THE REGULATION OF HUMAN MUSCLE STEM CELLS

THE REGULATION OF HUMAN MUSCLE STEM CELLS IN RESPONSE TO MUSCLE DAMAGE AND AGING

Bу

BRYON R. McKAY, B.Kin. (Hon.), M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

© Copyright by Bryon R. McKay, September 2011

DOCTOR OF PHILOSOPHY (2011)

McMaster University

(Kinesiology)

Hamilton, Ontario Canada

TITLE: The Regulation of Human Muscle Stem Cells in Response to Muscle Damage and Aging

AUTHOR: Bryon R. McKay, B.Kin (Hon.) (McMaster University), M.Sc. (the University of Western Ontario)

SUPERVISOR: Dr. Gianni Parise

SUPERVISORY COMMITTEE: Dr. Stuart M. Phillips

Dr. Mark A. Tarnopolsky

NUMBER OF PAGES: xx, 262

ABSTRACT

Skeletal muscle exhibits a remarkable capacity for growth and regeneration in response to physiological stimuli. This extensive plasticity is, in part, due to a tissue-resident stem cell called the satellite cell. Satellite cells respond to myotrauma by upregulating a class of transcriptional networks which orchestrate myogenic specification. This process is controlled by four main transcription factors known as the myogenic regulatory factors: Myf5, MyoD, MRF4 and Myogenin. Satellite cells respond to molecular cues released from the muscle fiber or inflammatory cells in response to muscle damage. Although several regulators have been implicated in the control of the satellite cell response to exercise or damage, very few of these have been examined in humans. Insulin-like growth factor-1 (IGF-1) and Interleukin-6 (IL-6) have been demonstrated to enhance satellite cell proliferation in animal and cell culture models. IGF-1 has also been shown to induce myogenic differentiation, however little is known about IGF-1 and IL-6 in humans, in response to physiological levels of muscle damage. Myostatin has been identified as a negative regulator of muscle growth and an inhibitor of satellite cells in mice. To date no data exists regarding the relation of myostatin to the satellite cell response to exercise and in the context of aging. The work outlined in this thesis provides support for the proposed divergent effects of the IGF-1 splice variants on satellite cell function. IGF-

iv

1 appears to be preferentially spliced as IGF-1Ec during the proliferative phase of the myogenic program while IGF-1Ea and Eb appear as the predominant splice variants during the initiation of differentiation based on the expression of the MRFs. Furthermore, the localization of IGF-1 with Pax7 in muscle-cross sections in the post-exercise time-course lends support to the importance of IGF-1 in the myogenic response to myotrauma. This thesis also provides novel evidence to support the role of IL-6 in the regulation of satellite cell proliferation in response to acute muscle damage in humans. These data confirm that IL-6 imparts its action on the satellite cell via the JAK2/STAT3 pathway. In addition, for the first time, myostatin is demonstrated to be altered by acute exercise in both young and older adults and this effect is most notable in the satellite cell compartment. In addition, these data implicate myostatin as a contributing factor to age-related satellite cell dysfunction in response to exercise (or myotrauma).

ACKNOWLEDGEMENTS

I would like to thank Gianni for taking me on as a PhD student even though I had no prior experience with muscle satellite cells or any of the lab techniques involved in molecular physiology! Thank you for always making time to talk to me whether it was about my data, lab frustrations or life. You have been a true friend and mentor throughout my time here at McMaster. I would not be here if it was not for you - Thank you for all of the opportunities you have given me, I look forward to future talks and collaborations with you.

I would like to thank Dr. Stuart Phillips and Dr. Martin Gibala for sparking my initial interest in muscle research when I was a young impressionable undergraduate student. You have both been excellent mentors to me throughout all of my degrees. I would also like to thank Dr. Mark Tarnopolsky for his guidance and assistance during my PhD as well as motivating me to not give up on my quest to become a physician/researcher. You have been an excellent role model, thank you.

I would also like to thank Todd Prior for all of his technical assistance and support over the past 4 years. I do not think any graduate student going through the physiology program in Kinesiology can thank Todd enough for all of his help.

I would like to thank my past and present members of the Parise Lab Mike, Adam, Jeff, Leeann, Kyle, and David for being good friends, mentors and colleagues. I thank you for all of your help with lab techniques, study designs, life talks, jokes and friendships. You have all made working in the lab a rewarding and fulfilling experience. I would also like to thank the students of the EMRG lab as a whole for always being there to lend a hand; I value all of the friendships we have made, and wish everyone the best of luck in the future.

Finally, I would like to thank my friends and family for all of their support over these 10 years of university, especially my mother Marilyn. You have kept me sane and focused and provided unwavering love and support which I am forever indebted. I would also like to thank Audra for providing me with the love and drive to pursue my dreams. I look forward to spending the next exciting chapter of my life with you.

ABBREVIATIONS

1RM	1 repetition maximum
ActRIIb	Activin receptor IIb
ANGII	Angiotensin II
ANOVA	Analysis of variance
Bcl-2	B-cell lymphoma two
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BMDC	Bone marrow-derived stem cell
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CD45	Cluster of differentiation 45
CD56	Cluster of differentiation 56
Cdk	Cyclin-dependant kinase
cDNA	Cyclic deoxyribonucleic acid
СК	Creatine kinase
COPD	Chronic obstructive pulmonary disease
CSA	Cross-sectional area
CSF	Cerebral spinal fluid
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECC	Eccentrically-biased exercise
EGF	Endothelial growth factor
Erk	Extracellular-signal-regulated kinase

FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FSTL-1	Follistatin-like-1
G ₁	Growth phase one
G ₂ /M	Growth phase two/mitosis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF-8	Growth and differentiation factor eight
gp130	Glycoprotein 130
Gy	Grey dose of radiation (1Joule of radiation per kilogram of mass)
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
IF	Immunofluorescence
IGF-1	Insulin-like growth factor one
IHC	Immunohistochemistry
IL-6	Interleukin six
IL-6Rα	Interleukin six receptor alpha
JAK	Janus activated protein kinase
JNK	c-Jun n-terminal kinase
LIF	Leukemia inhibitory factor
MAPK	Mitogen activated protein kinase
MF	Myofiber
MGF	Mechano growth factor
МНС	Myosin Heavy chain

MKK	Mitogen activated protein kinase kinase kinase
MLC	Muscle-lengthening contraction
MN	Myonuclei
MRF4	Myogenic regulatory factor four
MRFs	Myogenic regulatory factors
mRNA	Messenger ribonucleic acid
MSTN	Myostatin
Myf5	Myogenic factor five
MyoD	Myogenic determination factor
N-CAM	Neural-cell adhesion molecule
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory
ОМ	Older men
P.I.	Propidium iodide
Pax7	Paired box transcription factor seven
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinases
PIC	PW1+ interstitial cell
pSTAT	Phosphorylated signal transducers and activators of transcription
PW1	Paternally-expressed gene 3
Rb	Retinoblastoma protein
S	DNA synthesis phase

SC	Satellite cell
Sca-1	Stem cell antigen one
SOCS	Suppressors of cytokine signaling
STAT	Signal transducers and activators of transcription
TAK	TRAF activated kinase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
YM	Young men

TABLE OF CONTENTS

ABSTRACTiv			
ACKNOWLEDGEMENTSvi			
ABBREVIATIONSvii			
LIST OF FIGURES			
LIST OF TABLES			
CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIPxvii			
CHAPTER 1			
1.1 OVERVIEW AND HISTORY OF MUSCLE STEM CELLS. 1 1.1.1 Origins of skeletal muscle. 2 1.1.1 Muscle stem cells – 'the satellite cell'. 3 1.1.3 Pax7 and the myogenic program. 5 1.1.4 Detection of muscle stem cells <i>in vivo</i> . 10 1.2 MUSCLE STEM CELLS AND EXERCISE. 14 1.2.1 Exercise induced myotrauma. 17 1.2.2 Growth factors and the muscle stem cell response. 19 1.2.3 Inflammation and the muscle stem cell response. 22 1.3 MUSCLE STEM CELLS AND AGING. 24 1.3.1 Muscle stem cells and aging – intrinsic factors. 26 1.3.2 Muscle stem cells and aging – extrinsic factors. 27 1.3.3 Aging, myostatin and muscle stem cells. 28 1.4 PURPOSE OF THE THESIS. 31 1.5 REFERENCES. 34			
CHAPTER 2: Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans			
CHAPTER 3: Association of interleukin-6 signaling with the muscle stem cell response following muscle-lengthening contractions in humans84			
CHAPTER 4: Myostatin is associated with the induced inhibition of muscle stem cell activity in aging humans			
CHAPTER 5: Integrative Discussion			

5.1 INTRODUCTION	194
5.2 IGF-1 AND FUTURE WORK	194
5.3 INTERLEUKIN-6: THE PLEIOTROPIC REGULATOR	200
5.4 MYOSTATIN, AGING AND THE PRESERVATION OF MUSCLE MASS.	205
5.5 NON-CANONICAL PATHWAYS FOR MYOSTATIN SIGNALING	207
5.6 INTEGRATIVE SIGNALING: THE IN VIVO DILEMMA	209
5.7 CONCLUSION	212
5.8 REFERENCES	213
APPENDIX I: Satellite cell number and cell-cycle kinetics in response acute myotrauma in humans: immunohistochemistry versus flow	se to
cytometry	218
APPENDIX II: Preliminary data	261

LIST OF FIGURES

FIGURES	FIGURES FROM GENERAL INTRODUCTION				
Figure 1:	The myogenic program11				
Figure 2:	Identification of human satellite cells13				
Figure 3: myon	Myonuclear domain theory and satellite cell-mediated uclear accretion15				
Figure 4:	Molecular regulators of myogenesis21				
FIGURES	FROM CHAPTER 2				
Figure 1:	Serum IGF-1 concentration76				
Figure 2:	MyoD and Myf5 mRNA expression77				
Figure 3:	MRF4 and Myogenin mRNA expression78				
Figure 4:	IGF-1 Ea, Eb and Ec mRNA expression79				
Figure 5:	Correlations of IGF-1Ea, Eb and Ec with the MRFs80				
Figure 6:	Immunofluorescent co-localization of IGF-1 with Pax781				
FIGURES FROM CHAPTER 3					
Figure 1:	Circulating and muscle IL-6118				
Figure 2:	Muscle satellite cell response119				
Figure 3:	Satellite cell IL-6 protein expression120				
Figure 4:	JAK2/STAT3 Activity121				
Figure 5:	Response of downstream target genes122				
Figure S1 (MLC	: Muscle stem cell response to muscle lengthening contractions)				
Figure S2 length	2: Satellite cell IL-6 protein expression following muscle nening contractions				

Figure S3: Satellite cell co-localization with proliferating cell nuclear antigen (PCNA) following muscle lengthening contractions
Figure S4: Satellite cell co-localization with pSTAT3126
Figure S5: Control images127
FIGURES FROM CHAPTER 4
Figure 1: Stem cell response to acute exercise178
Figure 2: Cell-cycle analysis and gene expression179
Figure 3: Characterization of MyoD1 in muscle stem cells
Figure 4: Whole-muscle myostatin protein and gene expression182
Figure 5: Immuno-detection of myostatin and muscle stem cells183
Figure 6: Fiber-type assessment of myostatin co-localization185
Figure S1: Localization of ActRIIb186

- Figure S3: MyoD1 immunofluorescent controls......188
- Figure S4: Hi resolution image of MyoD1 co-localization with Pax7.....189

- Figure S8: Gene expression and nuclear/cytoplasmic protein......193

FIGURES FROM CHAPTER 5

Figure 1:	e 1: The proposed signaling pathways of IGF-1Ea and MGF (IGF		
1Ec)	······	197	
Figure 2:	IGF-1 gene splicing in response to exercise1	199	
Figure 3:	Known and hypothesized cellular functions of myostatin2	208	

LIST OF TABLES

TABLES FROM GENERAL INTRODUCTION

Table 1: Change in satellite cell number following acute and chronic exercise	.16
TABLES FROM CHAPTER 2	
Table 1: qRT-PCR primer sequences	73
TABLES FROM CHAPTER 3	
Table 1: qRT-PCR primer sequences1	15
TABLES FROM CHAPTER 4	
Table 1: Subject characteristics1	75
Table 2: Myonuclear domain and satellite cell content1	176
Table S1: qRT-PCR primer sequences1	177

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIP

Chapter 2

Publication

McKay BR, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G. Coexpression of IGF-1 Family Members with Myogenic Regulatory Factors Following Acute Damaging Muscle Lengthening Contractions in Humans. *J Physiol.* **2008** Nov 15;586(Pt 22):5549-60

Contributions

The principle investigator was GP. Experiments were conceived by GP and BRM. Experimental procedures and tissue collection were carried out by CEO, MAT, SMP and GP. Tissue preparation, RNA isolation and reverse transcription were conducted by BRM and CEO. RT-PCR, gene analysis, immunofluorescent analysis and correlations were conducted by BRM. The manuscript was written by BRM and edited by GP, SMP and MAT, all authors contributed to the final version of the manuscript.

