TEMPORAL PATTERN OF TYPE II FIBRE-SPECIFIC SATELLITE CELL EXPANSION TO EXERCISE CORRELATES WITH HUMAN MUSCLE HYPERTROPHY: POTENTIAL ROLE FOR MYOSTATIN

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TITLE: Temporal Pattern of Type II Fibre-Specific Satellite Cell Expansion to Exercise Correlates with Human Muscle Hypertrophy: Potential Role for Myostatin

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Abstract

Introduction: The extent of skeletal muscle (SKM) hypertrophy in response to resistance training is highly variable in humans. To explain the nature of this variability we hypothesized that since the myogenic stem cell population, the satellite cell (SC), mediates SKM hypertrophy, variability in the SC response to resistance exercise would account for observed variability in hypertrophy. Furthermore, we examined the role of myostatin (MSTN) in regulating SC function and subsequent SKM growth in humans.

Methods: 23 males (aged 18-35yrs) underwent 16wk of progressive, whole body RT, in addition to two intensity matched (80%1RM) bouts of resistance exercise. Muscle biopsies were obtained from the *vastus lateralis* before, 24h and 72h after an acute exercise bout prior to (Pre-1, 24-1, 72-1) and following (Pre-2, 24-2, 72-2) training.

Results: Following training increases of 7.9%$\pm$1.6 (range of -1.9 – 24.7%) and 21.0%$\pm$4.0 (range of -7.0 to 51.7%) in quadriceps volume (cm^3) and myofibre cross-sectional area (μm^2) respectively were observed. Myonuclear domain (1478.6$\pm$55.5 μm^2/nuclei pre-TR to 1464.5$\pm$70.2 μm^2/nuclei post-TR), and the analogous SC domain (50144.8$\pm$319.2 μm^2/SC pre-TR to 48787.2$\pm$3260.9 μm^2/SC post-TR) , were preserved, with an increase in the number of nuclei per fibre (4.0$\pm$0.2 pre-TR to 4.7$\pm$0.2 post-TR) observed with training, providing evidence of nuclear contribution to hypertrophy. The SC response to a single bout of exercise, demonstrated an elevation in slow-twitch type one fibre associated SC (MHCI-SC) content of 43.4% 24h post-exercise pre-training, that

shifted to increased fast-twitch type two fibre associated SC (MHCII-SC) content of 47.9% 72h post-exercise following training. Analysis of individual SC responses revealed a correlation between the relative change in MHCII-SC content between 24-72h pre-training and the percentage increase in quadriceps lean tissue mass assessed by MRI (r^2=0.663, p=0.001). The extent of MSTN co-localization with SC (Pax7), a potential regulator of the SC response, illustrates a step-wise decrease in the acute time-course following exercise, such that the number of MSTN negative SC at 24 and 72h post-exercise is correlated to SC expansion between Pre-24h and Pre-72h in the pre- and post-training time-courses respectively.

Conclusion: The temporal pattern of the SC response to exercise appears to become altered training; while individual capacity to invoke the SC response is predictive of SKM hypertrophy and may be limited by MSTN protein expression.

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List of Abbreviations

ACTN3	-	alpha-actinin 3
ActRIIB	-	activin receptor type IIB
AIDS	-	acquired immune deficiency syndrome
BrdU	-	bromodeoxyuridine
cdk2	-	cyclin-dependent kinase 2
CK	-	creatine kinase
CSA	-	cross-sectional area
DAPI	-	4’,6-diamidino-2-phenylindole
DEXA	-	dual energy x-ray absorptiometry
E-Protein HEB	-	E-protein HeLa E-box binding protein
ECC	-	eccentric contraction
eEF2	-	eukaryotic elongation factor 2
FSTL-1	-	follistatin-like 1
FSTN	-	follistatin
GDG-8	-	growth-differentiation factor 8
HGF	-	hepatocyte growth factor
HIV	-	human immunodeficiency virus
IGF-1	-	insulin-like growth factor 1
IHC	-	immunohistochemistry
IL-15RA	-	interleukin-15 receptor alpha
IL-6	-	interleukin-6
MHC	-	myosin heavy chain

MHCI	-	myosin heavy chain type I
MHCII	-	myosin heavy chain type II
MHC1-SC	-	myosin heavy chain type I fibre associated satellite cell
MHC2-SC	-	myosin heavy chain type II fibre associated satellite cell
MPS	-	muscle protein synthesis
MRFs	-	myogenic regulatory factors
MRI	-	magnetic resonance imaging
MSTN	-	myostatin
mTOR	-	mammalian target of rapamycin
mTORC1	-	mammalian target of rapamycin complex 1
NCAM	-	neural cell adhesion molecule
NO	-	nitric oxide
OCT	-	optimal cutting temperature
Pax7-DTA	-	Pax7 – diphtheria toxin A
Rb	-	Retinoblastoma
RM	-	repetition maximum
p70 S6K1	-	ribosomal protein S6 kinase 1
SC	-	satellite cell

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Review of the Literature

Skeletal muscle function and maintenance

Skeletal muscle (SKM) is a contractile multinucleated tissue, with the primary functions of locomotion, respiration, metabolism and homeostasis. The importance of SKM is highlighted in the context of disease, disuse and aging, where decline in lean tissue mass is proportional to all cause mortality (60). An established consequence of aging is the progressive decline in SKM mass and strength, referred to as sarcopenia, which can start as early as the fifth decade of life (47). Furthermore, muscle wasting has been associated with an increased risk of morbidity/mortality in patients with a number of diseases including acquired immunodeficiency syndrome (AIDS), cancer, heart and kidney failure (34, 62). Therefore, it is critically important to identify the underlying mechanisms associated with muscle growth and maintenance for the purposes of developing effective countermeasures to prevent the loss of lean tissue mass.

The basic contractile units of SKM, myofibres, are composed of actin and myosin filaments, grouped into bundles surrounded by connective tissue (9). Myofibres contain a contractile apparatus based on the expression of different myosin heavy chain (MHC) isoforms, that can be broken down into fatigue resistant/slow contracting and non-fatigue resistance/fast contracting fibres, referred to as type 1 (MHCI) and type 2 (MHCII) fibres respectively. Upon neural stimulation, calcium release into the cytosolic space results in cyclic cross-bridge formation between the thick MHC and thin actin filaments, causing them to slide over each other, ultimately contracting the muscle, in a process referred to as the

'sliding filament theory' (36). The relative proportion of MHCI and MHCII fibres distributed within a particular muscle dictates the contractile properties of that muscle. Characteristic to SKM regardless of fibre type is a high degree of vascularisation, innervation at the neuromuscular junction, connective tissue and myofibres that combine to form a contractile framework that can be translated into movement due to tendinous junctions with the skeletal system (9).

SKM demonstrates the remarkable ability to undergo complete regeneration as well as extensive adaptation to a wide range of stimuli. A well characterized response to repeated overload perturbations, such as resistance training, is the accretion of lean tissue mass and associated strength gains (25). A dose-response relationship between training volume and SKM hypertrophy has also been described (6, 29). In the early phases of training, gains in strength are primarily attributed to an enhancement in neural activation and better coordinated myosin cross-bridge formation (77), while later in a training program additional gains in strength are a result of increased myofibre volume (46). Subsequently, marked increases in SKM protein content, myofibre cross-sectional area (CSA), lean tissue mass observed through magnetic resonance imaging (MRI) and dual energy x-ray absorptiometry (DEXA) are observed following resistance training (17, 24), and serve as measures of SKM hypertrophy.

Heterogeneous response of human muscle adaptation to training

It is well classified that chronic resistance type training leads to increased muscle size (29); but there is a large degree of inter-individual variation in response to resistance exercise training (3, 35, 68). While several well

documented adaptations to exercise training have been established including increased aerobic capacity, improved insulin sensitivity, gains in lean tissue mass/strength or reductions in blood pressure, what may go unnoticed in some cases is that up to 10 – 20% of the study population show no physiological response to training (91). Gains in lean tissue mass following resistance training can range from no change to a 60% increase in muscle size (35, 91). Given the importance of SKM as outlined above, an inability to gain SKM in response to resistance training is of particular concern. Through dissecting the underlying factors that contribute to the variability in muscle adaptation in response to training, investigators are provided with an opportunity to examine the physiological mechanisms that govern human SKM adaptation.

A number of factors have been identified that may influence the hypertrophic response to training. In particular, the deletion, rather than insertion, of the angiotensin-converting enzyme gene has been associated with enhanced strength gains in response to a nine week training program (26). Further individual genetic polymorphisms in exons four and seven of the interleukin-15 receptor-α (IL-15RA) gene were associated with hypertrophic gains following 10wk of resistance training, accounting for 3.5% and 7.1% of the variation, respectively (71). Additionally, the mutant (polymorphism) R577X genotype of the α-actinin 3 (ACTN3) gene, encoding a Z disc protein in myofibres has been associated with enhanced performance in athletes (101). Specifically, in women homozygous for the ACTN3 R577X genotype, greater strength gains accounting for 2% of variation, following 12wk of resistance training were

observed (10). These studies identify genetic variation as an underlying factor in the regulation of individual hypertrophic responses to resistance training and continued investigation aimed to identify further genotypic candidates is warranted.

Another factor that may be influential in hypertrophic capacity is the ability to stimulate mammalian target of rapamycin (mTOR) signalling in response to acute bouts of resistance exercise. Specifically, mTOR complex 1 (mTORC1) activation has been implicated in stimulating muscle protein synthesis (MPS) following muscle contraction in human SKM (4). Blockade of mTORC1 signal transduction through treatment with rapamycin effectively inhibits the acute ~40% increase in MPS traditionally observed 1-2hr following an exercise bout (21). Phosphorylation of mTORC1 downstream targets, including p70-S6 kinase 1 (S6K1) and eukaryotic elongation factor 2 (eEF2), was also reduced with rapamycin treatment, further confirming a role for mTORC1 signalling in upregulating MPS (21). Interestingly, associations between the degree of phosphorylation of downstream mTORC1 targets and the extent of SKM hypertrophy following 16wk of resistance training has been observed (49). Moreover, it has been suggested that local mechanisms within SKM, rather than systemic regulation, are responsible for the induction of mTORC1 signalling in response to resistance exercise (98), ultimately contributing to elevated MPS. Recently, the role of epigenetic modifiers such as microRNA species have been described for their role as post-transcriptional regulators of gene products in response to exercise (91). microRNAs are ~22 nucleotides in length, and

demonstrate the ability to block translation of protein coding genes, influencing the extent of MPS (91). Following 12wk of resistance training, differential microRNA expression in the top and bottom 20% of responders (based on the extent of SKM hypertrophy observed) revealed differential expression of the microRNA species miR-378, miR-29a, miR-26a and miR-451 (18). Interestingly, miR-378 expression was positively correlated with gains in lean tissue mass (18). Collectively, these results suggest that the magnitude of local stimulation of mTORC1 signalling in SKM following an acute exercise bout, in conjunction with individual microRNA expression profiles, have a role in regulating MPS and ultimately hypertrophy. In conclusion, a number of factors including genetic variation (66), genetic polymorphisms (10, 71), ability to activate specific signalling cascades (21) to enhance MPS, and microRNA expression profiles (18, 91) have been identified for their role in governing the hypertrophic response. Though these studies may explain some degree of the variability associated with individual capacity for muscle adaptation with training, a consensus in the literature has yet to be reached, and further investigation is warranted.

The Myogenic Stem Cell Population – Satellite Cells

The plasticity demonstrated by SKM is thought to be due, in part, to the presence of a tissue-specific myogenic stem cell population, referred to as satellite cells (SC). Initially identified by Mauro in 1961 (48), SC were described based on their niche specific location beneath the basal lamina juxtaposed to myofibres on the surface of the sarcolemma. The primary role of SC is to serve

as the myogenic stem cell population integral to the maintenance and regeneration of muscle following injury or overload (9). Morphological features characteristic of quiescent SC include an increased nuclear-cytoplasmic ratio and heterochromatin content, reduced organelle content and transcriptional activity. SC are normally quiescent; however, in response to high intensity resistance exercise, SC become activated, proliferate and subsequently differentiate where they contribute to the myonuclear content of a muscle fibre (9). Furthermore, a subset of SC, once activated, have the ability to return to a quiescent state for the purpose of maintaining the SC pool, a process referred to as self-renewal (9, 31). Thus SC satisfy all criteria of a "stem cell" population, fulfilling the criteria of self-renewal, in addition to the capacity to differentiate into a specialized cell type.

The series of events demonstrated by SC from quiescence through to terminal differentiation is termed the myogenic programme (Figure I).

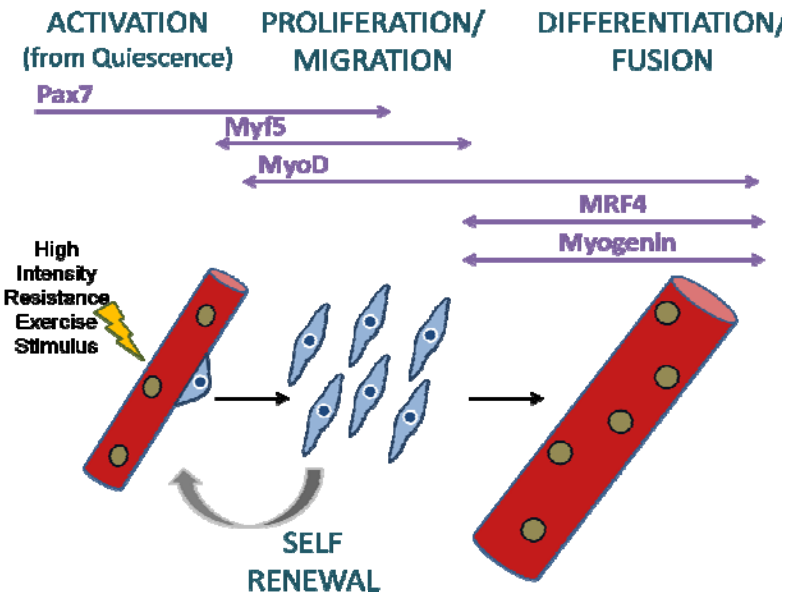

Figure I. THE MYOGENIC PROGRAMME
Following activation, a quiescent SC (Pax7^{+}) will upregulate Myf5 inducing multiple rounds of proliferation. MyoD is subsequently elevated aiding in the transition, where expression of MRF4 and myogenin commit the myoblasts to state of terminal differentiation. Differentiation can involve the fusion of myoblasts to each other in the formation of nascent myotubes, or fusion to existing myofibres, ultimately contributing to muscle growth, regeneration and maintenance.

Progression through the myogenic programme is largely regulated by a network of transcription factors, referred to as the myogenic regulatory factors (MRFs), which include Myf5, MyoD, MRF4 and myogenin. The MRFs are basic helix-loop-helix transcription factors with the ability to bind enhancer regions of DNA for genes encoding cell cycle machinery as well as genes directing terminal differentiation and muscle specific gene expression (9, 31). Traditionally, Myf5 and MyoD have been associated with SC commitment to the myogenic lineage and cell division, while MRF4 and myogenin are involved in driving the process of terminal differentiation (9). Additionally, upstream of Myf5 and MyoD regulation is Pax7, a transcription factor largely involved in SC specification by restricting alternate developmental programs and is commonly used as a marker for SC identification (81). In response to SKM stress or injury, SC activation is marked by the upregulation of Myf5 and MyoD as SC (commonly referred to as myoblasts or muscle precursor cells from this point), proliferate. In addition to myogenic commitment (75), Myf5 is known to induce SC proliferation, as predominance of Myf5 expression in primary myoblasts of $MyoD^{-/-}$ cells (MyoD knockout models) demonstrates enhanced proliferation (76). In contrast, MyoD has been implicated in the transition of SC from proliferation to differentiation (9), as further work with $MyoD^{-/-}$ cells displays reduced capacity for muscle regeneration and altered differentiation kinetics *in vivo* (59). When placed in low serum conditions, traditionally favourable for differentiation, $MyoD^{-/-}$ myoblasts continue to proliferate producing few multinucleated myotubes (14), while transfection with MyoD restores this differentiation capacity (14, 76). Specifically,

MyoD has been shown to form a heterdimeric complex with the beta isoforms of the E-protein, HeLa E-box binding protein (HEB) (65). The resultant MyoD-HEB beta complex has the capacity to bind the E1 E-box region of the myogenin promoter contributing to transcriptional activation, providing a direct link for MyoD in regulating the transition from proliferation to differentiation (65).

