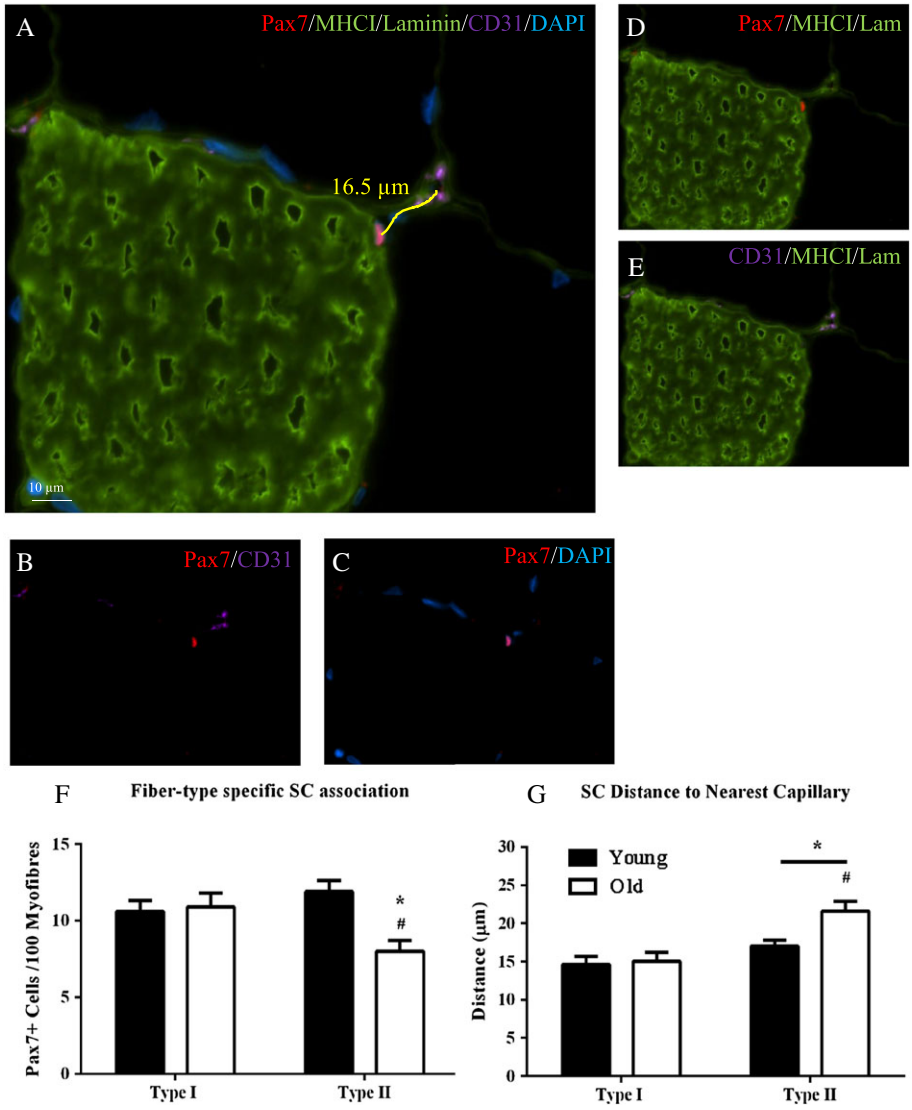


Figure 2 The number of type I and type II satellite cells per muscle fibre. (A) Representative image of a MHCI/laminin/CD31/Pax7 stain of a muscle cross section. Single channel views of (B) Pax7/CD31, (C) Pax7/DAPI, (D) Pax7/MHCI/laminin, and (E) CD31/MHCI/laminin. (F) The number of type I and type II 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).

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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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