Chapter 3

Publication

McKay BR, DeLisio M, Johnston, APW, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G. Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. *PLoS One*. 2009 Jun 24;4(6):e6027

Contributions

The principle investigator was GP. Experiments were conceived by BRM and GP. Experimental procedures and tissue collection were carried out by CEO, MAT, SMP and GP. Tissue preparation, RNA isolation and reverse transcription were conducted by BRM, MD and APWJ. RT-PCR and gene analysis was conducted by BRM and APWJ. Tissue sectioning, immunofluorescent analysis and correlations were conducted by BRM. Protein isolation was conducted by BRM and MD. Protein analysis and western blotting was conducted by MD. The manuscript was written by BRM and edited by GP, MD, APWJ, SMP and MAT, all authors contributed to the final version of the manuscript.

Chapter 4

Publication

McKay BR, Ogborn DI, Bellamy L, Tarnopolsky MA, Parise G. Myostatin-Induced Inhibition of Muscle Stem Cell Activity in Aging Humans. *Aging Cell:* In Submission

Contributions

The principle investigator was GP. Experiments were conceived by BRM and GP. Experimental design, procedures and tissue collection were carried out by BRM, DIO, MAT and GP. Tissue preparation, RNA isolation and reverse transcription were conducted by BRM, DIO and LB. RT-PCR and gene analysis was conducted by BRM. Serum creatine kinase assay was conducted by LB. Tissue sectioning, immunofluorescent and immunohistochemical analysis and was conducted by BRM. Primary myoblast isolation and immunocytochemistry was conducted by BRM. Protein isolation and western blotting was conducted by DIO. The manuscript was written by BRM and edited by GP, DIO and MAT, all authors contributed to the final version of the manuscript.

Appendix I

Publication

McKay BR, Toth KG, Tarnopolsky MA, Parise G. Satellite Cell Number and Cell-cycle Kinetics in Response to Acute Myotrauma in Humans: Immunohistochemistry versus Flow Cytometry. *J Physiol.* 2010 Sept. 1; 588 (17):3307-20

Contributions

The principle investigator was GP. The principle investigator was GP. Experiments were conceived by BRM and GP. Experimental design, procedures and tissue collection were carried out by BRM, KGT, MAT and GP. Tissue preparation was conducted by BRM and KGT. Serum creatine kinase assay was conducted by KGT. Tissue sectioning, immunofluorescent and immunohistochemical analysis and was conducted by BRM. Mononuclear cell isolation, flow cytometry and fluorescence activated cell sorting preparation and analysis was conducted by BRM. The manuscript was written by BRM and edited by GP, KGT and MAT, all authors contributed to the final version of the manuscript. The authors acknowledge Nicole McFarlane and Dr. Doug Borham for their assistance with flow cytometry and Fluorescence activated cell sorting.

Chapter 1

General Introduction

1.1 OVERVIEW AND HISTORY OF MUSCLE STEM CELLS:

Skeletal muscle is a multinucleated contractile tissue responsible for locomotion and plays an integral role in the maintenance of homeostasis. It is the largest organ in the body, and by virtue of its size relative to other organs, is the most metabolically active tissue (based on absolute energy consumption) and the principle site of protein storage in the body. Skeletal muscle is also the largest depot of glucose storage in the body and thus plays a key role in glucose homeostasis. It is a dynamic tissue capable of complete regeneration in response to overt damage (i.e. toxin-induced myotrauma) and possesses a remarkable ability for adaptation in response to physiological stimuli (i.e. exercise). For example, in response to an increase in energy demand (such as aerobic exercise) skeletal muscle is capable of upregulating oxidative machinery and inducing mitochondrial biogenesis, increasing the metabolic capacity of myofibers [1]. Alternately, in response to heavy loads, it is capable of increasing cross-sectional area (hypertrophy) and increasing the abundance of contractile proteins, thus increasing size and strength [2]. The extensive plasticity of skeletal muscle is thought to be, at least in part, to the presence of a tissue-specific class of stem cells, which is responsible for muscle maintenance and tissue repair [3]. In addition, interstitial progenitor cells [4], circulating hematopoietic stem cells [5], and muscle-specific stems cell known as side population cells [6] residing in

skeletal muscle may also contribute to muscle plasticity. Although the potential contribution of these stem-cells to myogenesis has been explored, the precise mechanisms and complex signals which govern their regulation remain largely unknown.

1.1.1) ORIGINS OF SKELETAL MUSCLE:

During embryogenesis all skeletal muscles, with the exception of the head, are derived from precursor cells from a region of paraxial mesoderm called the somites. The somite contains two sub-domains – epaxial, which gives rise to the musculature of the back and hypaxial which gives rise to the musculature of the limbs, abdomen and thorax [7]. The molecular events which orchestrate the myogenic specification of the mesodermal precursor cells are a combination of positive and negative signals emanating from the structures surrounding the dermomyotome, such as the neural tube and notochord [8]. The main signaling molecules thought to regulate myogenesis in the embryo are Wnt proteins, Sonic hedge hog, noggin (positive signals) and bone morphogenic proteins (negative signals). These signals regulate the expression of the myogenic regulatory factors (MRFs) and thus myogenic commitment. Following waves of migration and proliferation, precursor cells begin to form premuscle masses in the limb buds, eventually giving rise to multinucleated primary muscle fibers [9]. During embryonic specification and fetal development, the multinucleated fibers develop contractile properties (upregulation of myosin heavy chains and actin filaments) and mature into functional muscle fibers. Importantly, during the later stages of

muscle development, a distinct subpopulation of precursor cells thought to have originated from the somites [10,11] resists differentiation and withdraws from the cell-cycle, remaining on the periphery of the myofiber as a quiescent cell, maintaining its potency as a progenitor cell [12,13]. Late fetal and normal postnatal muscle development and adaptation is dependent on these progenitor cells for myonuclear addition to support increases in cytoplasmic volume as the muscle fibers mature and hypertrophy [14,15].

1.1.2) MUSCLE STEM CELLS – 'THE SATELLITE CELL':

Central to the capacity for muscle to regenerate is the tissue-resident stem cell which has been named 'the satellite cell' based on its anatomical location between the basal lamina and the sarcolemma of the muscle fiber [3]. Although the initial study by Alexander Mauro did not classify the function of these sublaminar cells, he speculated that satellite cells may contribute to muscle regeneration [3]. Over the next 10 years, extensive work confirmed that satellite cells were capable of entering the cell-cycle and contributing their nuclei to existing myofibers, suggesting that these cells function as resident stem-cells in adult muscle [16,17]. This notion gained further support from studies using isolated single fibers to illustrate that the satellite cell indeed was the source of myoblasts which were able to proliferate and fuse together to form multinucleated myotubes [18,19]. Although it was demonstrated that satellite cells give rise to myoblasts which were able to differentiate and form nascent myofibers, this evidence was not sufficient to prove satellite cells met the basic definition of a

McMaster University

stem cell. To fulfill the criteria of a stem cell, a single cell must be able to produce progeny capable of differentiating into a functional tissue and also exhibit the capacity to self-renew, thus repopulating the satellite cell pool [20]. The source of satellite cell renewal however, was controversial. Several studies investigating the efficacy of transplanted satellite cells into irradiated muscle revealed that the majority of myogenic donor cells die upon engraftment, making studying the contribution of satellite cell-derived cells as a source of renewal difficult [20]. In addition, a body of evidence exists to support that circulating progenitors such as hematopoietic stem cells [5,21] and interstitial stem cells [22] can contribute to the repopulation of the satellite cell niche, suggesting satellite cells can be repopulated by multipotent progenitors in addition to mechanisms of self-renewal. Recently, however, Collins et al. [23] illustrated that as few as seven satellite cells (on an intact single fiber) transplanted into radiation-ablated muscle were able to give rise to sufficient fusion-competent progeny to generate thousands of myonuclei. Importantly, they confirmed that these transplanted satellite cells were also capable of extensive self-renewal and repopulation of the satellite cell pool thus substantiating the stem cell properties of satellite cells [23]. This study extends conclusive evidence to the long-theorized stem cell nature of the muscle satellite cell. It is important to note however, that atypical progenitors such as CD45⁺:Sca1⁺ cells [22], PW1⁺ interstitial cells (PICs) [4], bone marrow derived stem cells (BMDC) and hematopoietic stem cells [5.24] can contribute to muscle repair. However, there is little evidence illustrating that any of these cell

populations contribute to post-natal muscle repair under normal conditions to any significant level. It is likely that in the absence of normally functioning satellite cells or in the case of extreme muscle damage (e.g. cardiotoxin injection), these cells may contribute to muscle regeneration. It is important to note, however, that the role of atypical muscle progenitors in human skeletal muscle is entirely unknown.

A common factor that may dictate the myogenic specification of satellite cells and atypical progenitors alike is the transcription factor Pax7. It appears that cells that progress down the myogenic lineage, regardless of their origin (PIC, BMDC, satellite cell), upregulate Pax7 during myogenic commitment [4,25,26], suggesting Pax7 may be required for myogenic specification.

1.1.3) PAX7 AND THE MYOGENIC PROGRAM:

In post-natal muscle tissue the majority of muscle stem cells (termed satellite cells from here on) exist in a quiescent state between the endomysium or basal lamina and the sarcolemma of the myofiber [3]. In neonatal muscle, satellite cells constitute approximately 30% of all myonuclei; however this number stabilizes in adult muscle to approximately 2-4% of myonuclei [9,27]. Muscle fibers are generally stable, requiring only minor myonuclear turnover due to subtle acquired structural or nuclear damage [28]. Estimates for myonuclear turnover suggest that approximately 1-2% of myonuclei are replaced on a weekly basis in rat muscle [29]. If we extend the observation that a rat extensor digitorum (EDL) longus has approximately 2.2 million myonuclei [30], then the

McMaster University

average satellite cell-mediated myonuclear turnover in the EDL is ~22 000 myonuclei per week. Therefore even in the absence of myotrauma the role of the satellite cell is indispensible. Furthermore, muscle lacking functional satellite cells due to exposure to radiation is unable to undergo significant hypertrophy in response to chronic overload [31]. This demonstrates that satellite cell-mediated myonuclear addition is necessary to support significant skeletal muscle hypertrophy. The process orchestrating the progression of a quiescent satellite cell to a terminally differentiated muscle cell is known as the myogenic program [32].