Importantly, SC have the ability to revert back to a quiescent state within the satellite cell niche. This is thought to be accomplished by a portion of the activated SC population downregulating Myf5 and MyoD expression and reverting back to a mitotically quiescent phenotype (9, 76, 100). This process of self-renewal is of great necessity for the maintenance of a myogenic stem cell pool and ultimately endows skeletal muscle with the capacity to respond and repair upon subsequent insults. It has also been proposed that the portion of the SC pool responsible for self-renewal does not express MRFs (specifically Myf5) at any point in time (42). Rather it is in the direction of SC division relative to the myofibre that dictates SC fate; with an apical-basal cell division (perpendicular to the myofibre) generating a basal $Pax7^{+}/Myf5^{-}$ cell for self-renewal, and an apical $Pax7^{+}/Myf5^{+}$ cell restricted to the fate of differentiation (42). Due to the difficulty associated with real time measurement of cell fate following apical-basal cell division, limited knowledge exists on this topic and further investigation is warranted prior to reaching a definitive conclusion.

When the proliferative phase of myogenesis has yielded sufficient expansion of the SC pool, a downregulation Myf5 and concomitant increase in MRF4 and myogenin expression is observed. This is accomplished as MyoD

and MRF4 function as upstream regulators of myogenin (70), ultimately promoting terminal differentiation. Although a role for MRF4 in myofibre maturation has been suggested (105), defective embryonic muscle development of MRF4 and myogenin mutant mouse models has limited further study of these genes in myogenesis and further investigation is merited.

A number of molecular markers are available for the detection of SC *in vivo.* Such markers include neural cell adhesion molecule (NCAM) (8, 56), c-met (15, 56), M-caderin (12, 13, 15) as well as expression of the MRFs, MyoD and Myf5 for the detection of activated SC (9, 31). The most commonly used marker by our research group is Pax7, a paired-box transcription factor that is responsible for postnatal maintenance of the SC population (81). Pax7 is expressed during quiescence and early on following activation/proliferation in 97% of all satellite cells and myoblasts (81), and has been identified as the most consistent marker of SC *in vivo*, in comparison to c-met and NCAM in human skeletal muscle (56). The detection of SC with markers enable the use of immunohistochemistry/ immunofluorescence and flow cytometry analysis, simplifying the process of SC quantification in the basal state, or following perturbations such as acute bouts of exercise and in response to training.

Response of SC to High Intensity Resistance Exercise – Acute and Chronic

There is a growing body of literature describing the response of SC to various intensities of resistance exercise in humans, implicating a role for myonuclear contribution during muscle adaptation. Investigation of the "SC response" to acute exercise or following training involves the enumeration of SC

number before and after the perturbation, and subsequently calculating the degree of SC pool expansion. Traditionally, SC in muscle cross-sections are identified based on co-localization of the various SC markers listed above with a nuclear marker (such as 4',6-diamidino-2-phenylindole – DAPI), as well as localization beneath the laminin border within the SC niche. Additionally, inclusion of antibodies raised against MHCI and MHCII allows for fibre type specific SC quantification (Figure II). SC content is then expressed relative to myofibre number (i.e. # SC per 100 fibres), or as a percentage of total myonuclei (53, 56, 67).

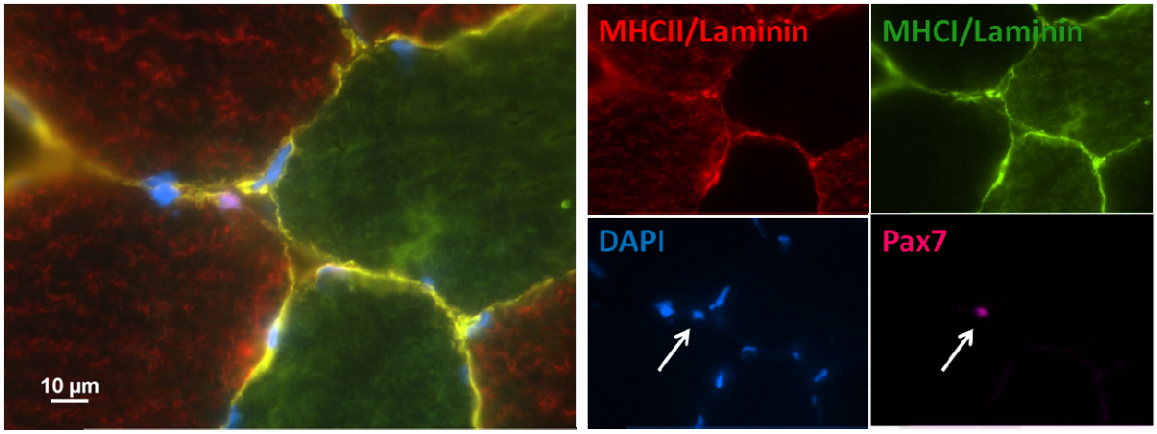

Figure II. SC IDENTIFICATION WITH PAX7
SKM cross sections are prepared from biopsy tissue samples frozen in optimal cutting temperature (OCT), at a thickness of 7µm. Antibodies raised against Pax7 (pink), Laminin (green and red – appears yellow in merge), MHCI (green), and MHCII (red), in addition to DAPI (blue) recognise SC, the niche location, fibre types, and nuclei respectively, allowing for the fibre type specific quantification of SC.

The SC response to a single high intensity resistance based exercise bout is traditionally measured throughout an acute time-course following exercise. A number of investigations have attempted to classify this time-course to determine the time point of peak SC pool expansion, as well as the duration of SC pool elevation post-exercise. A summary of the degree of SC pool expansion in

response to a single high intensity resistance based exercise bout is outlined in Table 1.

Table 1. Acute SC Response to Single High Intensity Resistance Exercise Bout

CHANGE IN SATELLITE CELL #	EXERCISE PROTOCOL (ECC = eccentric contraction)	SC MARKER	SUBJECT GROUP	REFERENCE
<24h Post-EX				
No Change	(4h) – 300 ECC	NCAM	Young Males	O'Reilly 2008 (64)
No Change	(4h) – 300 ECC	Pax7	Young Males	McKay 2009 (53)
No Change	(1 & 3h) – 300 ECC	Pax7	Young Males	Toth 2011 (92)
No Change	(Immed. & 9h) – Endurance + Resistance (40-75% 1RM)	NCAM	Young Males	Snijders 2012 (83)
24h Post-EX				
141% ↑	92 ECC	NCAM	Young Males	Dreyer 2006 (20)
~35% ↑	Resistance EX 80% 1RM	NCAM	Young/Old Males/Females	Petrella 2006 (67)
36% ↑	300 ECC	NCAM	Young Males	O'Reilly 2008 (64)
155% ↑	300 ECC	Pax7	Young Males	McKay 2009 (53)
36% ↑ 28% ↑ 80%	300 ECC	Pax7 NCAM c-Met	Young Males	McKay 2010 (56)
27% ↑	300 ECC	Pax7	Young Males	Toth 2011 (92)
44% ↑ (MHCI-SC)	Resistance EX 75% 1RM	Pax7	Young Males	McKay 2012 (55)
48h Post-EX				
No Change	50 unilateral leg drop-downs 2x80 ECC	NCAM	Young Males	Crameri 2004 (16)
46% ↑ (MHCI & MCHII SC)	Resistance EX 75% 1RM	Pax7	Young Males	McKay 2012 (55)
72h Post-EX				
80% ↑	300 ECC	NCAM	Young Males	O'Reilly 2008 (64)
184% ↑	300 ECC	Pax7	Young Males	McKay 2009 (53)
>72h Post-EX				
192% ↑	(96h) – 50 unilateral leg drop-downs 2x80 ECC	NCAM	Young Males	Crameri 2004 (16)
No Change	(120h) – 300 ECC	NCAM	Young Males	O'Reilly 2008 (64)
~110% ↑	(120h) – 300 ECC	Pax7	Young Males	McKay 2009 (53)
96% ↑	(192h) – 200 ECC	Pax7	Young Males	Mikkelson 2009 (61)

There appears to be no detectable change in SC content prior to 24h post-exercise. This is in agreement with the duration to complete a single round of cell division, as following activation, the cell cycle of SC is estimated to be ~17h (103). At the 24h time point following exercise, an increase in SC content is observed, as all studies with the inclusion of a muscle biopsy 24h post-exercise demonstrate SC pool expansion. Based on the data provided in Table 1, a peak in the SC response may be estimated to occur ~72h post-exercise, with a sustained elevation until ~120h post-exercise. Furthermore, based on the data provided in Table 1, the intensity of the exercise perturbation (or rather the degree of muscle damage resultant from exercise bout) may be proportional to the magnitude of the SC response observed, however this has not been investigated directly. More recently, attention has focused on quantification of the SC response in a fibre type specific manner (55, 94, 95). Though limited data is available, work from our laboratory demonstrates a sustained response of type 1 fibre associated SC (MHCI-SC), significantly elevated at 24h and 48h post-exercise 44% and 46% respectively, in comparison to type 2 fibre associated SC (MHCII-SC), only elevated 46% 48h post-exercise (55). Further investigation regarding fibre type specificity and the degree of variability between individuals within the SC response is warranted. Of particular interest is an investigation by Petrella and colleagues (67) where the degree of SC pool expansion 24h post-exercise was compared between participants who displayed robust or minimal hypertrophy following 16wk of resistance training. Responders and non-responders displayed 40% and 9.7% increases in myofibre CSA respectively.

Importantly, the degree of SC expansion (~35% increase) was consistent between the groups (67). These data are in agreement with the concept of an increase in SC content post-exercise. However, additional analysis with a more complete time-course following exercise to allow for closer examination of potential variability in the SC response to exercise is required, and may explain the differential capacity for hypertrophy with training. Furthermore, the majority of studies to date have employed very high intensity exercise protocols, primarily incorporating eccentric (muscle lengthening) contractions that have yielded valuable results with regards to SC characteristics. However, from a more applied view, it is important to classify the SC response to resistance based exercise designed to mimic a traditional workout or training session.

The influence of resistance training on SC content has been examined in a limited number of investigations, the results of which are summarized in Table 2.

Table 2. Change in SC Content in Response to Prolonged Resistance Training

CHANGE IN SATELLITE CELL #	EXERCISE PROTOCOL	SATELLITE CELL MARKER	SUBJECT GROUP	REFERENCE
46% ↑	10wk	NCAM	Young Females	Kadi 2000 (39)
35% ↑	9wk	Electron Microscopy	Young/Old Males/Females	Roth 2001 (73)
19% ↑ 31% ↑	30d 90d	NCAM	Young Males	Kadi 2004 (38)
49% ↑	16wk	NCAM	Young Males	Petrella 2006 (67)
+38% ↑ (MHCII SC)	14wk	NCAM	Old Males	Verney 2008 (95)
75% ↑ (MCHII SC)	12wk	NCAM	Old Males	Verdijk 2009 (94)
70% ↑	Trained (Powerlifters) vs Un-trained (Controls)	NCAM	Young Males	Kadi 1999 (37)
+100% ↑	Trained (Powerlifters) vs Un-trained (Controls)	NCAM	Young Males	Eriksson 2005 (23)

Collectively, an increase in SC content appears evident with training. However, particular attention should be paid to the timing of the post-training biopsy as the acute expansion in SC number following exercise is known to last several days (64). Therefore, some debate exists regarding training status and SC content. Additionally, two investigations have examined the change in SC content with training in a fibre-type specific manner, proposing a preferential increase in MHCII-SC content (94, 95). This result is of particular interest, as an increase in type 2 fibre CSA is often reported with training (7, 32, 86). Furthermore, in sarcopenic (age associated muscle wasting) conditions, a greater reduction in type 2 fibre size is observed with a concomitant decrease in the MHCII-SC pool (55, 94). Collectively, these studies suggest a role for myonuclear contribution in the process of muscle adaptation and growth, identifying the response of MCHII-SC as a key factor in the regulation of this process.

In summary, a single bout of high intensity resistance exercise is sufficient to induce a SC response, resulting in activation and proliferation as SC progress through the myogenic programme. However, further investigation to determine the influence of training status on basal SC content and on the acute SC response to exercise in a fibre type specific manner is required. The magnitude and duration of the SC response may be dependent on the intensity of exercise that is preformed to induce the response, and the degree of myotrauma that is resultant.

Myonuclear Contribution during Hypertrophy

Since myofibres are terminally differentiated and unable to divide the SC has been identified as the endogenous source of cells, capable of participating in muscle maintenance, growth and repair. Great debate is centered on the necessity of SC in the process of muscle hypertrophy. The myonuclear domain theory, elegantly establishes a role for myonuclear contribution in hypertrophy. Myonuclear domain is defined as the volume of cytoplasm maintained by the gene products of a single myonucleus (63). In response to an overload stimulus, some degree of hypertrophy in the absence of myonuclear contribution is made possible by the upregulation of transcriptional/translational networks by existing myonuclei. However, once the capacity of existing myonuclei is reached, myonuclear addition (through SC contribution) is required to achieve further gains in lean tissue mass. Thus, a proportional increase in myofibre volume and myonuclear content is observed, such that myonuclear domain is maintained (63, 74). A theoretical ceiling in myonuclear domain size of 2000μm^2/myonucleus has been proposed (67), however, reports of variation in myonuclear domain in response to resistance training (41) contribute to the ongoing debate of myonuclear contribution during hypertrophy.

To further outline the role of SC in muscle hypertrophy are investigations that have ablated the SC pool through administration of local gamma irradiation (1). Irradiation has been shown to inhibit SC proliferation, differentiation, and fusion (33, 97), and when administered prior to an overload stimulus (synergist ablation) muscle hypertrophy is prevented (1). One of the main criticisms of the

use of gamma irradiation is its' off target affects on other cell types (50); however, additional work has been done to confirm that endothelial cell function (63) and transcriptional/translational activity are sustained within irradiated muscle (1, 69, 72).

In further support of a role for SC in SKM hypertrophy, is a growing body of literature describing an elevation in SC number prior to the observation of significant hypertrophy. A variety of overload stimuli, including synergist ablation (5, 78, 84), testosterone (82), clenbuterol treatment (85), stretch overload (45, 99) and exercise (39, 44) have been administered previously, with the consistent report of an increase in SC number preceding hypertrophy. In contrast to this are a handful of studies that report muscle hypertrophy with no increase in myonuclear content following treatment with β-adrenergic agonists (commonly clenbuterol) (for a complete list of these studies refer to (50)). However, the majority of these studies utilize total muscle DNA content as a measure of myonuclear content, which has been proven inaccurate as other cell types including fibroblasts, endothelial and inflammatory cells are not considered (40).