The quiescent satellite cell exhibits a high nuclear-to-cytoplasmic ratio with an abundance of heterochromatin compared to the myonuclei and contains relatively few organelles [9,27]. Upon activation, satellite cell morphology changes and is characterized by a reduction in heterochromatin, an increase in organelle abundance, an expansion of the cytoplasm and an induction of transcriptional activity [27,33]. A well-defined series of highly-conserved transcriptional networks are responsible for the control of activation, proliferation and differentiation and are known as the myogenic regulatory factors (MRFs). The MRFs are basic helix-loop-helix (bHLH) transcription factors, which form heterodimeric DNA binding complexes within enhancer elements of genes that encode aspects of the cell-cycle machinery or genes that drive terminal differentiation [34–37]. There are four main MRFs that control normal myogenesis: Myf5, MyoD, MRF4 and myogenin. Myf5 is expressed during the

McMaster University

initiation of post-natal myogenesis and is the first MRF gene that is upregulated upon satellite cell activation [33,38]. Both Myf5 and MyoD are upregulated in proliferating satellite cells [33,39] and it appears cells that down-regulate MyoD but retain Myf5 represent cells withdrawing from the myogenic program, contributing to self-renewal of the satellite cell pool [40,41]. Cells from mice lacking MyoD (MyoD^{-/-} mice) display a normal proliferative capacity and Myf5 expression but these cells exhibit a greater susceptibility to self-renew as opposed to progressing through the myogenic program [37,41]. Furthermore, it appears MyoD is necessary for the transition from proliferation to differentiation as myoblasts from MyoD^{-/-} mice exhibit impaired differentiation and fail to upregulate MRF4 [35]. Transfecting MyoD^{-/-} cells with MyoD restored the ability of myoblasts to differentiate illustrating that MyoD directs cells from proliferation to terminal differentiation [35,40]. Specifically, MyoD forms a complex with the Eprotein HEBß and binds to the E1 E-box of the promoter region of the myogenin gene leading to transcriptional activation of myogenin, providing direct evidence for the role of MyoD in myogenic progression [36].

After sufficient rounds of proliferation, myoblasts begin to down-regulate Myf5 and upregulate MRF4 and myogenin. Both MRF4 and myogenin are involved in the specific events that orchestrate myogenic differentiation [42]. Both MyoD and MRF4 act upstream of myogenin [34,42], inducing the expression of myogenin and initiating terminal differentiation. The temporal expression of these MRFs was confirmed in culture demonstrating that MRF4 upregulation

precedes myogenin and the upregulation of myogenin is immediately followed by terminal differentiation of myoblasts into nascent myotubes [43]. Thus upon activation, a quiescent satellite cell begins to express Myf5, followed by both Myf5 and MyoD. Following proliferation, the majority of proliferating satellite cells (myoblasts) down-regulate Myf5 and upregulate MRF4 followed by the upregulation of myogenin leading to terminal differentiation and fusion of these cells either with the muscle fiber or by fusion with each other to form nascent myotubes. A subset of myoblasts may lose (or never upregulate) MyoD and withdraw from the cell-cycle, repopulating the satellite cell pool through selfrenewal.

Regulation of the post-natal myogenic program is thought to be controlled by a specific homeodomain protein Pax7. Pax7 is a member of the paired-box (Pax) transcription factor family and has been shown to be essential for myogenic specification of satellite cells [26]. Pax7 null mice (Pax7^{-/-}) have normal embryonic myogenesis mainly due to the increased activity of the Pax7 orthologue: Pax3 [7]. However, Pax7^{-/-} mice demonstrate decreased body weight, inhibited growth and die prematurely [26]. Although initially these mice were thought to lack satellite cells completely [26], it was later confirmed that they do in fact possess satellite cells, albeit at low numbers [44] and these cells are rapidly lost as the animals age [13]. Thus it appears that Pax7 is instrumental in the regulation of satellite cells but may not be necessary for myogenic specification as satellite cells from Pax7^{-/-} mice may still upregulate MyoD [45]. It

McMaster University

is important to note, however, that the observation of satellite cells in Pax7 null mice may be due to a persistent expression of Pax3 (necessary for embryonic myogenic development), which is not thought to be involved in normal adult myogenesis [45,46]. Although the precise role of Pax7 in myogenic specification remains somewhat unclear, it remains a critical regulatory factor in the normal progression of satellite cells through the myogenic program. Pax7 associates with a specific histone methyltransferase complex that directs the methylation of histone H3K4 to induce chromatin modifications, which stimulates the transcription of Myf5, thus regulating entry into the myogenic program [47]. In addition Pax7 appears to be necessary for satellite cell self-renewal [48]. In a series of studies examining the potential role of Pax7 in satellite cell self-renewal, the forced expression of Pax7 induced cells to exit the cell-cycle and return to guiescence. Furthermore, Pax7 was found to be absent in myogenin positive cells illustrating a potential reciprocal inhibition between Pax7 and myogenin, suggesting Pax7 must be down-regulated in order for differentiation to proceed [48,49]. Further work by Zammit et al. [50] demonstrate that Pax7 is coexpressed with MyoD in proliferating myoblasts and that the down regulation of Pax7 in these cells coincided with the progression of cells into terminal differentiation, while a sub-set of cells down-regulate MyoD but retain Pax7 and withdraw from the cell-cycle, thus replenishing the satellite cell pool. Collectively, these studies suggest that Pax7 is involved in the co-ordination of normal postnatal myogenesis. Pax7 is instrumental in the initiation of the myogenic program

through the transcriptional activation of Myf5 and maintenance of the satellite cell pool by increasing cell survival and preventing differentiation, thus favouring selfrenewal [45,47,48]. The myogenic program and the temporal expression of Pax7 and the MRFs are depicted in Figure 1.

Although regulation of the myogenic program by the MRFs has been extensively examined, the molecular signals responsible for the initiation of the myogenic program are unclear and almost entirely unknown in the human. The study of human satellite cell function, is however, particularly difficult due to the challenge of accurately identifying this population *in vivo*.

1.1.4 DETECTION OF MUSCLE STEM CELLS IN VIVO:

Since the first characterization of the satellite cell in 1961 [3], the gold standard for the detection of satellite cells remains electron microscopy. However, due to the cost and limited access to equipment, the majority of human investigations use light and fluorescence microscopy for the study of this cell population. Numerous cell surface and intracellular antigens have been used for the detection of satellite cells such as neural cell adhesion molecule (N-CAM, CD56), hepatocyte growth factor receptor C-Met, M-cadherin, syndecan-3, syndecan-4 and Pax7 [51–54]. It is important to note, however, that these markers are not necessarily specific to satellite cells. For example, N-CAM is expressed on intramuscular nerves, motor unit end terminals, Schwann cells and regenerating myofibers [55–58], making the use of N-CAM alone for the identification of satellite cells difficult to interpret.



Figure 1. THE MYOGENIC PROGRAM: Upon activation, a quiescent satellite cell (SC, Pax7⁺) upregulates the myogenic regulatory factor (MRF) Myf5 which induces proliferation. MyoD is also upregulated in activated SCs, advancing the progression of the proliferating SCs (referred to as myoblasts). SCs begin to down regulate Myf5 and Pax7 and upregulate MRF4 and Myogenin which direct the progression of myoblasts through to terminal differentiation. Terminal differentiation occurs when the myoblasts either fuse with each other to form nascent myotubes or with the myofiber. A subset of myoblasts, rather than upregulating MRF4 and Myogenin, down-regulate MyoD and retain Pax7 expression, exit the cell-cycle and return to quiescence, thus replenishing the satellite cell pool.

Furthermore, the relative expression of C-met, Pax7 and N-CAM appear to be heterogeneous within the satellite cell pool, with C-met being lowly expressed on quiescent human satellite cells, making accurate quantification difficult with the

McMaster University

use of C-met alone [59]. N-CAM and Pax7 however appear to be the most consistently expressed satellite cell markers in human muscle, being co-localized on approximately 95% of satellite cells detected via immunohistochemistry (IHC) [58–61] and verified via flow cytometry (see appendix I) [59]. Thus the use of Pax7 or N-CAM seems to be the best tool for the identification of satellite cells in humans. However, the use of Pax7 appears to be the most specific marker in human muscle, rarely demonstrating non-specific binding with other structures in muscle cross-sections in our lab. In order to ensure the specificity of staining, especially when using a surface marker such as N-CAM, which does not always co-localize with the nucleus (as Pax7 does), the use of laminin or dystrophin to visualize the anatomical location of N-CAM is necessary (see Figure 2). The use of a nuclear marker such as DAPI is also necessary to confirm that the satellite cell marker is actually associated with a cell.

Thus the use of multiple antigen labeling is the most accurate method for the detection of the satellite cell pool. However, multiple labeling makes it difficult to also associate satellite cells with other markers such as MRFs or fiber specific proteins (i.e. myosin heavy chains). The use of serial sectioning is often used to probe for multiple antigens within a sample [60,62], although this process is tedious and time consuming.



Figure 2. IDENTIFICATION OF HUMAN SATELLITE CELLS: The use of N-CAM alone makes it difficult for proper identification of satellite cells as some staining appears nuclear (arrows) while often the staining appears peri-nuclear (arrow and question mark). The use of laminin helps to locate the positive cells beneath the basal lamina (lower panel arrows).

The use of flow cytometry for satellite cell quantification and multiple antigen detection is an emerging tool for human research (see appendix I) [59]. With flow cytometry, future studies will be able to probe satellite cells isolated from muscle biopsies for the presence of regulatory molecules such as hepatocyte growth factor (HGF) or interleukin-6 (IL-6) which will undoubtedly prove to be a powerful tool for understanding the regulation of human satellite cells *in vivo*.

1.2 MUSCLE STEM CELLS AND EXERCISE:

In multinucleated muscle fibers, it is generally accepted that each myonucleus is responsible for governing a given volume of cytoplasm [14,63]. This so-called myonuclear domain is thought to have a finite size, beyond which additional myonuclei are needed to support additional cytoplasm [14,64]. Resistance exercise is generally accompanied with an increase in cytoplasmic volume (fiber hypertrophy). Modest hypertrophy may be achieved through increased protein synthesis without the addition of new myonuclei [65], however for substantial increases in myofiber size, satellite cell-mediated myonuclear accretion must occur [15,64]. This theory is supported by a study by Adams et al. [31], where satellite cell ablation by a 25 Gy dose of radiation prevented the hypertrophic response to 3 months of compensatory overload, while nonirradiated control mice displayed significant hypertrophy [31]. In that study, satellite cell content was not directly measured however total muscle DNA content was markedly elevated in non-irradiated mice, where DNA content remained at pre-treatment levels in the irradiated group, illustrating a lack of myonuclear accretion in the irradiated group [31]. Muscle fibers are post-mitotic thus the addition of genetic material to the myofiber must come from an exogenous source. The maintenance of the myonuclear domain in addition to

the regular replacement of exhausted or damaged myonuclei (normal myonuclear turnover) is one of the main functions of satellite cells in post-natal muscle [15,29]. Figure 3 is a schematic diagram of the myonuclear domain theory and the role of satellite cell-mediated myonuclear addition.



Figure 3. MYONUCLEAR DOMAIN THEORY AND SATELLITE CELL-MEDIATED MYONUCLEAR ACCRETION: In the basal state, each myonuclei is responsible for the maintenance of a definite area of cytoplasm (dashed boxes). Moderate hypertrophy can be achieved (through exercise) by increasing the cytoplasmic volume thus increasing the myonuclear domain to a maximal "ceiling" [64]. For significant myofiber hypertrophy to occur, satellite cells (SCs) must activate, proliferate and fuse with the myofiber to contribute their nuclei, therefore increasing nuclear abundance and the number of myonuclear domains.

In humans, exercise training studies enumerate satellite cell number

before and after exercise training to quantify the satellite cell response. Although

this provides valuable insight into the function of satellite cells and their contribution to hypertrophy, this approach provides little information regarding the factors that regulate satellite cell function. A series of acute exercise studies utilizing a model of eccentrically-biased (ECC) muscle contractions to induce some microscopic or detectable level of muscle damage (myotrauma) have been used to investigate the acute response of the satellite cells [51,66,67]. An example of the findings, with regards to the expansion of the satellite cell pool from both exercise training and acute ECC studies, are shown below in Table 1.

N-CAM70% vs. untrainedN/ATrained vs. Untrained Power lifters[68]N-CAM46% increase10-wk strength TRYoung Females[69]N-CAM31% increase14-wk strength TRYoung Males[70]N-CAM100+% vs.N/ATrained vs.[71]untrainedUntrained Power untrainedUntrained Power lifters[66]N-CAM192% increase96h post acute ECCYoung Males[72]N-CAM192% increase96h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h post acute ECCYoung Males[52]Pax7 (IHC)36% increase24h post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)27% increase24h post acute ECCYoung Males[51] <tr< th=""><th>Satellite Cell Marker</th><th>Change in Satellite Cell Number</th><th>Exercise Protocol</th><th>Subject Group</th><th>Reference</th></tr<>	Satellite Cell Marker	Change in Satellite Cell Number	Exercise Protocol	Subject Group	Reference
N-CAM46% increase10-wk strength TRYoung Females[69]N-CAM31% increase14-wk strength TRYoung Males[70]N-CAM100+% vs.N/ATrained vs.[71]untrainedUntrained Powerlifters[72]N-CAM192% increase96h post acute ECCYoung Males[72]N-CAM141% increase24h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)27% increase24h post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]Pax7 (IHC)27% increase24h post acute ECCYoung Males[62]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	70% vs. untrained	N/A	Trained vs. Untrained Power lifters	[68]
N-CAM31% increase14-wk strength TRYoung Males[70]N-CAM100+% vs.N/ATrained vs.[71]untrainedUntrained PowerliftersN-CAM192% increase96h post acute ECCYoung Males[72]N-CAM141% increase24h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM27% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]157% increase72h post acute ECCYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]Pax7 (FC)27% increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)27% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	46% increase	10-wk strength TR	Young Females	[69]
N-CAM100+% vs. untrainedN/ATrained vs. Untrained Power lifters[71]N-CAM192% increase96h post acute ECCYoung Males[72]N-CAM141% increase24h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]Pax796% increase72h post acute ECCYoung Males[61]Pax7 (IHC)36% increase8d post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	31% increase	14-wk strength TR	Young Males	[70]
N-CAM192% increase96h post acute ECCYoung Males[72]N-CAM141% increase24h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]157% increase72h post acute ECCYoung Males[52]Pax796% increase72h post acute ECCYoung Males[61]Pax7 (IHC)36% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (IHC)28% increase24h post acute ECCYoung Males[59]Pax7 (IHC)27% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	100+% vs. untrained	N/A	Trained vs. Untrained Power lifters	[71]
N-CAM141% increase24h post acute ECCYoung Males[66]N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]157% increase72h post acute ECCYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase8d post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]N-CAM (IHC)28% increase24h post acute ECCYoung Males[59]N-CAM (IHC)27% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[52]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	192% increase	96h post acute ECC	Young Males	[72]
N-CAM27% increase8d post 36-km runYoung Males[67]N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]157% increase72h post acute ECCYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase8d post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]N-CAM (IHC)28% increase24h post acute ECCYoung Males[59]N-CAM (FC)27% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	141% increase	24h post acute ECC	Young Males	[66]
N-CAM80% increase72h post acute ECCYoung Males[51]Pax778% increase24h postYoung Males[52]157% increase72h post acute ECCYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]N-CAM (IHC)28% increase24h post acute ECCYoung Males[59]N-CAM (FC)27% increase24h post acute ECCYoung Males[59]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	27% increase	8d post 36-km run	Young Males	[67]
Pax778% increase 157% increase24h post 72h post acute ECCYoung Males[52]Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (FC)36 % increase24h post acute ECCYoung Males[59]N-CAM (IHC)28% increase24h post acute ECCYoung Males[59]N-CAM (FC)27% increase24h post acute ECCYoung Males[62]Pax727% increase24h post acute ECCYoung Males[62]	N-CAM	80% increase	72h post acute ECC	Young Males	[51]
Pax796% increase8d post acute ECCYoung Males[61]Pax7 (IHC)36% increase24h post acute ECCYoung Males[59]Pax7 (FC)36% increase24h post acute ECCYoung Males[59]N-CAM (IHC)28% increase24h post acute ECCYoung Males[59]N-CAM (FC)27% increase24h post acute ECCYoung Males[62]Pax727% increase24h post acute ECCYoung Males[62]	Pax7	78% increase 157% increase	24h post 72h post acute ECC	Young Males	[52]
Pax7 (IHC)36% increaseYoung Males[59]Pax7 (FC)36% increase24h post acute ECC100 minorN-CAM (IHC)28% increase24h post acute ECC100 minorN-CAM (FC)27% increase27% increase100 minorPax727% increase24h post acute ECCYoung Males[62]	Pax7	96% increase	8d post acute ECC	Young Males	[61]
Pax7 (FC)36 % increase24h post acute ECCN-CAM (IHC)28% increaseN-CAM (FC)27% increaseC-Met (IHC)80% increasePax727% increase24h post acute ECCYoung Males[62]	Pax7 (IHC)	36% increase		Young Males	[59]
N-CAM (IHC)28% increaseN-CAM (FC)27% increaseC-Met (IHC)80% increasePax727% increase24h post acute ECCYoung Males[62]	Pax7 (FC)	36 % increase	24h post acute ECC		
N-CAM (FC)27% increaseC-Met (IHC)80% increasePax727% increase24h post acute ECCYoung Males[62]	N-CAM (IHC)	28% increase			
C-Met (IHC)80% increasePax727% increase24h post acute ECCYoung Males[62]	N-CAM (FC)	27% increase			
Pax727% increase24h post acute ECCYoung Males[62]	C-Met (IHC)	80% increase			
	Pax7	27% increase	24h post acute ECC	Young Males	[62]

 TABLE 1: Change in Satellite Cell Number Following Acute and Chronic Exercise

 Satellite Cell
 Everying Protocol

(ECC = eccentric exercise, TR = training, IHC = immunohistochemistry, FC = flow cytometry)
Although these studies employ a variety of different levels of exercise intensity (90 maximal ECC vs. 300 maximal ECC) and different post-exercise biopsy timepoints, the collective conclusion from these studies is that following acute heavy exercise, muscle satellite cells enter the myogenic program and begin to proliferate in as little as 24h. However, in the majority of these studies, no mechanisms for the expansion of the satellite cell pool were examined.

The rapid expansion of the satellite cell pool following exercise is preceded by cellular events within the satellite cell niche [73], myofiber [74] and surrounding extracellular matrix [75], which provide cues for the satellite cell to activate and enter the cell-cycle. The underlying mechanisms of the satellite cell response to exercise in humans are currently unknown; however, some evidence suggests that the induction of muscle damage from acute exercise may help drive the myogenic response (i.e. induces a satellite cell response).

1.2.1: EXERCISE INDUCED MYOTRAUMA:

It has been established that resistance exercise is sufficient to induce a satellite cell response, however the mechanisms that drive this response are not well understood. It appears that muscle contractions that lead to fiber ultrastructural damage can induce a satellite cell response. In a study by Roth et al. [76], subjects that were determined to have the greatest level of muscle ultrastructural damage had a markedly higher number of active satellites cells compared to subjects with lower levels of induced damage [76]. Although it is speculated that the myotrauma, *per se,* can induce a myogenic (satellite cell)

response, whether the fiber damage itself drives the myogenic response or associated, but distinct signaling molecules drive this response remains unknown. Hepatocyte growth factor (HGF) is a one of the few molecules shown to definitively induce satellite cell activation [77,78]. HGF is normally sequestered in the extracellular matrix and can be released in response to structural stress [75,77]. HGF may also be released by the satellite cells in an autocrine/paracrine fashion in response to mechanical strain, further inducing an activation cascade [77,79]. HGF release appears to be a result of mechanical stress in the muscle and provides a causative link between mechanical damage to the muscle fiber and activation of the satellite cell pool, however the role of HGF in human muscle is less clear. HGF in circulation was significantly elevated 4h after 300 maximal eccentrically-biased muscle contractions in young males, which was associated with a significant increase in N-CAM⁺ cells 24h post-exercise [51]. Although HGF protein in the muscle was not increased after exercise the HGF activator, HGFA, was significantly elevated 24h after exercise [51]. This is the only study to attempt to classify the role of HGF and the satellite cell response in humans in vivo, and provides some support to the notion that HGF is a factor in the regulation of satellite cells in response to muscle contraction-induced damage. It remains a central theory that factors related to muscle damage or exercise, per se, induce the satellite cell response, however these 'factors' remain to be elucidated. Although there is relatively little known about the events that induce the satellite cell to become active, new data is emerging implicating secreted

growth factors and inflammatory cytokines as molecular signals that influence the myogenic program and thus the satellite cell response.

1.2.2: GROWTH FACTORS AND THE MUSCLE STEM CELL RESPONSE:

Following exercise, muscle fibers produce a host of growth factors such as insulin-like growth factor-1 (IGF-1) and HGF. IGF-1 has been demonstrated to activate protein synthetic machinery by its downstream interaction with the mammalian target of rapamycin (mTOR) [80] and is a potent mitogen believed to be involved with cell recruitment to aid in tissue repair [81]. The expression of the MRFs appears to be regulated, in part, by the activity of locally produced isoforms of IGF-1 [81,82]. IGF-1 is a growth factor found in circulation, produced in the liver and locally by the muscle fiber itself [81]. Three specific isoforms of IGF-1: IGF-1Ea, IGF-1Eb and IGF-1Ec, have been described in muscle and each may contribute to some extent to muscle regeneration/repair [82-84]. IGF-1Ea transcribes the full-length IGF-1 protein, which is identical to that produced by the liver [84]. IGF-1Eb may be involved in hypertrophy or muscle regeneration; however, the physiological role of IGF-1Eb in human skeletal muscle is currently unknown. IGF-1Ec or mechano growth factor (MGF) is a splice variant of IGF-1Ea that may have both autocrine and paracrine functions, stimulating satellite cell proliferation and muscle hypertrophy following muscle stretch or muscle damage [84,85].

IGF-1 has been shown to activate cultured porcine satellite cells through an mTOR dependent mechanism [86] and enhance the proliferative potential of

murine satellite cells by activating the phosphotidylinositol 3'-kinase/Akt signaling pathway [87]. In response to exercise or injury, IGF-1 splice variants increase within 24 h injury in rats [85,88]; however, results from human studies are conflicting.

MGF expression following isometric knee exercise can increase in as little as 2.5 h [89], while maximal electrical stimulation does not appear to induce a change in MGF mRNA expression [90]. Information regarding the response of IGF-1Ea to exercise is equivocal, with some animal and human work demonstrating significant increases in IGF-1Ea expression as early as 24 h post exercise or muscle damage [85,88,89,91], while others showed a marked decrease in IGF-1Ea and total IGF-1 from 4-12 hours post exercise that remained unchanged after 96 h [90,92]. Although animal data suggest IGF-1 and the IGF-1 splice variants are involved in satellite cell proliferation and differentiation, there is a paucity of data regarding the role of IGF-1 in regulating the myogenic program. However based on human and animal studies, it is clear that exercise induces an increase in IGF-1 expression, thus IGF-1 may be a critical regulator of the myogenic response.

Other growth factors have been shown to influence the proliferative phase or differentiation of satellite cells in animals or in cell culture. Figure 4 is a schematic representation of the molecular regulators of the myogenic program. These extracellular signaling molecules include basic fibroblast growth factor (bFGF), which has been demonstrated to induce activation/proliferation and

inhibit differentiation [93], nitric oxide (NO), which has been implicated in satellite cell activation, acting up-stream of HGF to mediate the release of HGF [79], Delta-1, which increases proliferation [94] and myostatin and TGF- β , which inhibits activation, differentiation and maintains quiescence [95,96].