Recent work has utilized a novel mouse model, Pax7-diphtheria toxin A (Pax7-DTA), that allows for >90% ablation of $Pax7^{+}$ cells *in vivo* following tamoxifen and DTA treatment (51). Interestingly, ablation of SC prior to synergist ablation still yielded a doubling in plantaris muscle weight after two weeks of overload, that is maintained up to six weeks in comparison to controls (51). Although muscle integrity at the two week time point appears intact, with consistent specific force measures demonstrated between SC ablated and

control animals, this data is not provided for the extended six week time point. Furthermore, an extended time-course is required to define if these increases are maintained. The estimated myonuclear content of the rat EDL muscle is 2.2 million (96), with an anticipated basal rate of myonuclear turnover of 1-2% per week (79). These data equate to a turnover of ~22 000 myonuclei per week, therefore, even in the absence of hypertrophy, basal SC contribution appears to have a key role in the maintenance of muscle integrity.

Unfortunately, specific data pertaining to human SKM is largely correlative in nature. Relationships between myonuclear content and fibre size, (23, 37) have been described. These data suggest a role for myonuclear contribution of SC during hypertrophy. What remains unknown is the degree of individual variability associated with the SC response to exercise, and if any relationship between the degree of SC pool expansion and hypertrophy exists. Additional investigation in this area, with focus on the mechanisms mediating the SC response to exercise is warranted.

In summary, a great deal of evidence supporting a role for SC contribution in the process of SKM hypertrophy exists. Studies focused on the time-course of the SC response preceding hypertrophic gains, irradiation induced ablation of the SC pool as well as examination of the rate of myonuclear turnover support a maintenance of the myonuclear domain following hypertrophy through SC contribution to myofibres (63). Debate exists due to inconsistent reports with regards to a constant myonuclear domain (41), specifically in cases of hypertrophy induced by pharmacological treatment (50) and genetic modification

(51). These data bring into question the relevance and applicability of investigating the necessity of SC contribution to hypertrophy over what occurs during muscle adaptation under normal physiological conditions.

Regulation of SC Response to EX

Although the SC response to resistance based exercise is becoming more established, the cytokines and growth factors that propel SC to enter the myogenic program remain unknown. It is thought that the degree of myotrauma, or muscle damage resultant from high intensity muscle contraction is associated with either direct SC activation, or indirectly through initiating a cytokine-induced signalling response. Muscle damage may include sarcolemma disruption leading to the detection of creatine kinase (CK) in the serum, Z-band streaming, inflammation, swelling/disruption of t-tubules, or damage to the myofibrillar contractile components (28). Previously, an association between the degree of ultrastructural muscle damage following resistance exercise and the proportion of activated SC has been described (73). Additionally, it has been suggested that type 2 myofibres are more susceptible to damage following resistance exercise (28). Collectively, these results suggest a role for myotrauma in regulating the SC response to high intensity resistance exercise. A potential explanation regarding the preferential hypertrophy of type 2 fibres (7, 32, 86) and enhanced expansion of the MHCII-SC pool with training (94, 95) is provided with a greater degree of damage observed in type 2 fibres (27).

A number of signalling molecules have been identified as responsive (up- or down-regulated) to high intensity exercise, and investigated as potential

regulators of SC function. Hepatocyte growth factor (HGF), is one of the few known activators of SC (2, 89), normally sequestered in the extracellular matrix, HGF is released in response to structural stress (87) providing a link between muscle damage and SC activation. Importantly, the role of HGF in SC activation has been described in humans, where an increase in serum HGF 4h, and HGF activator protein in SKM 24h post-exercise were associated with expansion of the SC pool (64). A number of other signalling molecules have been linked with the process of SC activation/proliferation, including but not limited to basic fibroblast growth factor (bFGF) (19), insulin like growth factors -1 (IGF-1) (54), nitric oxide (NO) (88), Delta-1 (11), interlukin-6 (IL-6) (53) and, of particular interest, myostatin (MSTN) (55).

MSTN, a transforming growth factor-beta (TGF-β) family member, also referred to as growth and differentiation factor 8 (GDF-8), has been established as a negative regulator of muscle growth (43). Initially identified in cattle (58), MSTN knockout in multiple species (including a human case study) results in gross muscle hypertrophy with an overall increase in body weight of 2-3 fold in comparison to their wild-type counterparts (57, 58, 80). MSTN signalling (Figure III) is initiated by an extracellular protein binding to the cell surface activin type 2B receptor (ActRIIB), to initiate phosphorylation, dimerization and translocation of Smad2/3/4 complexes to the nucleus, where DNA binding in the promoter regions of genes known to influence muscle growth occurs (22). Additionally, inhibitors of MSTN signalling have been identified, including follistatin (FSTN)

and follistatin-like-1 (FSTL-1) proteins for their ability to bind MSTN inhibiting it from contact with ActRIIB (22).

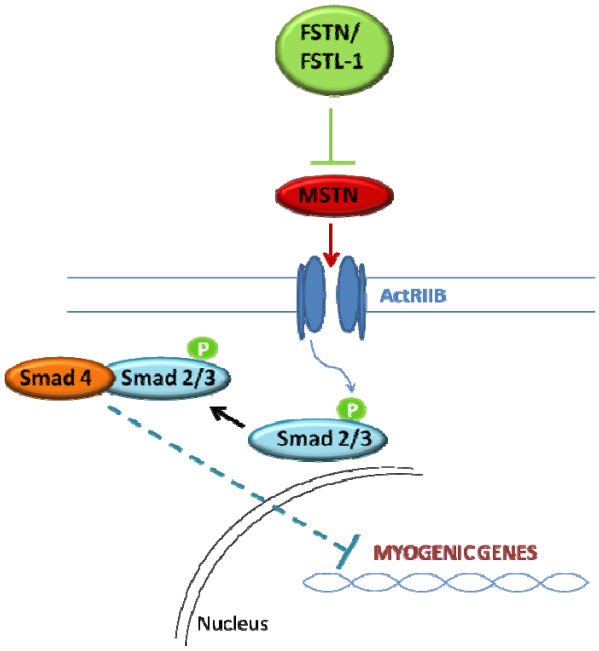

Figure III. MYOSTATIN SIGNALLING
The MSTN signalling cascade is initiated by MSTN binding to the ActRIIB. Following receptor binding, intracellular phosphorylation of Smad2/3 occurs, allow complex formation to occur between Smad2/3 and Smad4. The Smad2/3/4 complex then translocates to the nucleus inhibiting the expression of genes associated with the myogenic program and muscle growth. This process is inhibited when FSTN or FSTL-1 bind MSTN, (maintaining MSTN in an immature form), preventing MSTN binding to the ActRIIB.

The negative effect of MSTN on muscle growth appears to be conserved in humans, with initial studies describing elevated MSTN serum concentrations in conjunction with muscle wasting as a result of age (102), and human immunodeficiency virus (HIV) infection/AIDS (30). Additionally, elevated MSTN protein concentration has been described in the supernatant of primary myoblast cultures isolated from Duchenne muscular dystrophy patients in comparison to controls (104). Importantly, MSTN has been linked as a direct regulator of SC function, negatively influencing multiple aspects of myogenesis. In the absence of MSTN (MSTN knockout mouse models), an increase in the number of active and proliferating SC were observed, assessed based on CD34 positivity and SC bromodeoxyuridine (BrdU) incorporation respectively (52). Additionally, treatment of C2C12 cells with MSTN yields impaired progression of cells into S-

phase, arresting cells in the G1-phase of the cells cycle (90). The negative effect of MSTN on SC proliferation is attributed to elevated p21 protein, in addition to reduced cyclin-dependent kinase 2 (cdk2) levels contributing to hypophosphorylation of retinoblastoma (Rb) protein, all contributing to a blockade in cell cycle progression (90). Furthermore, a dose-response relationship of increased MSTN concentration resulting in decreased myotube formation from human primary cell cultures has been described (93). Recent work from our lab has demonstrated an acute reduction of MSTN protein localization in SC *in vivo* following a single high intensity resistance exercise bout, while aging appears to result in a reduced ability to decrease the proportion of SC expressing MSTN following exercise (55). Collectively, these results identify MSTN as a negative regulator of muscle growth, with the direct ability to inhibit SC function (activation/proliferation/differentiation). Additionally, the extent of MSTN suppression on SC function is responsive to exercise, with a reduction in MSTN protein expression in SC post-exercise. Further investigation to learn the degree of variation, potential association with the magnitude of hypertrophy, and fibre type specificity of SC specific MSTN expression in response to exercise is warranted.

Rational for Research

Though accretion of lean tissue mass and strength are well documented adaptations in response to repeated bouts of resistance exercise (29), a large degree of variation in the hypertrophic response to training is observed between individuals (35). A variety of factors have been identified as regulators of the

hypertrophic process (10, 21, 66, 71, 91, 98), including a relationship between myonuclear content and fibre size (23, 37) as well as SC content (51). Though these studies may explain a small degree of the variability associated with individual capacity for muscle adaptation with training, a consensus in the literature has yet to be reached, and further investigation is warranted.

Given the post-mitotic state of myofibres, an exogenous nuclear source is required for SKM maintenance, repair, and growth, which is provided by the tissue specific stem cell pool resident in SKM, the SC. Progression through myogenesis can be triggered through high intensity resistance exercise, where SC become activated from their normally quiescent state, proliferate and subsequently differentiate, donating their nuclei to re-establish SKM architecture (9). Although there is debate regarding the necessity of SC in the process of SKM hypertrophy (50, 51, 63), there is a growing body of literature describing the response of SC to various intensities of resistance exercise in humans (summary provided in Table 1). Though a variety of signalling molecules have been implicated as regulators of various aspects of myogenesis, the degree of individual variability within these regulators, and the SC response itself to high intensity resistance exercise, require further investigation.

MSTN has been identified as a negative regulator of muscle growth, displaying gross SKM hypertrophy and a double-muscle phenotype in MSTN knockout species (57, 58). Importantly, MSTN signalling is conserved in humans, with a direct inhibitory role on myoblast activation, proliferation and differentiation (52, 90, 93). Similarly, recent work from our lab has demonstrated

an acute reduction in SC specific MSTN protein expression in human SKM *in vivo* following a single high intensity exercise bout (55), though the degree of variability associated with this response remains unknown.

Statement of Research Question and Hypothesis

The purpose of this investigation is to determine the ability of the acute SC response to resistance exercise to predict individual capacity of muscle adaptation (hypertrophy) to training. Additionally, this work aims to characterize the role of MSTN in regulating SC function and subsequent SKM growth in humans. It is hypothesized that individuals with an elevated acute SC response (based on expansion of the SC pool and progression of SC through the myogenic program) will also demonstrate the greatest increases in lean tissue mass. Furthermore, it is thought that SC specific MSTN protein expression will be suppressed to a greater extent post-exercise in these individuals, allowing for enhanced SC proliferation, differentiation, and subsequent muscle growth.

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Temporal pattern of type II fibre-specific satellite cell expansion to exercise correlates with human muscle hypertrophy: potential role for myostatin

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Key Points Summary

- A large inter-individual variability exists in the degree of skeletal muscle hypertrophy observed with resistance training in humans.
- Here we provide further evidence for satellite cell (SC) contribution to hypertrophy following 16wk of resistance training in young males, demonstrating the adaptability of the SC response to a single exercise bout in both fibre type specificity and temporal pattern.
- We report that an individual's ability to invoke the SC response beyond 24h post-exercise is related to training induced muscle growth.
- The SC response may be limited by the degree to which myostatin protein expression within SC is suppressed post-exercise.
- These results identify SC as a contributor to muscle hypertrophy in humans which may be regulated, at least in part, by myostatin.

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Abstract

The extent of skeletal muscle hypertrophy in response to resistance training is highly variable in humans. To explain the nature of this variability, we focused on the myogenic stem cell population, the satellite cell (SC) as a potential mediator of hypertrophy. Twenty-three males (aged 18-35yrs) underwent 16wk of progressive, whole body resistance training, resulting in changes of 7.9%±1.6 (range of -1.9 – 24.7%) and 21.0%±4.0 (range of -7.0 to 51.7%) in quadriceps volume and myofibre cross-sectional area (CSA) respectively. The SC response to a single bout of resistance exercise (80% 1RM), resulted in an expansion in type one fibre associated SC (MHCI-SC) content of 43.7%±10.4 24h post-exercise pre-training, that shifted, post-training, to an increase in type two fibre associated SC (MHCII-SC) content of 47.6%±21.2 72h post-exercise. Analysis of individual SC responses revealed a correlation between the relative change in MHCII-SC content between 24-72h pre-training and the percentage increase in quadriceps lean tissue mass assessed by MRI (r=0.663, p=0.001). The proportion of SC co-localized with MSTN decreased progressively in the acute time-course following exercise and correlated with SC expansion between Pre-24h (r=0.563, p=0.012) and Pre-72h (r=0.454, p=0.045) in the pre- and post-training time-courses. In conclusion, the SC response to exercise appears to become more specific with training; while individual capacity to invoke the SC response is predictive of training induced muscular hypertrophy and may be limited by the degree of MSTN co-localization.

Word count: 230

Abbreviations β2M, beta-2-microglobulin; CSA, cross-sectional area; DAPI, 4',6'-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; DSHB, Developmental Studies Hybridoma Bank; FBS, fetal bovine serum; FSTL-1, follistatin-like-1; GDF-8, growth-differentiation factor 8; MHCI-SC, myosin heavy chain I fibre associated satellite cell; MHCII-SC, myosin heavy chain II fibre associated satellite cell; MRI, magnetic resonance imaging; MSTN, myostatin; OCT, optimal cutting temperature; RIN, RNA integrity number; SC, satellite cell; TGF-β, transforming growth factor beta

Introduction

A common observation following a resistance exercise training program is the wide inter-individual variation in skeletal muscle hypertrophy despite exposure to exercise of the same relative intensity (Hubal *et al.*, 2005; Bamman *et al.*, 2007; Petrella *et al.*, 2008). Accretion of lean tissue following resistance training has been reported to range from between a -2% and 59% change in muscle size (Hubal *et al.*, 2005). The basis for the variability in hypertrophic responses to training is poorly understood; however factors such as genetic variation (Pescatello *et al.*, 2006), genetic polymorphisms (Riechman *et al.*, 2004; Clarkson *et al.*, 2005), the ability to activate specific signalling cascades to enhance muscle protein synthesis (Drummond *et al.*, 2009), and microRNA expression (Davidsen *et al.*, 2011) have been identified as potential control points in determining the hypertrophic response.

Skeletal muscle possesses an endogenous stem cell pool, termed satellite cells (SC) that reside outside of the plasma membrane and beneath the basal lamina. Although the necessity of SC in mediating skeletal muscle hypertrophy (McCarthy & Esser, 2007; O'Connor & Pavlath, 2007; McCarthy *et al.*, 2011), is a hotly debated topic, there is a growing body of evidence describing the response of SC to various intensities of resistance exercise in humans, implying a role for nuclear addition during muscle fibre adaptation (Crameri *et al.*, 2004; Dreyer *et al.*, 2006; Mackey *et al.*, 2007; O'Reilly *et al.*, 2008; McKay *et al.*, 2008, 2009, 2012; Mikkelsen *et al.*, 2009; Toth *et al.*, 2011). SC are normally quiescent; however, in response to high intensity resistance exercise, SC become activated, proliferate and subsequently differentiate donating their nuclei to support muscle growth (Hawke & Garry, 2001; Le Grand & Rudnicki, 2007). Only one

report in humans (Petrella *et al.*, 2006), has attempted to dissect the individual variability in the myogenic response to acute resistance exercise or following resistance training. A consistent increase in SC number (~35%) from baseline to 24h post exercise was reported that did not correlate with the degree of hypertrophy observed following a 16 week training period (Petrella *et al.*, 2006).