Figure 4: MOLECULAR REGULATORS OF MYOGENESIS: Following muscle damage, several regulators are released which influence the myogenic program, positive regulators are illustrated with a green arrow and negative regulators are illustrated with a red line. Recently, angiotensin II (ANGII) has been implicated as an activator of quiescent satellite cells *in vivo* and in culture [97]. The actions of ANGII appear to regulate satellite cells through the angiotensin II receptor sub-type 1 (AT1), and may also play an instrumental role in satellite cell migration following muscle injury [97]. Platelet-derived growth factor (PDGF) and endothelial growth factor (EGF) also

appear to increase satellite cell proliferation in culture [9], however the majority of these factors have not been investigated *in vivo*. Therefore the contribution of these factors to normal human post-natal myogenesis is unclear.

1.2.3: INFLAMMATION AND THE MUSCLE STEM CELL RESPONSE:

Myotrauma due from unaccustomed exercise or injury may induce damage to contractile proteins and myofibrils, or larger ultrastructural disruptions, which initiates a significant inflammatory response [98,99]. Macrophage infiltration, which is responsible for the removal of cellular debris, is generally accompanied by lymphocytes, which coordinate the inflammatory response and importantly release factors that stimulate satellite cell proliferation [100,101]. Invading T-lymphocytes can bind adhesion molecules on damaged muscle fibers and release a host of cytokines including leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). In addition, the damaged muscle fiber itself secretes several cytokines and growth factors that may act as a chemoattractant for inflammatory and satellite cells, augmenting the rapid repair response [97,102–105]. In humans, administration of non-steroidal anti-inflammatory medication (NSAID) after damaging exercise inhibits the exercise-induced increase in satellite cells observed in controls [61,67], illustrating that inflammation may be a key factor regulating the satellite cell response.

Cytokines released by inflammatory cells have been shown to act on satellite cells *in vitro* by increasing proliferation [101]. Of the factors released, members of the interleukin-6 family of cytokines, IL-6 and LIF appear to be

instrumental in regulating satellite cell proliferation [101,106,107]. Both LIF and IL-6 bind the gp130 receptor, activating Janus-activated kinase (JAK) 2, which induces the phosphorylation of signal-transducers and activators of transcription (STAT) 3 [106,108]. Phosphorylated STAT3 (pSTAT3) forms a homodimer (or heterodimer with other STAT proteins), which translocates to the nucleus and induces the transcription of target genes such as Cyclin D1 and cMyc, which are instrumental in the initiation of the cell-cycle [108]. Importantly pSTAT3 also targets the IL-6 gene, thus the release of IL-6 and LIF induce the production of IL-6 in target cells, creating an autocrine/paracrine loop influencing satellite cell proliferation [108]. Until recently these findings had only been validated in cell culture models. In a landmark study by Serrano and colleagues [109], IL-6 was shown to be an essential regulator of satellite-cell function in vivo in a murine animal model. This study provided mechanistic evidence supporting the role of IL-6 as a regulator of satellite cell proliferation and that in the absence of IL-6 (IL-6^{-/-} mice), satellite cell proliferation and myonuclear accretion were severely blunted [109]. Furthermore, they reported that the deficiency in proliferation observed in the IL-6^{-/-} satellite cells could be rescued by the ectopic addition of IL-6 and that IL-6 mediated proliferation via the IL-6/STAT3 axis [109]. This study provides strong evidence to support the role of inflammatory cytokines in regulating muscle stem cell activity. However, the role of cytokines such as IL-6 in regulating human muscle stem cells in vivo has never been investigated.

The majority of mechanistic studies employ cell-culture or animal models to study potential satellite cell regulators. Thus with regards to key factors regulating the post-natal myogenic response in humans, there is a paucity of information, making it difficult to relate *in vitro* and animal work to humans. Clearly more research is needed to determine if factors such as IL-6 support satellite cell function in humans. In addition, elucidation of potential satellite cell regulators is imperative to develop treatment strategies for muscle wasting conditions such as cancer-associated cachexia and other atrophy conditions such as sarcopenia.

1.3 MUSCLE STEM CELLS AND AGING:

In humans, aging is a complex process and is associated with many physical and metabolic alterations that lead to the progressive loss of function over time [110]. Sarcopenia, which is an age-related condition that includes a progressive loss of muscle mass, leads to the loss of functional capacity and an increase in morbidity [111,112]. The progressive nature of age-related muscle loss leads to an increased incidence of injury and loss of autonomy, resulting in a significant loss in quality of life and increased risk of all-cause mortality [113,114]. Moreover, the economic impact of sarcopenia is staggering. Common agerelated injuries are often associated with falls due to a loss of strength and stability. Falls represent approximately 65% of injuries, 85% of injury-related hospital admissions, and 58% of injury related deaths in individuals over the age of 65. These statistics translate into actual Canadian health care costs of

approximately \$1 billion dollars annually [115]. Thus understanding the cellular mechanisms that lead to the onset and progression of sarcopenia is necessary to propose effective countermeasures.

The normal aging process has a profound effect on the structure and function of skeletal muscle. Aged muscle undergoes progressive atrophy, with a decreased cross-sectional area caused by a decrease in muscle fiber number and fiber size [116,117]. Muscle function also appears to be reduced in aged muscle, displaying a decrease in power and maximum force production [118]. In addition, aged muscle is more susceptible to injury compared to younger muscle and displays a decreased capacity for regeneration [116,119]. Satellite cells have been implicated as a significant factor contributing to the inability of the muscle to repair/remodel. An age-related impairment of the myogenic program (either impaired activation of or progression through the myogenic program) is hypothesized to be a major factor of satellite cell dysfunction, contributing to the onset or progression of sarcopenia. In adult muscle, satellite cells comprise only a small fraction of the total myonuclei (~2%) yet posseses an incredible proliferative capacity; capable of repairing extensive muscle injury. The size of the satellite cell pool is thought to remain relatively constant throughout adult life, however it has been suggested that in the later stages of life, the size of the satellite cell pool begins to decline, impairing the regenerative capacity of muscle. Results of studies comparing young and old muscle are equivocal, with some groups reporting a decline in satellite cells [120,121], while others report no

differences compared to young muscle [66,94]. Although it is unclear at present whether there is an age-related reduction in the satellite cell pool size, it is clear that aging is accompanied by an impairment in the response of satellite cells to myotrauma in humans [66] and animals [94]. Several studies have attributed both intrinsic factors of the satellite cells [122] as well as extrinsic factors such as age-related alterations to the satellite cell niche [94,123] as major influences on the dysfunction of this cell population with aging.

1.3.1: MUSCLE STEM CELLS AND AGING – INTRINSIC FACTORS:

Studies examining the ability of satellite cells isolated from aged muscle to proliferate in culture illustrate a basic decrease in the myogenic capacity of old cells. In culture, aged satellite cells and their progeny readily undergo apoptosis [121]. In the presence of pro-apoptotic agents such as tumor necrosis factoralpha (TNF- α), aged satellite cells demonstrate increased levels of activated caspases and TUNEL-positive cells as well as a 40% reduction in the antiapoptotic protein Bcl-2 [122]. These data suggest that under stress, aged satellite cells are less capable of entering the myogenic program and fail to resist cell-death, therefore impairing the regenerative response to injury. The sub-set of satellite cells that survive after becoming activated are able to proliferate and differentiate similarly to those of young muscle [120,121]. This suggests that if satellite cells can resist apoptosis, the intrinsic ability of individual cells to progress through the myogenic program is adequate. However, the amplification kinetics of the SC pool and total yield of SC progeny at a given time point is

significantly impaired in aged muscle [120,124]. In addition, the ability to repopulate the reserve pool following proliferation appears to be significantly reduced in aged satellite cells [125]. Interestingly, in aged animals, some muscle fibers are completely devoid of satellite cells, which may indicate some fibers have completely exhausted their satellite cell pool due to an impairment in self-renewal [120,121,125].

1.3.2: MUSCLE STEM CELLS AND AGING – EXTRINSIC FACTORS:

In addition to intrinsic impairments, the satellite cell niche and the systemic milieu have been identified as factors in age-related satellite cell dysfunction. The satellite cell niche or microenvironment that supports the satellite cell helps regulate satellite cell activity through physical contact and direct interaction with satellite cell membrane proteins [73,126]. Changes in the microenvironment associated with aging may impair satellite cell activation [94] and even influence muscle progenitors to adopt alternate lineages (i.e. become fibrotic [127] or adipogenic [128]) leading to the loss of regenerative capacity of cells in the aged niche [123]. Enhanced Wnt signaling in aged mice induces a shift from the normal myogenic fate to an increased propensity for fibrogenesis [127]. Furthermore, studies using heterochronic parabiotic pairings show a restored regenerative capacity of satellite cells in aged mice exposed to a young systemic environment [129]. The transmembrane protein Notch was shown to be implicated in age-induced satellite cell dysfunction, remaining inactivated following freeze-crush myotrauma, therefore preventing satellite cell activation

(as illustrated by 75% reduction in BRdU incorporation) [94]. The forced activation of Notch or the exposure to a young circulatory environment via heterochronic parabiotic pairing (or exposure to young serum in culture), restored the regenerative and proliferative response in aged muscle to the same levels observed in young animals [94,129]. In humans, Notch and Notch signaling gene expression were impaired in both isolated satellite cell cultures and in whole muscle homogenates in muscle biopsies taken from elderly men [130]. Interestingly, resistance exercise training attenuated the age-related impairment of Notch related gene expression, suggesting extrinsic factors can be manipulated through exercise [130]. It is important to note that when human primary myoblasts from young donors were cultured with serum from either young or elderly humans, proliferation and differentiation efficiencies were not altered [131]. However, in that study only 'young' satellite cells were tested in culture, thus it remains unclear whether a young circulatory environment can affect aged satellite cells in humans. While there is strong evidence from animal studies illustrating a deleterious effect of factors in aged serum on satellite cell function, a definitive study has yet to be performed using human donor cells.

1.3.3: AGING, MYOSTATIN AND MUSCLE STEM CELLS:

Additional circulating factors such as IL-6 and IGF-1 have been shown to change as a result of aging. IL-6 concentration in circulation may be increased in the elderly, which is associated with a loss of muscle strength [132,133]. Prolonged elevated levels of IL-6 was shown to induce muscle atrophy [134] and

thus may also negatively impact satellite cell function. Growth hormone receptor and IGF-1 protein content and circulating factors such as testosterone and IGF-1 also appear to be reduced in the elderly, which may reduce the anabolic capacity of aged muscle [65,135–137]. Perhaps the most potent of these circulating factors which influences muscle mass, which may also be elevated as a function of aging, is myostatin.

Myostatin (GDF-8) is a transforming growth factor- β (TGF- β) family member that plays an integral role in the growth and maintenance of muscle mass [138]. The function of myostatin is highly conserved across species [139] and the loss or mutation of the myostatin gene results in a 'double muscled' phenotype [138,139]. Muscle from myostatin null mice is typically 2-3 times larger than wild-type mice, which appears to be a consequence of both hypertrophy and hyperplasia of muscle fibers [138]. Postnatal blockade of myostatin signaling induces increases in muscle mass and strength, which is attributed to fiber hypertrophy in adult mice [140,141]. Myostatin works similarly to TGF- β signaling by binding to a type II serine/threonine kinase receptor activin IIb [142]. This activated complex phosphorylates receptor-regulated Smad proteins. Smad2 and Smad3 are the primary Smads, which mediate myostatin signaling [143]. This canonical myostatin signaling pathway has been shown to inhibit satellite cell proliferation by upregulating the cyclin dependent kinase (Cdk) inhibitor p21, which hypophosphorylates phospho-retinoblastoma (pRb) and prevents progression from G_1 to the DNA synthesis phase (S-phase) of the cell-

cycle [144]. Satellite cells in culture, treated with exogenous myostatin, illustrate a significant increase in p21 protein, a down regulation of Cdk2 and a downregulation in hyperphosphorylated Rb with a simultaneous increase in hypophosphorylated pRb, which prevents the upregulation of cell-cycle machinery and prevents the cells from passing the G₁ cell-cycle checkpoint and entering S-phase [95]. Myostatin may also signal via non-Smad mediated pathways (non-canonical), activating p38 mitogen activated protein kinase (MAPK) via the TAK1-MKK6 cascade [145] or by induction of the extracellular signal-regulated kinase (Erk) 1/2 signaling [146], inhibiting myoblast proliferation and differentiation.