Myostatin (MSTN, or growth-differentiation factor 8, GDF-8), a transforming growth factor-beta (TGF-β) family member, has been identified as a negative regulator of muscle growth (McPherron & Lee, 1997). Knockouts of MSTN in multiple species (including a human case study) resulted in gross muscle hypertrophy and an overall increase in body mass of 2-3 fold as compared to wild-type counterparts (McPherron *et al.*, 1997; McPherron & Lee, 1997; Schuelke *et al.*, 2004). Rodent studies have demonstrated that MSTN induces its effects, at least in part, by inhibiting MyoD expression, effectively impairing myoblast proliferation and terminal differentiation (Langley *et al.*, 2002). Importantly, a role for MSTN appears to be conserved in humans as recent work from our lab demonstrated an acute reduction in the co-localization of MSTN to SC (identified with Pax7) in human skeletal muscle *in vivo* following a single exercise bout. Furthermore, aging was found to be associated with an impaired ability to decrease the proportion of SC co-localized with MSTN following exercise (McKay *et al.*, 2012). Collectively, these results imply a role for MSTN in the regulation of human SC function and suggest that MSTN may regulate hypertrophy, at least in part, through the function of SC.

The purpose of this investigation was to determine whether the acute SC response to resistance exercise was related to the individual capacity for muscle

adaptation (hypertrophy) to training. Additionally, this study aimed to characterize the role of myostatin in regulating SC function and subsequent skeletal muscle growth in humans. We hypothesized that individuals with an elevated acute SC response, based on expansion of the SC pool and progression of SCs through the myogenic program, would also demonstrate the greatest increase in lean tissue mass. Furthermore, we hypothesized that myostatin would be suppressed to a greater extent post-exercise in individuals with the greatest SC response.

Methods

Ethical Approval

Consent to complete this investigation was obtained by the McMaster University Research Ethics Board. All procedures conformed to the standards for the use of human subject in research as outlined in the most recent revision of the *Declaration of Helsinki.* Additionally, informed written consent was obtained from all participants prior to commencement of the study.

Subjects

Twenty-three previously untrained healthy males (age 20-30 years) completed 16 weeks of whole body resistance training. Participants were recreationally active but had no formal weight training experience within the past year.

Exercise Training

The training program consisted of four supervised sessions per week, divided into two upper and two lower body sessions. The upper body sessions consisted of chest press, shoulder press, lat pull down, row, bicep curl and triceps extension exercise. The lower body sessions consisted of leg press, leg extension, leg curl, calf press, and abdominal

exercise. The program was designed to progress from two sets performed at 70% of 1RM to four sets performed at 85% of 1RM, with all sets performed to the point of momentary muscle exhaustion. At the conclusion of each workout, participants consumed a beverage containing 30 grams of whey protein, 25.9 g of carbohydrates and 3.4 g of fat (Musashi p30^{TM}, Notting Hill Victoria, Australia).

Acute Exercise Protocol

An intensity matched (80% 1RM) acute exercise bout was performed pre- and post-training, consisting of four sets and eight reps each of leg press, leg extension, calf press and leg curl. The final set was performed to volitional failure. All exercises (with the exception of leg press) were performed using HUR circuit training-type exercise equipment (HUR, Kokkola Finland).

MRI

Magnetic resonance imaging (MRI) scans of the quadriceps were completed by all participants prior to and following the training program. Prior to scanning, participants rested in the supine position for 1h to prevent the influence of fluid shift on muscle volume. Imaging was performed in a 3T HD scanner (Signa MRI System, GE Medical, Milwaukee, WI). Image acquisition in the axial plane was performed with the following parameters: repetition time/echo time = 2100ms/ 23.58 ms; field of view = 28 cm; matrix size = 320/320 reconstructed to 512/512 phase/frequency; slice thickness = 5 mm. Thigh image acquisition utilized an eight-channel torso coil with two excitations. There was a 10 mm gap between slices. Quadriceps volume was measured from the first slice where the rectus femoris was visible to the first slice where the gluteus maximus was visible, and calculated by multiplying the slice area by the distance between slices. The

area of each slice was determined with Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Time of day, joint angle and leg compression were matched in all pre- and post-training scans.

Sample Collection

Muscle biopsies were obtained from the *vastus lateralis* before, 24h and 72h after an acute exercise bout prior to (Pre-1, 24-1, 72-1) and following (Pre-2, 24-2, 72-2) 16 weeks of exercise training. Upon excision, the muscle samples were immediately dissected into pieces that were snap frozen in liquid nitrogen (gene expression analysis), embedded in optimal cutting temperature (OCT) compound for immunofluorescence analysis, or maintained as fresh tissue in growth media (Dulbecco's modified eagle medium - DMEM containing 20% fetal bovine serum - FBS) for flow cytometry analysis.

Flow Cytometry

Muscle samples from biopsies obtained prior to training (Pre-1, 24-1, 72-1) were prepared for flow cytometry analysis. Briefly, muscle samples were weighed prior to mulching with sterile surgical scissors in 35mm tissue culture plates. Single cell suspensions were achieved using enzymatic digestion. 400µl of Collagenase/dispase solution (10mg/ml Collagenase B, Roche Diagnotsics, Mannheim, Germany; 2.4U/ml dispase, Life Technologies, Garlsbad, CA, USA; containing 5µl/ml of 0.5M $CaCl_2$) was added to each plate, triturated for ~2min, and incubated at 37˚C for 9min, this step was then repeated with an incubation time of 5min. The sample was then filtered using a 70µm mesh filter and centrifuged at 800 g for 5min to obtain a pellet of mononuclear cells. Cells were fixed in ice cold 70% ethanol and stored at -20˚C. Samples were

prepared as previously described (McKay *et al.*, 2010) by incubation in Pax7 primary antibody (neat, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), Alexa Fluor 488 goat anti-mouse secondary antibody (1:500, Invitrogen, Carlsbad, CA, USA), and propidium iodide. Flow cytometry analysis was completed on a Beckman-Coulter Epics XL (Beckman-Coulter Inc., Brea, CA, USA) instrument operated by a trained technician.

Immunohistochemistry

Muscle cryosections, 7μm in thickness, were prepared from OCT embedded samples, allowed to air dry for 15-45 minutes and stored at -80˚C. Tissue sections were fixed in 4% paraformaldehyde (PFA) for 10min, washed 3x5min in PBST, blocked for 60min at RT (in PBS containing 2% bovine serum albumin, 5% FBS, 0.2% Triton x-100, 0.1% NaAzide, and 2% goat serum), and subsequently incubated in primary antibodies Pax7 (neat, DSHB), Laminin (1:250 or 1:750, Abcam, Cambridge, MA, USA), MHCI (neat, DSHB, Iowa City, IA, USA), MHCII (1:1000, Abcam, Cambride, MA, USA), and Myostatin (1:150, Millipore, Etobicoke, ON, Canada) for 2hr at RT or overnight at 4˚C. Secondary antibody detection included Pax7 (Alexa Fluor 594 goat anti-mouse, 1:500), Laminin (Alexa Fluor 488 goat anti-rabbit, 1:500), MHCI (Alexa Fluor 488 goat anti-mouse, 1:500), MHCII and Myostatin (Alexa Fluor goat anti-rabbit, 1:500), all from Invitrogen, Carlsbad, CA, USA, for 2hr at RT. Nuclei were labelled with DAPI (4',6-diamidino-2-phenylindole) (1:20000, Sigma-Aldrich, Oakville, ON, Canada), prior to cover slipping slides with fluorescent mounting media (DAKO, Burlington, ON, Canada). Images were taken with a Nikon Eclipse 90i microscope at 20x magnification and captured with a high-resolution QImaging fluorescent camera (Nikon Instruments,

Melville, NY, USA). SC quantity and myostatin positive cells were quantified using the Nikon NIS Elements AR 3.0 software (Nikon Instruments, Melville, NY, USA) on large scale images consisting of ≥100 fibres/subject/timepoint in a blinded fashion.

RNA Isolation

RNA was isolated from 15 – 25mg of muscle using the Trizol/RNeasy method. All samples were homogenized with 1mL of Trizol® Reagent (Life Technologies, Burlington, ON, Canada), in Lysing Maxtrix D tubes (MP Biomedicals, Solon, OH, USA), with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA) for a duration of 40 sec at a setting of 6m/sec. Following a five minute room temperature incubation, homogenized samples were stored at -80˚C for one month (samples may be stored up to one year), until further processing. After thawing on ice, 200µl of cholorform reagent (Sigma-Aldrich, Oakville, ON, Canada) was added to each sample, mixed vigorously for 15 sec, incubated at RT for 5 min, and spun at 12000g for 10min at 4˚C. The RNA (aqueous) phase was purified using the commercially available E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) as per manufacturer's instructions. RNA concentration (ng/µl) and purity (260/280) was determined with the Nano-Drop 1000 Spectorophotometer (Thermo Fisher Scientific, Rockville, MD, USA). RNA integrity reported via RNA integrity numbers (RIN scale of 0 – 10) were determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Toronto, ON, Canada). Samples were reverse transcribed using the commercially available high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in 20µl 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 25µl 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 realplex^2 Master Cycler epgradientS (Eppendorf, Mississauga, ON, Canada). Primer sequences and concentrations, as well as PCR conditions are shown in Table 1.

Table 1. Quantitative real time RT-PCR Primer Sequences/Concentrations and Thermocycler Conditions

Gene	Forward Sequence	Reverse Sequence	Primer Conc. (µM)	Anneal Temp. (˚C)
β2M	5'-ATGAGTATGCCTGCCGTGTGA-3'	5'-GGCATCTTCAAACCTCCATG-3'	2	58
Myf5	5'-ATGGACGTGATGGATGGCTG-3'	5'-GCGGCACAAACTCGTCCCCAAATT-3'	4	55
MyoD	5'-GGTCCCTCGCGCCCAAAAGATT-3'	5'-CAGTTCTCCCGCCTCTCCTACCTCAA-3'	4	55
Myogenin	5'-CAGTGCACTGGAGTTCAGCGCCAA-3'	5'-TTCATCTGGGAAGGCCACAGACACAT-3'	4	55
MRF4	5'-CCCCTTCAGCTACAGACCCAAACAAGAA-3'	5'-CCCCCTGGAATGATCGGAAACAC-3'	4	55
Pax7	5'-GCTCCGGGGCAGAACTACC-3'	5'-GCACGCGGCTAATCGAACTC-3'	4	60
Myostatin	5'-TGGTCATGATCTTGCTGTAACCTT-3'	5'-TGTCTGTTACCTTGACCTCTAAAA-3'	8	58
Follistatin	5'-AGTCCAGTACCAAGGCAGATGT-3'	5'-GGTCACACAGTAGGCATTATTGG-3'	4	60
FSTL-1	5'-AGAGGAGGAGATGACCAGATATG-3'	5'-CGCTGAAGTGGAGAAGATGC-3'	4	62

Statistical Analysis

Statistical analysis was performed using PASW Statistics 18 (SPSS, Quary Bay, HK) analysis software. Two-way-repeated-measures ANOVA tests (with one factor for acute time and one for training status), followed by Bonferonni post-hoc adjustments, paired two-tailed t-tests, and Pearson's correlations were performed where appropriate. Statistical significance was considered to be $p<0.05$ with data reported as mean ± standard error of the mean (SEM).

Results

Skeletal Muscle Mass and Nuclear Content

Sixteen weeks of whole body resistance training resulted in skeletal muscle hypertrophy/gains in lean tissue as quantified on the basis of MRI and muscle histology. Scans of the thigh region revealed a 7.9%±1.6 increase, with a range of -1.9 to 24.7% between individual responses (Fib. 1A/B). Furthermore, we observed an increase in myofibre CSA of 20.0%±3.7 and 13.9%±6.1 in MHCII and MHCI fibres respectively (range of -7.0 to 51.7% within all fibres) following training. Myonuclear domain size was maintained (1478.6±55.5 μm^2/nuclei pre-TR to 1464.5±70.2 μm^2/nuclei post-TR), with an increase in the number of nuclei per fibre (4.0±0.2 pre-TR to 4.7±0.2 post-TR) observed with training (Fig. 2A/B). Similar to myonuclear domain, a "satellite cell domain" was also calculated based on myofibre area (μm^2) per $Pax7^+$ cell, including all fibres as well as in a fibre type specific manner. In agreement with the myonuclear domain findings, SC domain was maintained with training (50144.8±319.2 μm^2/SC pre-TR to 48787.2±3260.9 μm^2/SC post-TR) (Fig. 2C).

Acute Satellite Cell Response to a Single Bout of Resistance Exercise

To quantify the SC response to a single exercise bout pre- (Pre-1, 24-1, 72-1) and post-training (Pre-2, 24-2, 72-2), immunofluorescent staining of Pax7 was performed. SC were quantified in a fibre type specific manner, with co-staining of antibodies raised against Pax7, laminin and the MHCI and MHCII isoforms all within the same tissue section (Fig. 3Aa-d). SC associated with MHCI and MHCII fibre types are denoted as MHCI-SC and MHCII-SC respectively. Analysis revealed that in the un-trained state expansion of the SC pool was specific to MHCI-SC elevated 43.4%±10.4 24h post-exercise, with no change in MHCII-SC content (Fig. 3B and D). Conversely, following training, the acute response appeared to be MHCII-SC specific increasing 47.9%±21.2, with a peak response 72h post acute-exercise, with no change in MHCI-SC content (Fig. 3C and E).

Temporal pattern of SC response as a predictor of muscle hypertrophy

To determine the relationship of the SC response to a single resistance exercise bout, relative change in SC content across the acute time-course was compared to relative gains in lean tissue mass for each individual. Acute exercise in the pre-training state provoked a highly variable MHCII-SC response across individuals (Fig. 4A). Careful examination of individual responses revealed a significant correlation between the relative changes in SC content between 24 and 72h pre-training and the percentage increase in quadriceps lean tissue mass assessed by MRI ($r=0.467$, $p=0.038$) (Fig. 4B). In other words, individuals who demonstrated an increase in SC content between 24 and 72h post-exercise also gained the greatest amount of lean tissue mass with training, while individuals with a decrease in MHCII-SC number from 24 to 72h post-exercise accrued lean tissue mass to a lesser extent. Interestingly, there was a

significant correlation between the change in MHCII-SC and change in muscle volume (r=0.663, p=0.001) (Fig. 4C), while the same association was not apparent for MHCI-SC (r=0.330, p=0.156) (Fig. 4D).

Association of Myostatin with SC following a single bout of exercise

Since MSTN is a known negative regulator of myogenesis, we analysed the co-localization of MSTN with SC in a fibre type specific manner by incubating tissue sections with antibodies recognizing Pax7, MSTN, laminin and MHCI. Quantification of MSTN was performed based on co-localization with Pax7 and DAPI and localization to the SC niche beneath the basal lamina (Fig. 5Aa-d).

Pre-training: The number of $Pax7^+/MSTN^-$ cells expressed per 100 fibres, revealed increases of 57.9%±18.7 and 136.6%±47.8 at 24 and 72h post-exercise respectively (Fig. 5B). This result mirrored that of MHCII-SC (elevated 65.6%±31.9 and 158.2%±50.9 at 24 and 72h), however, within MHCI-SC the number of $Pax7^+/MSTN^-$ cells per 100 fibres was only significantly elevated by 104.5%±16.8 at 72h post-exercise (Fig. 5C/D). Conversely, the percentage of $Pax7^+/MSTN^+$ cells progressively decreased across the acute time-course following a single exercise bout, with basal MSTN co-localized with 65.8%±2.5 of SC, decreasing to 48.4%±1.9 and 30.6%±2.0 24 and 72h post-exercise respectively (Supp. Fig. 1A). This relationship was conserved across both fibre types as the percentage of $MSTN^+$ Type I and II fibre associated SC was similar (Supp. Fig. 1B/C). The acute reduction in the proportion of SC co-localized with MSTN was supported by a 3.9 fold decrease in MSTN mRNA expression 24h post-exercise (Fig. 6A). In contrast to the consistent decrease at 24h post-exercise, MSTN mRNA expression at 72h was variable (Fig. 7B) and correlated to the degree of muscle

hypertrophy assessed by MRI (r=-0.549, p=0.008) (Fig. 6C). Furthermore, the observed reduction in the proportion of SC co-localized with MSTN occurred in parallel with an increase in the number of $Pax7^+$ cells progressing through the cell cycle as determined by flow cytometry analysis. Specifically, the percentage of SC in S-phase progressively increased by 33.9%±14.9 and 50.2%±23.5 24 and 72h following exercise (Fig. 5E/F). This also coincided with a 1.8 fold increase in Pax7 mRNA expression 24h post-exercise (Fig. 6D).

Post-training: Basally, the number of SC per 100 fibres negative for MSTN was elevated 59.4% in comparison to pre-training baseline values (Fig. 5B) Additionally, a 75.6%±38.5 increase in $Pax7^+/MSTN^-$ cell number was observed at 72h post-exercise compared to that measured prior to the acute bout in the post-trained state (Fig. 5B). This effect was driven by the number of MSTN negative, MHCII-SC at 72h post-exercise (92.5%±41.7 increase), as no changes were observed in the MHCI-SC pool (Fig. 5C/D). The elevation in basal SC number negative for MSTN following training was further supported by a 1.6 fold increase in follistatin-like-1(FSTL-1) mRNA expression, a known inhibitor of MSTN signalling (Fig. 6E). Similar to pre-training, a 1.5 fold decrease in MSTN mRNA expression was observed 24h post-exercise (Fig. 6A). Furthermore, the percentage of $Pax7^+/MSTN^+$ cells decreased from 59.0%±3.5 at baseline, to 45.5%±2.3 and 30.7%±3.1 24 and 72h post-exercise respectively (Supp. Fig. 1A). These data are consistently demonstrated in both MHCII and MHCI fibre types (Supp. Fig. 1B/C). These data also provide evidence to support the notion that SC specific co-localization of MSTN is reduced in response to a single exercise bout and that this effect persists in the trained state.

Myostatin as a regulator of SC expansion following exercise

To further classify a relationship between MSTN and SC function, the individual capacity to reduce the proportion of SC co-localized with MSTN was compared to the magnitude of SC expansion following an exercise bout. The number of MSTN negative SC 24 and 72h post-exercise was associated with SC expansion between Pre-24h ($r=0.563$, $p=0.012$) and Pre-72h ($r=0.454$, $p=0.045$) in the pre- and post-training time-courses respectively (Fig. 7A/B). Specific to MHCI fibres, the number of $Pax7^{+}/MSTN^{-}$ cells 24h post-exercise correlated with the magnitude of SC expansion between baseline to 24h following exercise, in the acute time course pre-training ($r=0.507$, $p=0.027$) (Fig. 7C). However, in the post-training acute time-course, specific to MHCII fibres, a trend for a correlation between the number of $Pax7^{+}/MSTN^{-}$ cells 72h post-exercise and MHCII-SC pool expansion between baseline to 72h post-exercise was observed ($r=0.414$, $p=0.069$) (Fig. 7D). Therefore, individuals with the greatest number of $Pax7^{+}$ cells negative for MSTN demonstrated the greatest acute increase in SC number following exercise. This data suggests that SC expansion in response to resistance exercise is associated with down-regulation of MSTN.

Discussion

For the first time, we report a relationship between the acute temporal SC response to exercise and the accretion of lean mass as a result of exercise training suggesting that the acute SC response may be, to some degree, predictive of muscle hypertrophy induced by exercise. We also demonstrate, for the first time in humans, that MSTN expression following acute exercise is inversely associated with the

accretion of lean mass as a result of resistance exercise training and that MSTN may regulate the expansion of the SC pool ultimately influencing hypertrophic capacity.

The desire to understand the underlying factors regulating skeletal muscle hypertrophy has become a significant interest to many groups. This interest is driven by the need to develop pharmaceutical and non-pharmaceutical therapeutic strategies for treating disease associated with muscle wasting and aging. To date, numerous factors have been identified for their role in regulating skeletal muscle hypertrophy yet these factors can only account for a small degree of the variation in muscle growth (Timmons, 2011). Whether the SC is a contributor to muscle hypertrophy has been hotly debated but the question remains unresolved. In this study we focused on the temporal response of SC to an acute bout of exercise as a potential predictor of skeletal muscle hypertrophy during a subsequent 16 week training study. While this is not the first investigation to correlate myonuclear content and muscle fibre CSA (Kadi et al., 1999; Eriksson et al., 2005) or demonstrate an increase in SC number in conjunction with hypertrophy (Kadi et al., 1999, 2004; Sinha-Hikim et al., 2003; Petrella et al., 2006); our study is the first to examine the association between acute expansion of the MHCII-SC pool in response to a single exercise bout (designed to mimic a typical resistance training session) with the degree of hypertrophy observed. Our results suggest that the ability to induce a SC response beyond 24h post-exercise specifically on MHC II positive fibres is a critical piece to maximally enhance muscle mass gains during training. It is conceivable that expansion of the MHCII-SC pool may ultimately provide a larger pool of cells to contribute myonuclei over a longer period of time resulting in a greater degree of hypertrophy over the training period. This relationship is not apparent

in the MHCI-SC, and may be a plausible explanation for the greater extent of myofibre hypertrophy observed in MHCII versus MHCI fibres (20%±3.7 versus 13.9%±6.1 increase in CSA). In agreement, previous investigations have identified a MHCII specific enhancement of the SC pool following resistance exercise (Verney et al., 2008; Verdijk et al., 2009). Collectively, these studies suggest a role for myonuclear contribution in the process of muscle adaptation and growth, identifying the response of MCHII-SC as a key factor in the regulation of this process.

MSTN has been recognized as one of the most potent regulators of muscle mass. Loss of MSTN function (including a human case study) results in gross muscle hypertrophy, a double muscling phenotype, and an overall increase in body weight of 2-3 fold in comparison to wild-type counterparts (McPherron *et al.*, 1997; McPherron & Lee, 1997; Schuelke *et al.*, 2004). These observations have led to significant enthusiasm for the development of MSTN inhibitors for therapeutic purposes. Interestingly, there has been very little evidence to support that MSTN plays a role in muscle hypertrophy in response to physiological stimuli. Reports in the literature, including data from our group, has demonstrated that MSTN gene expression is down-regulated in response to a resistance exercise bout (Raue *et al.*, 2006; McKay *et al.*, 2012), and 24h following remobilization after two weeks of immobilization in humans (Jones *et al.*, 2004). Despite these promising results there is no data in humans implicating MSTN as playing a relevant role in skeletal muscle hypertrophy in response to resistance exercise. In the current investigation we report a significant down-regulation of MSTN mRNA expression 24h post acute exercise (Fig. 6A). Perhaps more importantly, we report a correlation between MSTN mRNA expression 72h post-

exercise (pre-training) and lean tissue gains (MRI), revealing that those with the lowest MSTN gene expression 72h following an acute bout of exercise were the individuals who gained the most lean mass in the subsequent 16 week training program (Fig ????). This result highlights two important points. First, that MSTN gene expression may be a predictor of muscle hypertrophy in response to resistance exercise training in humans. Second, that analysis of individual responses at time-points where no significant group effects are observed may be the most important points to investigate in attempting to understand the individual variability in skeletal muscle hypertrophy. Furthermore, MSTN has been implicated in the regulation of SC function by impairing activation and differentiation, as previously demonstrated in animal (Langley *et al.*, 2002) and cell culture models (McCroskery *et al.*, 2003). Additionally, recent work from our lab demonstrated an acute down-regulation of the proportion of SC co-localizing with MSTN in response to an acute exercise bout in young and older men (McKay *et al.*, 2012). In the current study, we again demonstrated a decrease in the proportion of SC co-localizing with MSTN following an acute exercise bout and observed that the number of SC negative for MSTN (relative to fibre number) was elevated post-exercise. This observation was exclusive to MHCII fibres following training. We hypothesize that SC activation and progression through the myogenic program requires down-regulation of MSTN. Perhaps most interesting was the observation that the magnitude of the SC pool expansion was correlated to the number of MSTN negative SC following acute exercise (Fig. 7). This suggests that MSTN negatively regulates SC pool expansion. Collectively, the presence or absence of MSTN in SC may dictate SC expansion

following exercise, which according to our data and that of others (Langley et al., 2002; McKay et al., 2012) appears linked to skeletal muscle hypertrophy.

We evaluated skeletal muscle hypertrophy/muscle mass accretion using MRI and myofibre CSA. Like other reports before ours (Hubal et al., 2005; Bamman et al., 2007) we observed a great degree of individual variability in muscle growth whether assessed using MRI or CSA. The observation of unaltered myonuclear domain size in addition to an increase in the average number of nuclei per myofibre following training strongly suggests a role for nuclear accretion in the process of hypertrophy. This conclusion is further supported by an unchanged SC domain, which is analogous to a myonuclear domain, consisting of myofibre area per Pax7+ cell. Consistent with previous work (Eriksson et al., 2005; O'Connor & Pavlath, 2007; Mackey et al., 2011), our data suggests that as myofibre size increases there is a concomitant increase in the progenitor pool to maintain and support growth of the myofibre.

This study is the first to demonstrate a shift in both fibre type specificity as well as timing of the acute SC response as a result of resistance training. In the un-trained state, expansion of the SC pool was driven by greater proliferation of MHCI-SC peaking 24h post-exercise. Alternatively, following the 16 week training period, the acute MHCII-SC response was of greater magnitude, progressive and sustained out to 72h post-exercise. These results are supported by observations in sarcopenic muscle, where the reduction in muscle mass with aging was shown to be specific to the reduced size of MHCII fibres (Verdijk et al., 2009). In accordance with this observation a reduction in MCHII-SC content was observed (Verdijk et al., 2007; McKay et al., 2012). Interestingly, this phenomenon was reversible, as following 12wk of RT in older men, a

MHCII fibre specific increase in CSA (6 – 9%), accompanied by a 75% increase in MHCII-SC content (4.8±0.3 to 8.4±0.8 SC per 100 myofibres) was observed, with no change in MHCI-SC content (Verdijk et al., 2009). We hypothesize that an underlying mechanism governing the transition in fibre type specificity of the acute SC response, from type 1 pre-training to type 2 post-training is that of repeated type 2 fibre activation with training. Alterations in fibre recruitment with training may influence the SC response to exercise, given that stimulation of myofibres to induce contraction results in the release of cytokines, and local tissue disruption, which are known activators of SC activity. Collectively, these results suggest that prior to training MHCII fibre recruitment was irregular resulting in an emphasis on a MHCI-SC response to exercise. Throughout training, continued recruitment of fast motor units may result in a refined SC response that is MHCII fibre specific. It should be noted that a number of other factors, not just fibre activation, may be integral in regulating training induced adaptations of the acute SC response to exercise.

In summary, the results presented in this investigation identify the MHCII-SC response as a mediator of muscle mass in humans. Specifically, the ability to expand MHCII-SC beyond 24h in response to a single high intensity exercise bout is strongly related to hypertrophic potential with resistance training. The magnitude of the MHCII-SC response appears to be regulated by individual capacity for an acute reduction in SC specific MSTN protein expression following exercise. These data suggest that under physiological conditions the contribution of nuclei to myofibres by SC is an important event in the regulation of muscle hypertrophy.

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Author Contributions

Study conception and design: G.P., S.M.P., C.J.M., L.M.B. Sample collection: S.M.P., S.B., L.M.B., C.M., S.J., B.M. Data analysis: L.B., S.J., B.M., C.J.M. Manuscript preparation: L.M.B., G.P. Manuscript editing: L.M.B., G.P., S.J., S.M.P., C.J.M. All authors approved the final version for publication. The current study was completed at McMaster University, Hamilton, Ontario, Canada.

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Tables

Table 1. Quantitative real time RT-PCR Primer Sequences/Concentrations and Thermocycler Conditions

β2M, beta-2-microglobulin; MRF4, muscle-specific-regulatory-factor-4; FSTL-1, follistatin like-1.

Figures and Legends

Figure 1. Skeletal Muscle Hypertrophy following Training

SKELETAL MUSCLE hypertrophy was assessed with MRI scans of the quadriceps to calculate volume (cm^3); overall (A) and individual values (B) are demonstrated pre- and post-training. * denotes a significant difference from Pre-TR ($p < 0.05$).

Figure 2. Myonuclear Contribution to Muscle Hypertrophy

Myonuclear content and fibre size were quantified by visualization of nuclei (Pax7/DAPI) and myofibre borders (laminin) on muscle cross-sections. Calculations of (A) myonuclear domain (fibre area (μm^2) per nucleus), (B) nuclei per fibre, and (C) satellite cell domain (analogous to myonuclear domain; SC domain refers to the average fibre area (μm^2) per $Pax7^+$ cell), are reported. * denotes a significant difference from Pre-TR ($p < 0.05$).

Figure 3. Acute Satellite Cell Response to a Single Bout of Resistance Exercise

(A) Representative image (merge) of a Pax7/Laminin/MHCI/MCHII immunofluorescent stain. Arrow denotes a $Pax7^+$ cell associated with a type 2 fibre. Single channel views of (a) MHCII and laminin (red), (b) MHCI and laminin (green), (c) DAPI (blue), (d) Pax7 (pink) are provided, scale bar measures 10μm. The SC response to an acute bout of exercise was quantified in a fibre type specific manner, prior to and following 16wk of resistance training. The SC response of (B) MHCI-SC pre-training, (C) MHCI-SC post-training, (D) MHCII-SC pre-training, and (E) MHCII-SC post-training are expressed as SC number per 100 myofibres. * denotes a significant difference from respective Pre timepoint (Pre-1 for pre-training time-course, and Pre-2 for post-training time-course) ($p < 0.01$)

Figure 4. Temporal pattern of acute SC response as a predictor of muscle hypertrophy

(A) Variation of individual MHCII-SC response to a single exercise bout assessed pre-training, (SC content expressed as MHCII-SC per 100 fibres). A relationship between the acute SC response, from 24 to 72h post-exercise and the extent of SKELETAL MUSCLE hypertrophy (assessed via MRI) was established within (B) the entire SC pool ($r=0.467$, $p=.038$) and (C) MHCII-SC ($r=0.663$, $p=.001$), though it was not evident with MHCI-SC ($r=0.330$, $p=.156$). Significance was considered at $p<0.05$

Figure 5. Association of Myostatin with SC following a single bout of exercise

(A) Representative image (merge) of a MSTN/Pax7/Laminin/MHCI immunofluorescent stain. Arrow denotes a type 2 fibre associated $MSTN^{+}/Pax7^{+}$ cell. Single channel views of (a) Pax7 (red), (b) MSTN (yellow), (c) DAPI (blue), and (d) MHCI and Laminin (green) are provided, scale bar measures 10μm. Quantification of MSTN co-localization to SC ($Pax7^{+}$) was performed with the inclusion of (B) SC associated with all myofibres, (C) MHCII-SC, and (D) MHCI-SC, with data expressed as $MSTN^{-}/Pax7^{+}$ cells per 100 fibres. SC cell cycle kinetics in response to a bout of resistance exercise was analyzed through flow cytometry analysis of Pax7/PI staining in the acute time-course pre-training. Percentage of SC ($Pax7^{+}$) in (E) all cell cycle phases (G_o/G_l, S, G_2/M), and (F) S-phase are demonstrated. * denotes a significant difference from respective Pre timepoint (Pre-1 for pre-training time-course, and Pre-2 for post-training time-course) ($p < 0.01$). # denotes a significant difference between Pre-1 and Pre-2 ($p<0.01$).