In humans there is little work demonstrating a direct relationship between myostatin and muscle wasting; however there is some evidence to support the role of myostatin in muscle atrophy related to disease. Patients with HIV-associated muscle wasting demonstrate an elevation of serum and intramuscular concentrations of myostatin, suggesting that the upregulation of myostatin contributed to the overall loss of skeletal muscle [154]. Most of the evidence supporting a role for myostatin in regulating muscle mass is derived from animal studies. In the animal model of Duchenne muscular dystrophy (*MDX* mouse model), the blockade of myostatin signaling in postnatal *MDX* mice induced an 11-30% increase in muscle mass [140]. Furthermore, the lifelong lack of myostatin (*Mstn^{-/-}* mouse model) appears to reduce the aged phenotype compared to age-matched wild-type controls [147] suggesting myostatin is a key

contributor to sarcopenia in rodents. In humans, three days of unloading (withdrawal of weight bearing via unilateral lower limb suspension) increased myostatin protein and mRNA compared to the control limb, suggesting that myostatin is involved in the initiation of muscle atrophy [148]. The increase in myostatin was accompanied by increased gene expression of atrogin-1 and MuRF-1, which are E3 ubiquitin ligases, instrumental in the regulation of muscle atrophy [148–150]. Exercise has been shown to modulate myostatin by decreasing gene expression; however acute exercise does not appear to be sufficient to induce a decrease in myostatin protein in whole muscle [151]. Interestingly, 16 weeks of resistance exercise lead to a 44% increase in the fulllength latent myostatin complex while myostatin mRNA decreased approximately 44% over the 16 week training intervention [151]. Acute resistance exercise or exercise training did not appear to alter circulating myostatin concentrations although a significant degree of variability was noted between subjects [151]. Although exercise may alter myostatin levels at the whole muscle level, the precise mechanisms of how myostatin regulates human muscle satellite cell activity, in vivo, remains unknown. In addition, no data exist on the role of myostatin in regulating satellite cell function as a consequence of human aging.

1.4 PURPOSE OF THIS THESIS:

The research experiments discussed as part of this thesis were designed to investigate the role of key signaling molecules and their relationship with myogenic regulation in humans. In the first two studies, the role of two main

regulatory molecules (IGF-1 and IL-6) were analyzed by utilizing an experimental protocol known to induce a significant amount of myotrauma including ultrastructural damage (z-disk streaming, desmin disruption, increase in circulating muscle creatine kinase-CK) and a robust inflammatory response (macrophage and neutrophil infiltration) in order to induce a myogenic response [59,99,152]. The role of IGF-1 in the context of human satellite cell regulation has never been examined. Therefore, we first hypothesized that muscle specific IGF-1 gene expression would be upregulated in response to contraction-induced myotrauma and that the three different splice variants (IGF-1Ea, Eb, and Ec) would be differentially regulated. We also hypothesized that IGF-1Ec (MGF) may be upregulated early during the proliferative phase of the satellite cell response as MGF has been demonstrated to inhibit differentiation in myoblast cultures [153]. We analyzed MRF mRNA to determine the relationships among the IGF-1 splice-variants and the MRFs. We used immunohistochemistry to confirm the colocalization of IGF-1 with the satellite cells.

Interleukin-6 was recently implicated as a key regulator of satellite cell function in mice [109]. The role of IL-6 in muscle has generally been thought to alter metabolism or accompany exercise induced inflammation [102]. With regards to human satellite cell function no data exists on the role of IL-6 in the regulation of the satellite cell response to myotrauma. Therefore, using the same experimental protocol we investigated the role of IL-6 in regulating muscle satellite cell proliferation. We quantified the mRNA response of IL-6 and IL-6

related genes and investigated the role of STAT3 as a potential mechanism for the hypothesized IL-6-mediated satellite cell proliferation. We hypothesized that via the Jak2/STAT3 axis, IL-6 would induce the transcription of cell-cycle regulatory genes. We used immunohistochemistry to investigate the satellite cellspecific association of IL-6 and pSTAT3 with the myogenic response.

Myostatin regulates satellite cell function in mice through the inhibition of cell-cycle regulatory proteins [95]. It has also been hypothesized as a contributor to the progression of sarcopenia [147]. Finally due to speculation that myostatin is involved in satellite cell dysfunction with age, we examined a group of young (21y) and older (70y) adults before and after an acute bout of heavy resistance exercise, which was sufficient to induce a significant increase in blood creatine kinase (CK). We hypothesized that myostatin would be associated with a larger proportion of satellite cells in the elderly and that an increased proportion of myostatin positive satellite cells would be associated with an impaired myogenic response in the elderly. Based on recent findings of a fiber-type specific decline in satellite cell number [60], we further investigated the myostatin response in a fiber-type specific fashion. The results presented in this thesis provide evidence to support both IGF-1 and IL-6 as key regulators of human satellite cell proliferation and differentiation and also provide novel insight into the *in vivo* function of myostatin. Furthermore, we also describe myostatin as a potentially important regulator of satellite cell function following exercise which may be a key factor in age-related satellite cell dysfunction.

1.5 REFERENCE LIST:

- Irrcher I, Adhihetty PJ, Joseph AM, Ljubicic V, Hood DA (2003) Regulation of mitochondrial biogenesis in muscle by endurance exercise. Sports Med 33: 783-793.
- Mayhew DL, Kim JS, Cross JM, Ferrando AA, Bamman MM (2009) Translational Signaling Responses Preceeding Resistance Training-Mediated Myofiber Hypertropy in Young and Old Humans. J Appl Physiol
- 3. Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9: 493-495.
- Mitchell KJ, Pannerec A, Cadot B, Parlakian A, Besson V, Gomes ER, Marazzi G, Sassoon DA (2010) Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol 12: 257-266.
- 5. LaBarge MA, Blau HM (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 111: 589-601.
- Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401: 390-394.
- Buckingham M, Relaix F (2007) The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. Annu Rev Cell Dev Biol 23: 645-673.
- 8. Charge SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84: 209-238.
- 9. Hawke TJ, Garry DJ (2001) Myogenic satellite cells: physiology to molecular biology. J Appl Physiol 91: 534-551.
- Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75: 1351-1359.
- 11. Armand O, Boutineau AM, Mauger A, Pautou MP, Kieny M (1983) Origin of satellite cells in avian skeletal muscles. Arch Anat Microsc Morphol Exp 72: 163-181.
- 12. Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomes D, Tajbakhsh S (2005) Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev 19: 1426-1431.
- Relaix F, Rocancourt D, Mansouri A, Buckingham M (2005) A Pax3/Pax7dependent population of skeletal muscle progenitor cells. Nature 435: 948-953.
- 14. Hall ZW, Ralston E (1989) Nuclear domains in muscle cells. Cell 59: 771-772.

- 15. Petrella JK, Kim JS, Mayhew DL, Cross JM, Bamman MM (2008) Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. J Appl Physiol 104: 1736-1742.
- 16. Reznik M (1969) Thymidine-3H uptake by satellite cells of regenerating skeletal muscle. J Cell Biol 40: 568-571.
- 17. Moss FP, Leblond CP (1970) Nature of dividing nuclei in skeletal muscle of growing rats. J Cell Biol 44: 459-462.
- 18. Bischoff R (1975) Regeneration of single skeletal muscle fibers in vitro. Anat Rec 182: 215-235.
- 19. Bischoff R (1986) Proliferation of muscle satellite cells on intact myofibers in culture. Dev Biol 115: 129-139.
- 20. Partridge TA (2003) Stem cell route to neuromuscular therapies. Muscle Nerve 27: 133-141.
- 21. Ferrari G, Cusella-De AG, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279: 1528-1530.
- 22. Polesskaya A, Seale P, Rudnicki MA (2003) Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. Cell 113: 841-852.
- Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE (2005) Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122: 289-301.
- 24. Dreyfus PA, Chretien F, Chazaud B, Kirova Y, Caramelle P, Garcia L, Butler-Browne G, Gherardi RK (2004) Adult bone marrow-derived stem cells in muscle connective tissue and satellite cell niches. Am J Pathol 164: 773-779.
- 25. Seale P, Ishibashi J, Scime A, Rudnicki MA (2004) Pax7 is necessary and sufficient for the myogenic specification of CD45+:Sca1+ stem cells from injured muscle. PLoS Biol 2: E130.
- Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102: 777-786.
- 27. Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, Olsen S, Kjaer M (2005) The behaviour of satellite cells in response to exercise: what have we learned from human studies? Pflugers Arch 451: 319-327.
- Decary S, Mouly V, Hamida CB, Sautet A, Barbet JP, Butler-Browne GS (1997) Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. Hum Gene Ther 8: 1429-1438.

- 29. Schmalbruch H, Lewis DM (2000) Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. Muscle Nerve 23: 617-626.
- 30. Viguie CA, Lu DX, Huang SK, Rengen H, Carlson BM (1997) Quantitative study of the effects of long-term denervation on the extensor digitorum longus muscle of the rat. Anat Rec 248: 346-354.
- 31. Adams GR, Caiozzo VJ, Haddad F, Baldwin KM (2002) Cellular and molecular responses to increased skeletal muscle loading after irradiation. Am J Physiol Cell Physiol 283: C1182-C1195.
- Parise G, O'Reilly CE, Rudnicki MA (2006) Molecular regulation of myogenic progenitor populations. Appl Physiol Nutr Metab 31: 773-781.
- Cornelison DD, Wold BJ (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191: 270-283.
- 34. Cao Y, Kumar RM, Penn BH, Berkes CA, Kooperberg C, Boyer LA, Young RA, Tapscott SJ (2006) Global and gene-specific analyses show distinct roles for Myod and Myog at a common set of promoters. EMBO J 25: 502-511.
- Cornelison DD, Olwin BB, Rudnicki MA, Wold BJ (2000) MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Dev Biol 224: 122-137.
- 36. Parker MH, Perry RL, Fauteux MC, Berkes CA, Rudnicki MA (2006) MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation. Mol Cell Biol 26: 5771-5783.
- Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10: 1173-1183.
- Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151: 1221-1234.
- 39. Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17): 2895-2901.
- 40. Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA (1999) Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol 144: 631-643.
- Yablonka-Reuveni Z, Rudnicki MA, Rivera AJ, Primig M, Anderson JE, Natanson P (1999) The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol 210: 440-455.

- 42. Rhodes SJ, Konieczny SF (1989) Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev 3: 2050-2061.
- 43. Smith CK, Janney MJ, Allen RE (1994) Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol 159: 379-385.
- 44. Oustanina S, Hause G, Braun T (2004) Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J 23: 3430-3439.
- 45. Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M (2006) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 172: 91-102.
- 46. Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA (2006) Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol 172: 103-113.
- 47. McKinnell IW, Ishibashi J, Le GF, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ, Rudnicki MA (2008) Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol 10: 77-84.
- 48. Olguin HC, Olwin BB (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 275: 375-388.
- 49. Olguin HC, Yang Z, Tapscott SJ, Olwin BB (2007) Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. J Cell Biol 177: 769-779.
- 50. Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166: 347-357.
- O'Reilly C, McKay B, Phillips S, Tarnopolsky M, Parise G (2008) Hepatocyte growth factor (HGF) and the satellite cell response following muscle lengthening contractions in humans. Muscle Nerve 38: 1434-1442.
- 52. McKay BR, De Lisio M, Johnston AP, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G (2009) Association of interleukin-6 signalling with the muscle stem cell response following musclelengthening contractions in humans. PLoS One 4: e6027.
- 53. Cornelison DD, Filla MS, Stanley HM, Rapraeger AC, Olwin BB (2001) Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. Dev Biol 239: 79-94.
- 54. Sajko S, Kubinova L, Cvetko E, Kreft M, Wernig A, Erzen I (2004) Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. J Histochem Cytochem 52: 179-185.