Figure 6. Gene Expression Analysis

Whole muscle mRNA expression of (A) MSTN, (D) Pax7, and (E) FSTL-1 at baseline, 24 and 72 post-exercise in the pre-training (white bars) and post-training (black bars) state. (B) Variability associated with MSTN mRNA expression in the pre-training time course, such that (C) MSTN mRNA expression at 72h is correlation to muscle hypertrophy assessed via MRI ($r=-0.549$, $p=0.008$). * indicates a significant difference versus respective Pre timepoint (Pre-1 for pre-training time-course, and Pre-2 for post-training time-course) ($p < 0.01$). # denotes a significant difference between Pre-1 and Pre-2 ($p<0.01$).

Figure 7. Myostatin as a regulator of SC expansion following exercise

MSTN regulation of the SC response to exercise is evident by the relationship between an increase in the number of $MSTN^-/Pax7^+$ cells per 100 fibres and SC pool expansion. In the pre-training time-course, SC expansion from Pre- to 24h post-exercise was associated with the number of (A) $MSTN^-/Pax7^+$ cells per 100 fibres ($r=0.563$, $p=.012$), and (C) MHCI fibre associated $MSTN^-/Pax7^+$ cells per 100 fibres ($r=0.507$, $p=.027$) 24h post-exercise. The post-training acute time-course demonstrates a similar relationship between SC expansion from Pre- to 72h post-exercise and the number of (B) $MSTN^-/Pax7^+$ cells per 100 fibres ($r=0.454$, $p=.045$), and (D) MHCII fibre associated $MSTN^-/Pax7^+$ cells per 100 fibres ($r=0.415$, $p=.069$ - trending) at 72h post-exercise. Significance was considered at $p<0.05$

Supplemental Figure Legend

Supplemental Figure 1. Reduction of Myostatin Co-localization with Satellite Cells in Response to a High Intensity Resistance Exercise Bout

A step-wise reduction in the percentage of $Pax7^{+}$ cells co-localized with MSTN is demonstrated over the acute time-course in response to exercise, and demonstrated within (A) all SC, (B) MHCII-SC, and (C) MHCI-SC. The reduction in SC specific MSTN co-localization follow exercise is observed both pre-training (white bars) and post-training (black bars). * denotes a significant difference versus respective Pre timepoint (Pre-1 for pre-training time-course, and Pre-2 for post-training time-course) ($p < 0.01$). # denotes a significant difference between Pre-1 and Pre-2 ($p<0.05$).

Figure 1

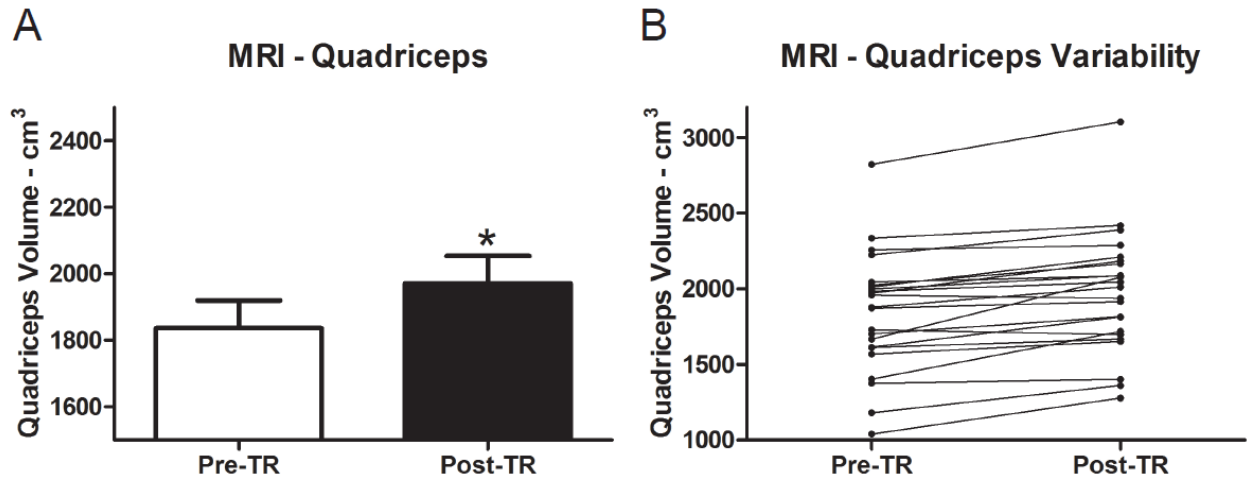

A
MRI - Quadriceps
Quadriceps Volume - cm3
2400
2200
2000
1800
1600
*
Pre-TR
Post-TR
B
MRI - Quadriceps Variability
Quadriceps Volume - cm3
3000
2500
2000
1500
1000
Pre-TR
Post-TR

Figure 2

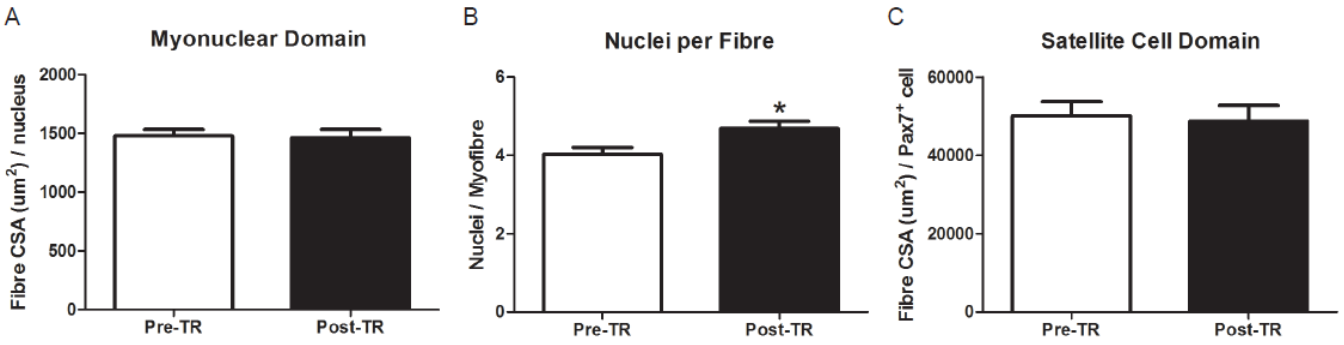

A
Myonuclear Domain
Fibre CSA (um2) / nucleus
2000
1500
1000
500
0
Pre-TR
Post-TR
B
Nuclei per Fibre
Nuclei / Myofibre
6
4
2
0
*
Pre-TR
Post-TR
C
Satellite Cell Domain
Fibre CSA (um2) / Pax7+ cell
60000
40000
20000
0
Pre-TR
Post-TR

Figure 3

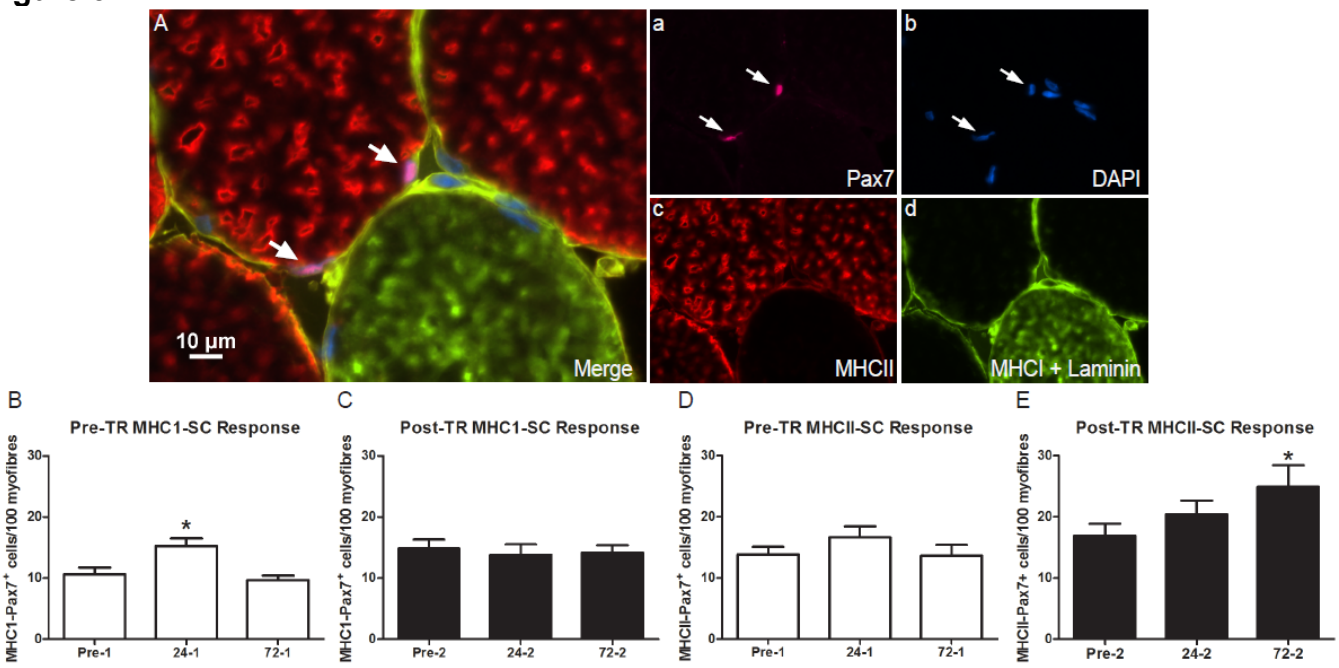

A
a
Pax7
b
DAPI
c
MHCII
d
MHCI + Laminin
10 µm
Merge
B
Pre-TR MHC1-SC Response
C
Post-TR MHC1-SC Response
D
Pre-TR MHCII-SC Response
E
Post-TR MHCII-SC Response
MHC1-Pax7+ cells/100 myofibres
MHCII-Pax7+ cells/100 myofibres
Pre-1
24-1
72-1
Pre-2
24-2
72-2
*

Figure 4

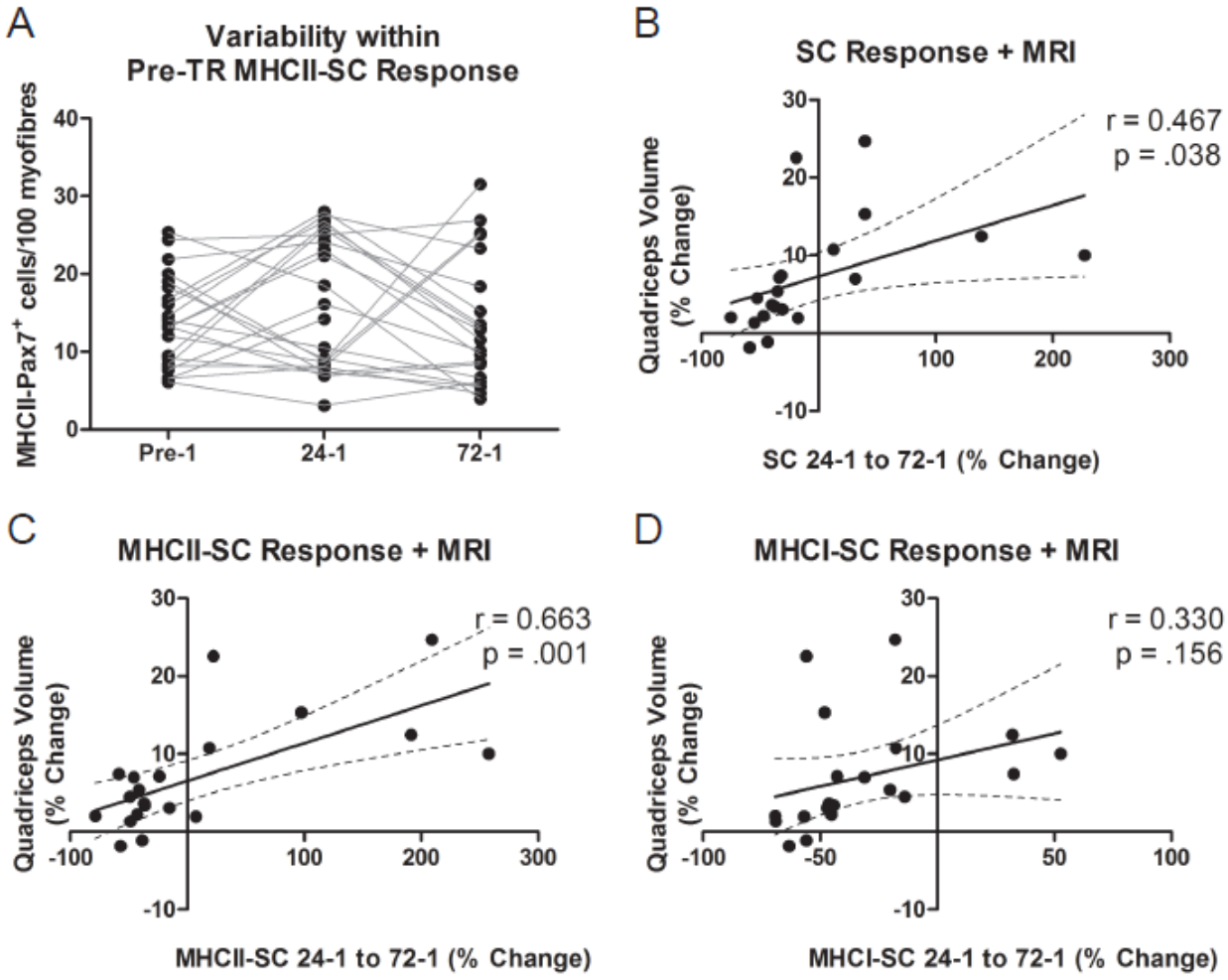

A
Variability within
Pre-TR MHCII-SC Response
MHCII-Pax7+ cells/100 myofibres
40
30
20
10
0
Pre-1
24-1
72-1
B
SC Response + MRI
r = 0.467
p = .038
Quadriceps Volume
(% Change)
30
20
10
-10
-100
100
200
300
SC 24-1 to 72-1 (% Change)
C
MHCII-SC Response + MRI
r = 0.663
p = .001
Quadriceps Volume
(% Change)
30
20
10
-10
-100
100
200
300
MHCII-SC 24-1 to 72-1 (% Change)
D
MHCI-SC Response + MRI
r = 0.330
p = .156
Quadriceps Volume
(% Change)
30
20
10
-10
-100
-50
50
100
MHCI-SC 24-1 to 72-1 (% Change)