- 55. Cashman NR, Covault J, Wollman RL, Sanes JR (1987) Neural cell adhesion molecule in normal, denervated, and myopathic human muscle. Ann Neurol 21: 481-489.
- 56. Illa I, Leon-Monzon M, Dalakas MC (1992) Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. Ann Neurol 31: 46-52.
- 57. Mechtersheimer G, Staudter M, Moller P (1992) Expression of the natural killer (NK) cell-associated antigen CD56(Leu-19), which is identical to the 140-kDa isoform of N-CAM, in neural and skeletal muscle cells and tumors derived therefrom. Ann N Y Acad Sci 650: 311-316.
- Lindstrom M, Thornell LE (2009) New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. Histochem Cell Biol 132: 141-157.
- 59. McKay BR, Toth KG, Tarnopolsky MA, Parise G (2010) Satellite cell number and cell cycle kinetics in response to acute myotrauma in humans: immunohistochemistry versus flow cytometry. J Physiol 588: 3307-3320.
- Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, van Loon LJ (2007) Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. Am J Physiol Endocrinol Metab 292: E151-E157.
- 61. Mikkelsen UR, Langberg H, Helmark IC, Skovgaard D, Andersen LL, Kjaer M, Mackey AL (2009) Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise. J Appl Physiol 107: 1600-1611.
- 62. Toth KG, McKay BR, De Lisio M, Little JP, Tarnopolsky MA, Parise G (2011) IL-6 induced STAT3 signalling is associated with the proliferation of human muscle satellite cells following acute muscle damage. PLoS One 6: e17392.
- 63. Cheek DB (1985) The control of cell mass and replication. The DNA unit-a personal 20-year study. Early Hum Dev 12: 211-239.
- 64. Allen DL, Roy RR, Edgerton VR (1999) Myonuclear domains in muscle adaptation and disease. Muscle Nerve 22: 1350-1360.
- 65. Petrella JK, Kim JS, Cross JM, Kosek DJ, Bamman MM (2006) Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. Am J Physiol Endocrinol Metab 291: E937-E946.
- Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA (2006) Satellite cell numbers in young and older men 24 hours after eccentric exercise. Muscle Nerve 33: 242-253.

- 67. Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH, Schroder HD, Langberg H (2007) The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. J Appl Physiol 103: 425-431.
- 68. Kadi F, Eriksson A, Holmner S, Butler-Browne GS, Thornell LE (1999) Cellular adaptation of the trapezius muscle in strength-trained athletes. Histochem Cell Biol 111: 189-195.
- 69. Kadi F, Thornell LE (2000) Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. Histochem Cell Biol 113: 99-103.
- Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, Andersen JL (2004) The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. J Physiol 558: 1005-1012.
- 71. Eriksson A, Kadi F, Malm C, Thornell LE (2005) Skeletal muscle morphology in power-lifters with and without anabolic steroids. Histochem Cell Biol 124: 167-175.
- 72. Crameri RM, Langberg H, Magnusson P, Jensen CH, Schroder HD, Olesen JL, Suetta C, Teisner B, Kjaer M (2004) Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. J Physiol 558: 333-340.
- Kuang S, Gillespie MA, Rudnicki MA (2008) Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2: 22-31.
- Vierck J, O'Reilly B, Hossner K, Antonio J, Byrne K, Bucci L, Dodson M (2000) Satellite cell regulation following myotrauma caused by resistance exercise. Cell Biol Int 24: 263-272.
- 75. Tatsumi R, Allen RE (2004) Active hepatocyte growth factor is present in skeletal muscle extracellular matrix. Muscle Nerve 30: 654-658.
- 76. Roth SM, Martel GF, Ivey FM, Lemmer JT, Tracy BL, Metter EJ, Hurley BF, Rogers MA (2001) Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training. J Gerontol A Biol Sci Med Sci 56: B240-B247.
- 77. Tatsumi R, Sheehan SM, Iwasaki H, Hattori A, Allen RE (2001) Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. Exp Cell Res 267: 107-114.
- Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice GM (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J Cell Physiol 165: 307-312.
- Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, Allen RE (2002) Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol Biol Cell 13: 2909-2918.

- Frost RA, Lang CH (2011) mTor signaling in skeletal muscle during sepsis and inflammation: where does it all go wrong? Physiology (Bethesda) 26: 83-96.
- 81. Mourkioti F, Rosenthal N (2005) IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
- 82. Chakravarthy MV, Abraha TW, Schwartz RJ, Fiorotto ML, Booth FW (2000) Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway. J Biol Chem 275: 35942-35952.
- Rotwein P, Pollock KM, Didier DK, Krivi GG (1986) Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. J Biol Chem 261: 4828-4832.
- 84. Yang S, Alnaqeeb M, Simpson H, Goldspink G (1996) Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. J Muscle Res Cell Motil 17: 487-495.
- 85. Hill M, Goldspink G (2003) Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J Physiol 549: 409-418.
- 86. Han B, Tong J, Zhu MJ, Ma C, Du M (2008) Insulin-like growth factor-1 (IGF-1) and leucine activate pig myogenic satellite cells through mammalian target of rapamycin (mTOR) pathway. Mol Reprod Dev 75: 810-817.
- 87. Galvin CD, Hardiman O, Nolan CM (2003) IGF-1 receptor mediates differentiation of primary cultures of mouse skeletal myoblasts. Mol Cell Endocrinol 200: 19-29.
- 88. Hill M, Wernig A, Goldspink G (2003) Muscle satellite (stem) cell activation during local tissue injury and repair. J Anat 203: 89-99.
- 89. Greig CA, Hameed M, Young A, Goldspink G, Noble B (2006) Skeletal muscle IGF-I isoform expression in healthy women after isometric exercise. Growth Horm IGF Res 16: 373-376.
- 90. Bickel CS, Slade J, Mahoney E, Haddad F, Dudley GA, Adams GR (2005) Time course of molecular responses of human skeletal muscle to acute bouts of resistance exercise. J Appl Physiol 98: 482-488.
- 91. Hameed M, Orrell RW, Cobbold M, Goldspink G, Harridge SD (2003) Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. J Physiol 547: 247-254.
- Psilander N, Damsgaard R, Pilegaard H (2003) Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. J Appl Physiol 95: 1038-1044.

- 93. DiMario J, Buffinger N, Yamada S, Strohman RC (1989) Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. Science 244: 688-690.
- 94. Conboy IM, Conboy MJ, Smythe GM, Rando TA (2003) Notch-mediated restoration of regenerative potential to aged muscle. Science 302: 1575-1577.
- McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (2003) Myostatin negatively regulates satellite cell activation and selfrenewal. J Cell Biol 162: 1135-1147.
- Allen RE, Boxhorn LK (1987) Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. J Cell Physiol 133: 567-572.
- Johnston AP, Baker J, Bellamy LM, McKay BR, De Lisio M, Parise G (2010) Regulation of muscle satellite cell activation and chemotaxis by angiotensin II. PLoS One 5: e15212.
- 98. Smith LL, Keating MN, Holbert D, Spratt DJ, McCammon MR, Smith SS, Israel RG (1994) The effects of athletic massage on delayed onset muscle soreness, creatine kinase, and neutrophil count: a preliminary report. J Orthop Sports Phys Ther 19: 93-99.
- 99. Beaton LJ, Tarnopolsky MA, Phillips SM (2002) Contraction-induced muscle damage in humans following calcium channel blocker administration. J Physiol 544: 849-859.
- 100. Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice GM (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J Cell Physiol 165: 307-312.
- 101. Cantini M, Massimino ML, Rapizzi E, Rossini K, Catani C, Dalla LL, Carraro U (1995) Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes. Biochem Biophys Res Commun 216: 49-53.
- 102. Pedersen BK, Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev 88: 1379-1406.
- Broholm C, Mortensen OH, Nielsen S, Akerstrom T, Zankari A, Dahl B, Pedersen BK (2008) Exercise induces expression of leukaemia inhibitory factor in human skeletal muscle. J Physiol 586: 2195-2201.
- 104. Gallucci S, Provenzano C, Mazzarelli P, Scuderi F, Bartoccioni E (1998) Myoblasts produce IL-6 in response to inflammatory stimuli. Int Immunol 10: 267-273.
- Tomiya A, Aizawa T, Nagatomi R, Sensui H, Kokubun S (2004) Myofibers express IL-6 after eccentric exercise. Am J Sports Med 32: 503-508.

- 106. Spangenburg EE, Booth FW (2002) Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation. Am J Physiol Cell Physiol 283: C204-C211.
- 107. Austin L, Bower J, Kurek J, Vakakis N (1992) Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. J Neurol Sci 112: 185-191.
- 108. Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signaling pathway. J Cell Sci 117: 1281-1283.
- 109. Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P (2008) Interleukin-6 is an essential regulator of satellite cellmediated skeletal muscle hypertrophy. Cell Metab 7: 33-44.
- 110. Roubenoff R (2003) Catabolism of aging: is it an inflammatory process? Curr Opin Clin Nutr Metab Care 6: 295-299.
- 111. Roubenoff R (1999) The pathophysiology of wasting in the elderly. J Nutr 129: 256S-259S.
- 112. Beccafico S, Puglielli C, Pietrangelo T, Bellomo R, Fano G, Fulle S (2007) Age-dependent effects on functional aspects in human satellite cells. Ann N Y Acad Sci 1100: 345-352.
- 113. Roubenoff R, Hughes VA (2000) Sarcopenia: current concepts. J Gerontol A Biol Sci Med Sci 55: M716-M724.
- Melton LJ, III, Khosla S, Crowson CS, O'Connor MK, O'Fallon WM, Riggs BL (2000) Epidemiology of sarcopenia. J Am Geriatr Soc 48: 625-630.
- 115. SMARTRISK (2009) *The economic burden of unintentional injury in Canada*. s I SMARTRISK,

http://www.smartrisk.ca/index.php/burden:

- 116. Grounds MD (1998) Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. Ann N Y Acad Sci 854: 78-91.
- 117. Koopman R, van Loon LJ (2009) Aging, exercise, and muscle protein metabolism. J Appl Physiol 106: 2040-2048.
- 118. Brooks SV, Faulkner JA (1988) Contractile properties of skeletal muscles from young, adult and aged mice. J Physiol 404: 71-82.
- 119. Brooks SV, Faulkner JA (1996) The magnitude of the initial injury induced by stretches of maximally activated muscle fibres of mice and rats increases in old age. J Physiol 497 (Pt 2): 573-580.
- 120. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z (2006) Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294: 50-66.
- 121. Collins CA, Zammit PS, Ruiz AP, Morgan JE, Partridge TA (2007) A population of myogenic stem cells that survives skeletal muscle aging. Stem Cells 25: 885-894.
- 122. Jejurikar SS, Henkelman EA, Cederna PS, Marcelo CL, Urbanchek MG, Kuzon WM, Jr. (2006) Aging increases the susceptibility of skeletal

muscle derived satellite cells to apoptosis. Exp Gerontol 41: 828-836.