Figure 5

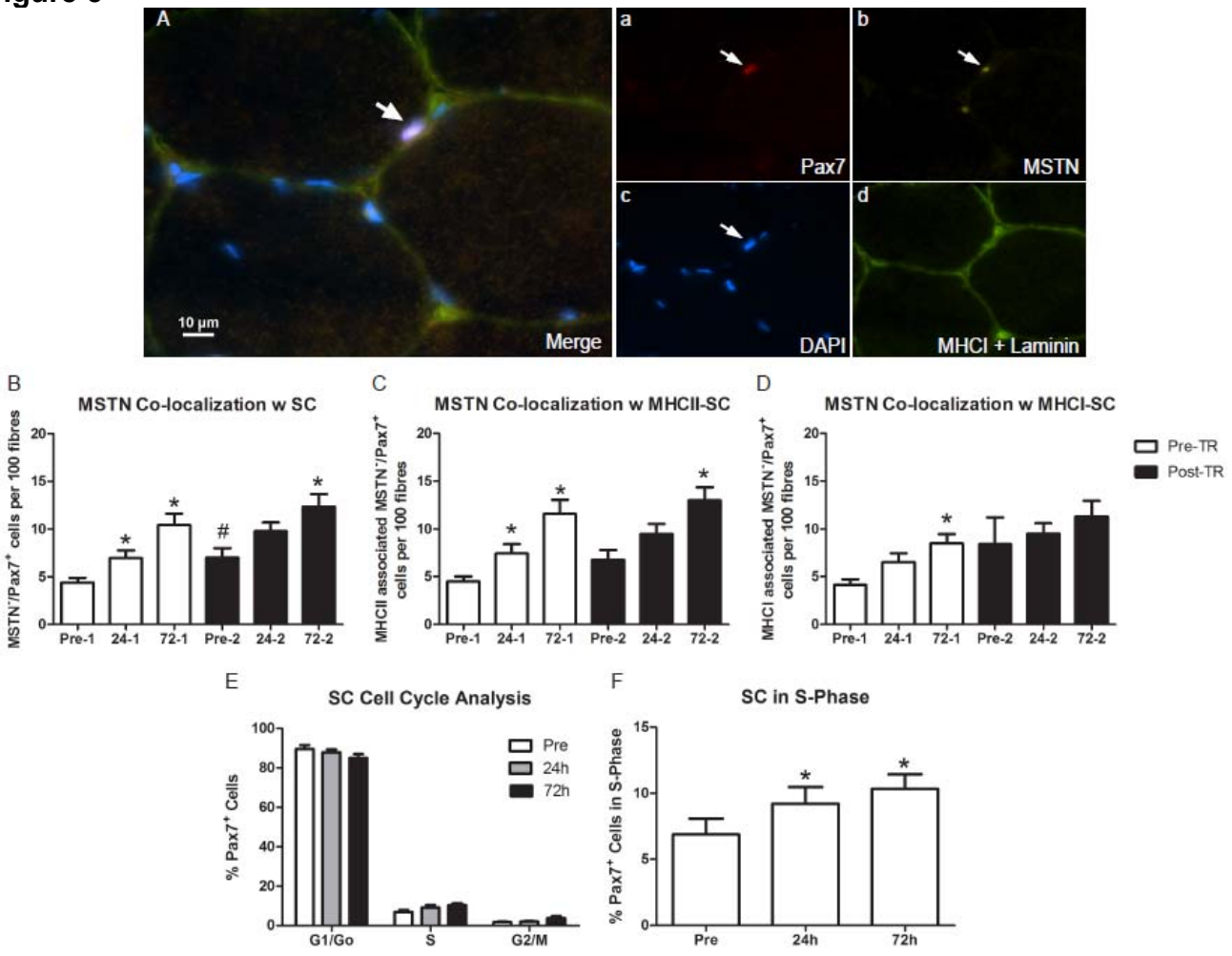

A
a
b
c
d
Pax7
MSTN
DAPI
MHCI + Laminin
Merge
10 µm
B
MSTN Co-localization w SC
MSTN+/Pax7+ cells per 100 fibres
C
MSTN Co-localization w MHCII-SC
MHCII associated MSTN+/Pax7+ cells per 100 fibres
D
MSTN Co-localization w MHCI-SC
MHCI associated MSTN+/Pax7+ cells per 100 fibres
Pre-TR
Post-TR
Pre-1
24-1
72-1
Pre-2
24-2
72-2
E
SC Cell Cycle Analysis
% Pax7+ Cells
Pre
24h
72h
G1/Go
S
G2/M
F
SC in S-Phase
% Pax7+ Cells in S-Phase

Figure 6

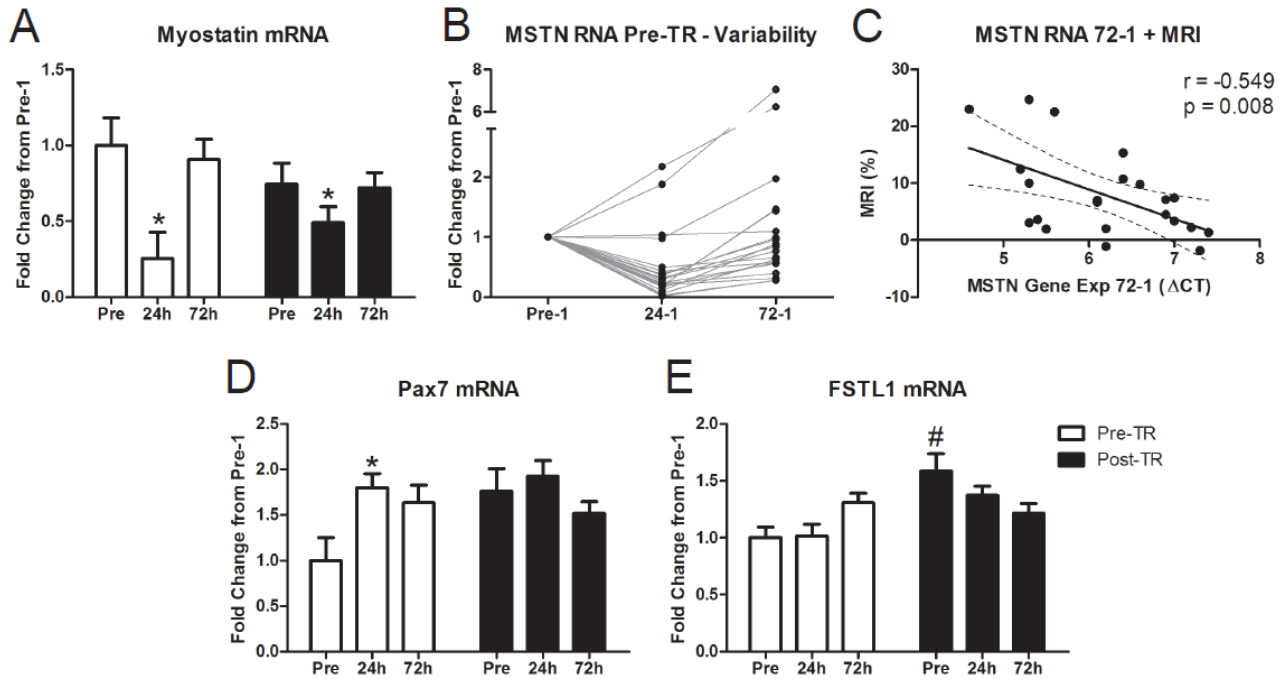
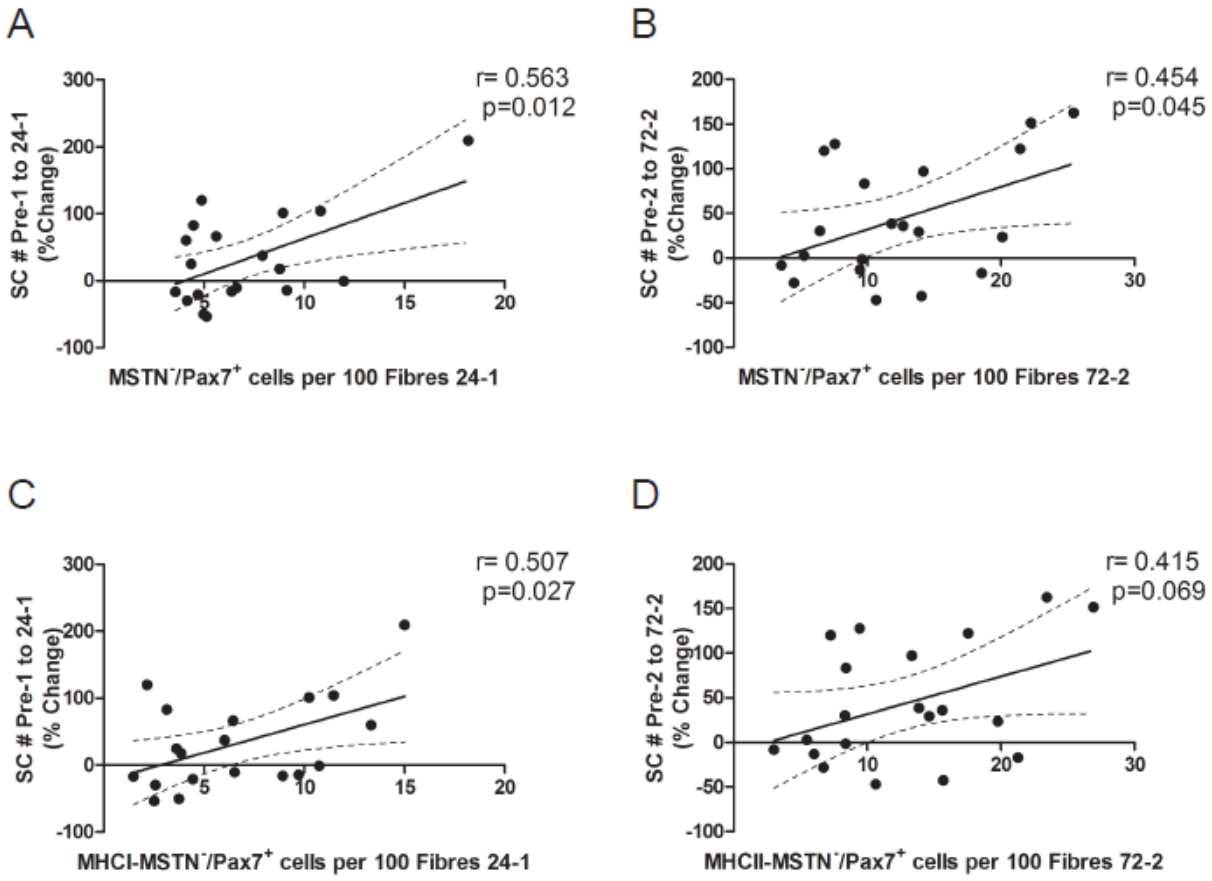
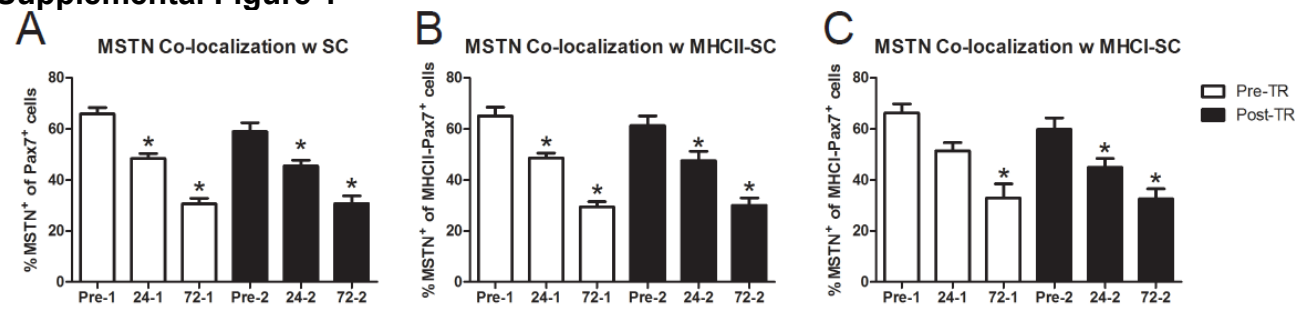

A
Myostatin mRNA
Fold Change from Pre-1
1.5
1.0
0.5
0.0
*
*
Pre
24h
72h
Pre
24h
72h
B
MSTN RNA Pre-TR - Variability
Fold Change from Pre-1
8
6
2
1
0
Pre-1
24-1
72-1
C
MSTN RNA 72-1 + MRI
r = -0.549
p = 0.008
MRI (%)
30
20
10
0
-10
5
6
7
8
MSTN Gene Exp 72-1 (ΔCT)
D
Pax7 mRNA
Fold Change from Pre-1
2.5
2.0
1.5
1.0
0.5
0.0
*
Pre
24h
72h
Pre
24h
72h
E
FSTL1 mRNA
Fold Change from Pre-1
2.0
1.5
1.0
0.5
0.0
#
Pre
24h
72h
Pre
24h
72h
Pre-TR
Post-TR

Figure 7


A
r= 0.563
p=0.012
SC # Pre-1 to 24-1 (%Change)
MSTN-/Pax7+ cells per 100 Fibres 24-1
B
r= 0.454
p=0.045
SC # Pre-2 to 72-2 (%Change)
MSTN-/Pax7+ cells per 100 Fibres 72-2
C
r= 0.507
p=0.027
SC # Pre-1 to 24-1 (% Change)
MHCI-MSTN-/Pax7+ cells per 100 Fibres 24-1
D
r= 0.415
p=0.069
SC # Pre-2 to 72-2 (% Change)
MHCII-MSTN-/Pax7+ cells per 100 Fibres 72-2

Supplemental Figure 1



Discussion

The purpose of this investigation was to assess whether individuals with an enhanced SC response to acute exercise would also demonstrate the greatest potential for muscle growth following resistance training. Furthermore, we hypothesized that MSTN would be suppressed to a greater extent post-exercise in individuals with the greatest SC response. The following major findings are outlined in this thesis:

1. The acute SC response to a single bout of high intensity resistance exercise becomes refined with resistance training.
2. The ability to sustain the MHCII-SC response to an acute bout of exercise (between 24-72h) is predictive of lean mass accretion as a result of resistance training.
3. The magnitude of the SC response to exercise may be limited by MSTN.

Skeletal Muscle Adaptation with Training

In response to 16wk of progressive, whole body resistance training, SKM hypertrophy was observed based on MRI (7.88% increase in quadriceps volume) and myofibre CSA (20.0% and 13.9% increases in MHCII and MHCI fibres respectively). Importantly, variation in the extent of hypertrophy was observed between individuals, with changes in quadriceps volume (MRI) and myofibre size ranging from -1.85 to 24.67%, and -7.0 to 51.7% respectively, allowing for identification of those with a robust response to resistance exercise and those with less of a response. Furthermore, in support of myonuclear addition during resistance exercise, we report (Figure 2A/B) a preservation in myonuclear

domain size, and increase in nuclei per fibre with training. Additionally, we are among the first to report a satellite cell domain (Figure 2C), of ~50 000μm^2 in size (based on the number of Pax7$^+$ cells per μm^2), that was also unchanged with training. Interestingly, we reported an association between SKM hypertrophy assessed via MRI and the SC response to exercise (discussed in more detail below), suggesting that the acute SC response was predictive of skeletal muscle hypertrophy. However, when the correlation was attempted with fibre CSA instead of MRI the correlation was non-significant.

There are a number of challenges with analysis of fibre CSA that may explain this apparent discrepancy. Specifically, each step including, maintenance of muscle integrity during the biopsy procedure, freezing of the muscle sample without freeze fracture, and orientation of the fibres in a perfectly cross-sectional fashion without error could all contribute to variability in the measurement. Given the robust changes in muscle size following training, myofibre CSA analysis was sufficient for the detection of overall and fibre type specific changes in fibre area; however, to accurately assess the degree of change experienced on an individual basis the use of myofibre CSA may be limited. As MRI is often considered the 'gold standard' in the measurement of muscle size (25, 29) due to high contrast detection between tissues of differing molecular properties, we feel the MRI data reported in this thesis (Figure 1A/B) most accurately reflects changes in muscle size resultant of training. Given the low error (1-5%) associated with the measure of muscle volume (29), inclusion of MRI analysis in

correlation with the SC response data adds strength to the validity of the reported results.

Acute Satellite Cell Response to Exercise

The general response of SC to an acute resistance exercise bout is becoming clear. Based on the data summarized in Table 1, an increase in SC content appears evident by 24h following exercise, while the peak time and duration of this response remains equivocal. In comparison to previous results from our lab (21–24, 27, 34) the SC response reported in this thesis is unique. In past studies we have reported a robust increase in overall SC content at 24h, which continued to rise and peak at 48 or 72h post exercise depending on the time points selected for analysis. However, in the current investigation a significant elevation in the overall SC pool was only apparent when SC content was analyzed in a fibre-type specific manner (Figure 3). Specifically, prior to training MHCI-SC content responded with an increase of ~43% 24h post-exercise returning to baseline at 72h. This response appeared altered and perhaps more focused with training, as MHCII-SC content increased steadily over the time-course peaking at 72h with an ~ 48% expansion. Speculation into the mechanisms responsible for why the SC response appeared less robust than in our previously reported experiments likely stems from the type of resistance exercise employed to induce the response. Traditionally, we have utilized an exercise protocol comprising 300 maximal eccentric contractions, designed to maximize muscle damage and subsequently the SC response. A single study from our lab has measured the SC response to a 1RM test immediately followed

by a 75%1RM exercise bout, demonstrating an increase in SC content 48h post-exercise (23). In the current investigation, as biopsies were not taken 48h post-exercise, we are unable to compare the SC response we observed to the later study. An additional difference in the current investigation, is that all exercise (with the exception of leg press) was performed utilizing the HUR fitness equipment. As the HUR equipment provides a very smooth motion with less "jerking" of the weights, we speculate the degree of muscle damage resultant from exercise may have been reduced. As the exercise protocol employed in the current work was designed to mimic a traditional resistance training session the applicability of the results are strengthened. Although we did not measure muscle damage in the current investigation our results infer that the magnitude of the SC response following exercise may be proportional to the degree of tissue damage.

A sustained acute SC response is predictive of hypertrophy and regulated by MSTN

We report a relationship between the capacity to sustain the acute SC response to a single bout of exercise, and the degree of SKM hypertrophy observed (Figure 4). Furthermore, we suggest that an underlying mediator of SC expansion is the ability to downregulate MSTN protein expression acutely post-exercise (Figure 5). A summary of these results is provided in Figure IV.

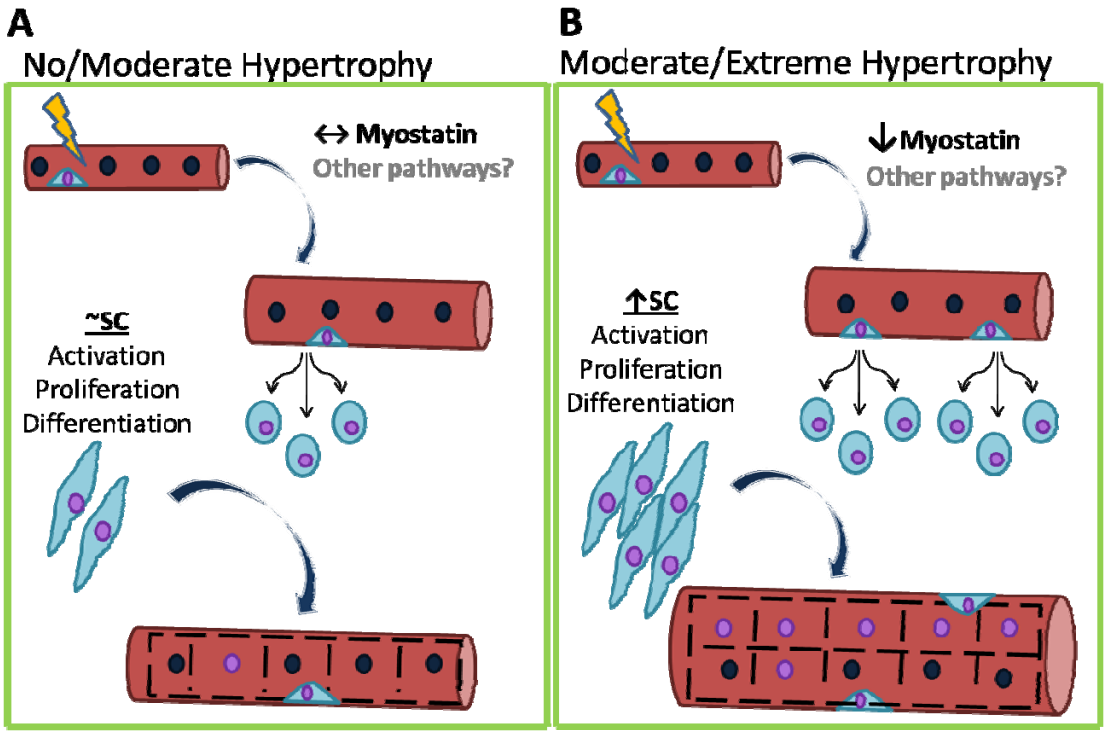


Figure IV. RESULTS SUMMARY
(A) Individuals who demonstrated little to no change in SC specific MSTN protein expression yielded a blunted SC response (elevated at 24h post-exercise only), ultimately contributing to a reduced capacity for SKM hypertrophy. (B) Alternatively, individuals who demonstrated a greater ability to reduce MSTN in SC acutely post-exercise elicited an enhanced SC response (elevated at 24 and 72h post-exercise) resulting in elevated accretion of muscle mass.

Individuals that displayed minimal change in SC specific MSTN protein expression post-exercise exhibited an attenuated expansion and duration of the SC pool. Although the response of MCHII-SC may have been elevated at 24h, in some individuals this expansion was not sustained at 72h resulting in minimal accretion of lean tissue mass with training (Figure IV A). Alternatively, those with enhanced capacity to reduce MSTN post-exercise displayed greater SC content negative for MSTN, and experienced a greater SC response, specifically within MHCII-SC that remained elevated 72h post-exercise (Figure IV B). Collectively, SC specific protein expression of MSTN appears to regulate the expansion and sustainability of the SC response following exercise, which in turn is predictive of

muscle hypertrophy with training. Therefore, it can be inferred that MSTN protein expression may indirectly dictate muscle growth through regulation of SC function in the acute time-course post-exercise.

The results of the current study, although restricted to the SC population, raise a compelling notion that the described phenomenon may apply to other progenitor cell populations. For example, it is well known that exercise can induce significant angiogeneic and neural adaptation, as well as remodelling of the extracellular matrix (13, 31, 33). The obvious question remains; can the acute response of other progenitor populations such as endothelial cells, Schwann cells or fibroblasts ultimately dictate the degree of adaptation in those specific tissues? Similarly, since these adaptations occur concomitantly, do a group of "responders" demonstrate a superior response in numerous cell populations, suggesting a predisposition to adaptation?

Although the contribution of SC to muscle hypertrophy is now better defined; there are other key factors known to regulate SKM hypertrophy, primarily muscle protein synthesis and breakdown (MPB) (18). Though it is not possible to measure the extent of muscle hypertrophy in response to a single bout of exercise, it is hypothesized that the cumulative response of both SC in combination with elevated MPS and suppressed MPB are complimentary in directing muscle growth. Given that the nuclear content of adult SKM is maintained through SC contribution (3, 11), myonuclei can be thought of as terminally differentiated SC. Therefore, it would be interesting to know whether individuals with a sustained SC response to exercise also experience similar

responses in MPS/MPB. The proposed common link between these processes could be MSTN signalling. Illustrated in Figure 6 (manuscript), our results suggest that a greater increase in the number of SC negative for MSTN contributes to the superior progression of MHCII-SC through the myogenic programme. Similarly, MSTN has been shown to inhibit activation of the Akt/mTOR/p70S6K signalling pathway (35), which is a known upstream regulator of MPS (8). Therefore, individual capacity to downregulate MSTN in both myonuclei and SC may ultimately contribute to enhanced hypertrophic potential. It is important to recognize that although MSTN was identified in this study as a regulator of SC activity, a number of other molecular regulators (also responsive to resistance exercise) have been identified, and likely also contribute to the regulation of hypertrophy.

Clinical Relevance

Although results generated in this study are from young healthy individuals it would be of clinical relevance to investigate whether individuals with an inhibited SC response to exercise are predisposed to muscle wasting conditions. Indeed, sarcopenia, aging and cachexia have all been linked to a reduction in SC number (15, 30, 36). Furthermore, aging has been associated with an impaired ability to decrease the proportion of SC co-localized with MSTN following exercise (23). Given the importance of MSTN as a negative regulator of muscle mass, specific targeting of MSTN signalling (inhibition of MSTN) for the purpose of sustaining the SC response may be of great clinical importance. Along these lines, the clinical outcome of muscle wasting conditions associated with elevated

MSTN levels (10, 14, 39, 40), including sarcopenia, cachexia, muscular dystrophy, or HIV/AIDs may be predicted by SC function.

Evidence is accumulating that implicates MSTN as a negative regulator of muscle growth, SC function, and maintenance of muscle mass and integrity throughout life. Therefore, the inhibition of MSTN signalling as a potential treatment modality for conditions of muscle wasting is receiving much attention. Promising results have been demonstrated in mouse models of muscular dystrophy coupled with genetic knockout of MSTN, yielding enhanced muscle size, decreased fibrosis and reduced lipid accumulation (1, 38). However, a number of clinical trials have been initiated using pharmacological inhibition of MSTN signalling in humans with mixed results. Treatment of muscular dystrophy patients with the MSTN inhibitor Myo-029 demonstrated no known safety risks; however, no benefit was conferred with regards to muscle size and function (37). Furthermore, while clinical trials involving treatment with another MSTN inhibitor, ACE-031 demonstrated promising results, trials were ceased due to numerous off target effects such as nose bleeds and excessive blood vessel growth (41). Therefore, though a growing body of evidence supports MSTN inhibition as a means to promote muscle growth, further work is required to optimize such pharmacological treatment in humans. As MSTN has been shown to have biological effects on multiple organ systems (12), targeting SC specifically may increase the efficacy of this treatment.

Debate of SC Contribution to Hypertrophy

A topic under constant debate is whether SC are necessary for the induction of SKM hypertrophy. Although interesting from a developmental perspective, it can be argued that this question is irrelevant from the perspective of physiology. Perhaps a more relevant question is "*Do* SC contribute to muscle hypertrophy"? Evidence in support of SC contribution to hypertrophy is centered around the maintenance of a myonuclear domain, such that proportional increases in myonuclear content and myofibre size are observed during muscle growth (26). Preservation of a constant myonuclear domain has been reported on numerous occasions, including the current investigation (Figure 2, manuscript), following an overload stimulus or training (26, 32). Previous data specific to human SKM illustrate that myonuclear content is associated with fibre size and SC content (9, 16, 28). Importantly, we provide further correlative data illustrating the capacity of sustaining the SC response to exercise to predict SKM hypertrophy with training (Figure 4, manuscript). However, it is important to acknowledge that instances of variation in myonuclear domain do exist (17), and that following anabolic steroid treatment (19) or SC ablation in the Pax7-DTA mouse model (20), hypertrophy in the absence of myonuclear contribution has been observed. In light of this, it may be concluded that SKM hypertrophy can occur in the absence of SC contribution under extreme conditions. A point to highlight is that there have been no reports of SKM hypertrophy without nuclear contribution under normal physiological conditions (i.e. in the absence of pharmacological treatment and genetic manipulation). Therefore, a more refined

question to direct future investigation is the necessity of SC in the maintenance of muscle integrity (size, structure and function).

Recently, a single study has indirectly investigated the necessity of SC in maintaining SKM integrity (20). This study utilized a genetic mouse model whereby the $Pax7^{+}$ (SC) pool was specifically ablated. In response to two and six weeks of synergist ablation induced overload, SKM hypertrophy of a similar magnitude was observed in both SC ablated and control mice. Additionally, specific force data as an indicator of muscle integrity was reported as maintained at the two week timepoint, however, this data is not provided following six weeks of overload. Though reported as unchanged, careful analysis of the muscle integrity/hypertrophy data reveals a potential trend to a decrease in muscle integrity and weight at the two and six week time-points respectively. It is important to note a limitation of this investigation is the likely insufficient sample size (n=6-8 and n=2-4 following two and six weeks of overload respectively) and duration of overload (six weeks). As it has recently been reported that an n=5 is desired when identifying changes in SKM hypertrophy specific to rodent models (2), this would suggest the analysis of SKM hypertrophy in SC ablated muscle at the six week timepoint is under powered, with the potential commitment of a type-2-error. Furthermore, a 17% increase in myonuclear size of Pax7 ablated mice following two weeks of synergist ablation was reported. Nuclear swelling has been reported in necrotic cells during the process of apoptosis (7). Collectively, these results suggest that though the process of SKM hypertrophy may be possible without myonuclear contribution, the long term maintenance of muscle

integrity may be compromised in the absence of a functional SC pool. Despite extensive investigation, under normal physiological conditions, there have been no reports of SKM hypertrophy in the absence of nuclear contribution. This notion is further supported by the data provided in the current investigation where an association between the sustained SC response to exercise and the extent of muscle hypertrophy is described. Moving forward, investigation directed towards the necessity of SC in the maintenance of muscle integrity, and the mechanisms that govern SC function will be of great clinical value.

Future Directions

A number of cytokines known to regulate SC function have been identified and are known to be responsive to high intensity exercise. Such mediators include but are not limited to HGF (27), bFGF (6), IGF-1 (22), Delta-1 (4), and IL-6 (21). Further investigation into the variability associated with the individual response of such molecules to determine whether additional variation can be explained regarding the SC response and subsequent muscle hypertrophy is warranted. Additionally, studies designed to include microarray data for the purpose of identifying new candidates that are differentially expressed between individuals with and without a high capacity for hypertrophy may lead to the discovery of additional regulators of muscle growth and SC function. Similar work currently under investigation has led to the identification of microRNA species (5) that offer some explanation regarding differential capacity for hypertrophy.

In addition to altered cytokine responses, training may result in an increased intrinsic capacity for SC to undergo proliferation and myogenesis. Through the sample collection phase of the current investigation we were able to isolate human primary myoblasts in both the trained and un-trained state within the same individuals. This offers a unique opportunity to investigate the basic proliferation/differentiation kinetics of cells in response to training. We hypothesize that in the trained state myoblast progression through the myogenic programme will be enhanced. Importantly, the rate of proliferation and degree of myoblast fusion/differentiation may be superior in individuals who demonstrated the greatest gains in lean tissue mass, offering further explanation to the inter-individual variation in SKM hypertrophy observed with resistance training.

Limitations

Though great value exists in the results reported in this thesis, it is important to recognize the limitations associated with this investigation. A number of associations between the SC response, MSTN reduction following exercise and the degree of hypertrophy observed with training are reported based on significant correlations. Although correlations may offer insight into associations between two outcomes, specific cause and effect conclusions cannot be discerned. It is important to acknowledge that cause and effect is not always possible in the experimental design of human research, as genetic modification and certain pharmacological treatments are not ethically possible. However, the advantage to human work is the applicability and relevance offered in the results obtained.

Further challenges associated with this investigation include participant compliance and the recruitment of a subject pool that is representative of the population of interest. To minimize variation, recruitment was restricted to healthy males aged 18-35yrs, who were unaccustomed to resistance training, and all exercise sessions were supervised. Given the training duration of 16wk, dietary consistency was a difficult factor to control and is recognized as a limitation. However, the existence of such uncontrollable factors as dietary inconsistency may strengthen the applicability of the results to the general population. Collectively, though factors exist that remain outside of the investigators control, the appropriate steps were taken to minimize unwanted variability between subjects.

Conclusion

In summary, the results presented in this thesis identify MHCII-SC content as a mediator of muscle mass in humans. Specifically, a relationship between the ability to sustain the expansion of MHCII-SC beyond 24h in response to a single high intensity exercise bout and the degree of SKM hypertrophy with resistance training is shown. Furthermore, the magnitude of the MHCII-SC response appears to be regulated by the capacity for an acute reduction in SC specific MSTN protein expression following exercise. These data contribute to the debate of SC necessity for muscle hypertrophy, providing further support for nuclear contribution during muscle growth. More importantly, the legitimacy of this debate is brought into question, refining the argument to what occurs under normal physiological conditions. Is the myogenic stem cell population, the SC,

necessary for the maintenance of SKM mass and integrity? Additionally, clinical implications regarding the preservation of muscle mass in the context of age or disease are suggested through targeting of MSTN signalling in an effort to sustain or enhance SC function.

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