- 123. Carlson ME, Conboy IM (2007) Loss of stem cell regenerative capacity within aged niches. Aging Cell 6: 371-382.
- 124. Corbu A, Scaramozza A, Badiali-DeGiorgi L, Tarantino L, Papa V, Rinaldi R, D'Alessandro R, Zavatta M, Laus M, Lattanzi G, Cenacchi G (2010) Satellite cell characterization from aging human muscle. Neurol Res 32: 63-72.
- 125. Day K, Shefer G, Shearer A, Yablonka-Reuveni Z (2010) The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev Biol 340: 330-343.
- 126. Gopinath SD, Rando TA (2008) Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell 7: 590-598.
- 127. Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317: 807-810.
- 128. Taylor-Jones JM, McGehee RE, Rando TA, Lecka-Czernik B, Lipschitz DA, Peterson CA (2002) Activation of an adipogenic program in adult myoblasts with age. Mech Ageing Dev 123: 649-661.
- 129. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433: 760-764.
- Carey KA, Farnfield MM, Tarquinio SD, Cameron-Smith D (2007) Impaired expression of Notch signaling genes in aged human skeletal muscle. J Gerontol A Biol Sci Med Sci 62: 9-17.
- 131. George T, Velloso CP, Alsharidah M, Lazarus NR, Harridge SD (2010) Sera from young and older humans equally sustain proliferation and differentiation of human myoblasts. Exp Gerontol 45: 875-881.
- 132. Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, Nevitt M, Harris TB (2002) Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. J Gerontol A Biol Sci Med Sci 57: M326-M332.
- 133. Pereira LS, Narciso FM, Oliveira DM, Coelho FM, Souza DG, Dias RC (2009) Correlation between manual muscle strength and interleukin-6 (IL-6) plasma levels in elderly community-dwelling women. Arch Gerontol Geriatr 48: 313-316.
- 134. Haddad F, Zaldivar F, Cooper DM, Adams GR (2005) IL-6-induced skeletal muscle atrophy. J Appl Physiol 98: 911-917.
- 135. Hameed M, Toft AD, Pedersen BK, Harridge SD, Goldspink G (2007) Effects of eccentric cycling exercise on IGF-I splice variant expression in the muscles of young and elderly people. Scand J Med Sci Sports

- 136. Musaro A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, Barton ER, Sweeney HL, Rosenthal N (2001) Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 27: 195-200.
- Leger B, Derave W, De BK, Hespel P, Russell AP (2008) Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res 11: 163-175B.
- 138. McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387: 83-90.
- 139. McPherron AC, Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94: 12457-12461.
- 140. Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS (2002) Functional improvement of dystrophic muscle by myostatin blockade. Nature 420: 418-421.
- 141. Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM (2003) Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun 300: 965-971.
- 142. Lee SJ, McPherron AC (2001) Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A 98: 9306-9311.
- 143. Huang Z, Chen X, Chen D (2011) Myostatin: A novel insight into its role in metabolism, signal pathways, and expression regulation. Cell Signal 23: 1441-1446.
- 144. Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R (2000) Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 275: 40235-40243.
- 145. Philip B, Lu Z, Gao Y (2005) Regulation of GDF-8 signaling by the p38 MAPK. Cell Signal 17: 365-375.
- 146. Yang W, Chen Y, Zhang Y, Wang X, Yang N, Zhu D (2006) Extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase pathway is involved in myostatin-regulated differentiation repression. Cancer Res 66: 1320-1326.
- 147. Siriett V, Platt L, Salerno MS, Ling N, Kambadur R, Sharma M (2006) Prolonged absence of myostatin reduces sarcopenia. J Cell Physiol 209: 866-873.
- 148. Gustafsson T, Osterlund T, Flanagan JN, von WF, Trappe TA, Linnehan RM, Tesch PA (2010) Effects of 3 days unloading on molecular regulators of muscle size in humans. J Appl Physiol 109: 721-727.

- 149. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL (2001) Atrogin1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci U S A 98: 14440-14445.
- 150. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704-1708.
- 151. Kim JS, Petrella JK, Cross JM, Bamman MM (2007) Load-mediated downregulation of myostatin mRNA is not sufficient to promote myofiber hypertrophy in humans: a cluster analysis. J Appl Physiol 103: 1488-1495.
- 152. Beaton LJ, Allan DA, Tarnopolsky MA, Tiidus PM, Phillips SM (2002) Contraction-induced muscle damage is unaffected by vitamin E supplementation. Med Sci Sports Exerc 34: 798-805.
- 153. Yang SY, Goldspink G (2002) Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett 522: 156-160.
- 154. Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S (1998) Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci U S A 95: 14938-14943

Chapter 2: Manuscript 1

The Journal of Physiology. 2008 Nov 15;586(Pt 22):5549-60

Co-expression of IGF-1 Family Members with Myogenic Regulatory Factors Following Acute Damaging Muscle Lengthening Contractions in Humans

Bryon R McKay¹, Ciara E. O'Reilly¹, Stuart M. Phillips¹, Mark A. Tarnopolsky² and Gianni Parise^{1, 3}

Departments of Kinesiology¹, Pediatrics² and Medical Physics & Applied Radiation Sciences³, McMaster University, Hamilton Ontario, L8S 4L8

Running Title: IGF-1, myogenic regulatory factors and exercise

Correspondence: Gianni Parise, PhD Departments of Kinesiology, and Medical Physics & Applied Radiation Sciences McMaster University Hamilton, Ontario, Canada L8S 4L8

Telephone:	(905) 525-9140 x27353
Fax:	(905) 523-6011
E-mail:	pariseg@mcmaster.ca

Abstract:

Muscle regeneration following injury is dependent on the ability of muscle satellite cells to activate, proliferate and fuse with damaged fibers. This process is controlled by the myogenic regulatory factors (MRF). Little is known about the temporal relation of the MRF to the expression of known myogenic growth factors (i.e. IGF-1) in humans following muscle damage. Eight subjects $(20.6\pm2.1\gamma)$; 81.4±9.8kg) performed 300 lengthening contractions (180°.s⁻¹) of their knee extensors in one leg on a dynamometer. Blood and muscle samples were collected before and at 4 (T4), 24 (T24), 72 (T72), and 120 hours (T120) postexercise. Mechano growth factor (MGF), IGF-1Ea, and IGF-1Eb mRNA were quantified. Serum IGF-1 did not change over the post-exercise time-course. IGF-1Ea and IGF-1Eb mRNA increased ~4-6 fold by T72 (p<0.01) and MGF mRNA expression peaked at T24 (p=0.005). MyoD mRNA expression increased ~2-fold at T4 (p<0.05). Myf5 expression peaked at T24 (p<0.05), while MRF4 and myogenin mRNA expression peaked at T72 (p<0.05). Myf5 expression strongly correlated with the increase in MGF mRNA ($r^2=0.83$; p=0.03), while MRF4 was correlated with both IGF-1Ea and Eb (r^2 =0.90; r^2 =0.81, respectively; p<0.05). Immunofluorescence analysis showed IGF-1 protein expression localized to satellite cells at T24, and to satellite cells and the myofiber at T72 and T120; IGF-1 was not detected at T0 or T4. These results suggest that the temporal response of MGF is likely related to the activation/proliferation phase of the myogenic program as marked by an increase in both Myf5 and MyoD, while

IGF-1Ea and Eb may be temporally related to differentiation as marked by an increase in MRF4 and myogenin expression following acute muscle damage.

Key Words: Insulin-like growth factor-1, mechano growth factor, myogenic regulatory factors, damaging lengthening contractions

Introduction:

Muscle repair/regeneration following injury is dependent on the action of muscle satellite (stem) cells to activate, proliferate and terminally differentiate. The paired box transcription factor Pax7 is expressed in guiescent and activated satellite cells and directs the induction of target genes such as Myf5 that regulate entry of satellite cells into the myogenic program [1,2]. Myogenesis is controlled by a group of transcriptional networks known as the myogenic regulatory factors (MRF) [3,4]. There are four MRF involved in adult myogenesis, MyoD, Myf5, MRF4 and myogenin. MyoD and Myf5 are transcription factors known to regulate myoblast proliferation while MRF4 and myogenin are transcription factors that regulate terminal differentiation (for review see [4]. The time course for MRF expression is well defined in cell culture; however, data from humans is less clear [5,6]. The myogenic commitment of satellite cells is marked by the presence of Myf5, which is expressed early after mitogenic activation. The proliferative phase of the myogenic program is associated with the coexpression of Myf5 and MyoD [7–9]. The Subsequent down-regulation of Myf5 and MyoD is associated with the

induction of differentiation, while the up-regulation of myogenin and MRF4 is known to direct terminal differentiation.

The MRF expression time-course in human tissue is not well defined, in part due to the varying models used to examine MRF expression, but also due to the fact that MRF expression profiles are confounded by non-satellite cell myofibrillar gene expression [5,6,10–12]. Although exercise has been used to examine MRF gene expression in humans [5,6,10,11], there is a paucity of information on the time-course of MRF expression following contraction-induced muscle damage. There is even less information on the temporal association between potential satellite cell regulators, such as IGF-1, and MRF expression in human tissue in the context of muscle repair and regenesis.

The expression of the MRF appears to be regulated, in part by the mitotic and myogenic activity of locally produced isoforms of insulin-like growth factor-1 (IGF-1) [13,14]. IGF-1 is a growth factor found in circulation (produced in the liver) and locally in muscle. Three specific isoforms of IGF-1, IGF-1Ea, IGF-1Eb and IGF-1Ec, have been described in muscle and each may contribute in some extent to muscle regeneration [13,15,16]. IGF-1Eb may be involved in hypertrophy or muscle regeneration; however, the physiological roll of IGF-1Eb in human skeletal muscle is currently unknown. IGF-1Ec or mechano growth factor (MGF) is a splice variant of IGF-1Ea that may have both autocrine and paracrine functions, stimulating satellite cell proliferation and muscle hypertrophy following muscle stretch or muscle damage [16,17]. IGF-1Ea and MGF expression have

been shown to increase as early as 24 h after exercise and injury in rats [17,18]; however, results from human studies are conflicting [5,19]. Information regarding the response of IGF-1Ea to exercise is equivocal [5,6,17–20]. There is little evidence regarding the expression of IGF-1Eb in human muscle following exercise, and there is no information on how the IGF-1 isoforms relate to MRF expression in humans.

The aims of the current study were to: 1) investigate the time-course of expression for IGF-1Ea, IGF-1Eb and MGF in human muscle following intense damaging muscle contractions; 2) investigate the time-course of expression for MyoD, Myf5, MRF4 and myogenin in human muscle following damaging exercise; and 3) determine if any there were relationships between the temporal expression of the IGF-1 isoforms and the MRF in the post-exercise period. Based on available data from humans we hypothesized that both MyoD and Myf5 would be expressed early following injury (within 24 h) and that MRF4 and myogenin expression would occur after Myf5 and MyoD. Further, MGF and IGF-1 expression would increase early after exercise and remain elevated for a number of days post exercise.

Methods:

Subjects:

Eight healthy males (age 20.6 \pm 2.1 y, height 180.5 \pm 5.2 cm, weight 81.4 \pm 9.8 kg) were recruited from the McMaster University community. Subjects underwent

a routine medical screening, completed a health questionnaire and were not involved in any lower body resistance training for at least 6 months prior to beginning the study. Subjects were told to refrain from exercising throughout the time-course of the study. All subjects were informed of the procedures and potential risks associated with the study and gave their written informed consent to participate. This study was approved by the Hamilton Health Sciences Research Ethic Board and conforms to all declarations on the use of human subjects as research participants.

Muscle Damage Protocol:

Maximal isokinetic unilateral muscle lengthening contractions of the *quadriceps femoris* were performed using the Biodex dynamometer (Biodex-System 3, Biodex Medical Systems, Inc., USA) at 180°.s⁻¹. For each subject, one leg was selected randomly to perform the exercise protocol described below. Movement at the shoulders, hips, and thigh were restrained with straps in order to isolate the knee extensors during the protocol.

Immediately prior to the intervention, subjects underwent a brief familiarization with the equipment, involving 5 to 10 submaximal lengthening contractions of the leg to be exercised. Subjects were required to perform 30 sets of 10 maximal knee extensions with one minute rest between sets, for a total of 300 lengthening contractions. During each set, investigators provided verbal encouragement for the subjects to complete and exert maximal force during each contraction. This

protocol has been previously shown to induce a significant level of skeletal muscle damage [21].

Muscle Biopsies:

Five percutaneous needle biopsies were obtained from the mid-portion of the vastus lateralis under local anesthetic (1% lidocaine). One muscle biopsy was obtained prior to the intervention. Baseline measures were generated from the pre-intervention biopsy (PRE) taken in the morning prior to beginning the exercise session. Four biopsies were obtained from the working leg at different time points following the intervention. Muscle biopsies and blood draws were taken concurrently, at 4 hours (T4), 24 hours (T24), 72 hours (T72), and 120 hours (T120) post-intervention from a new incision 3-5 cm proximal to the last biopsy site. Approximately 150 mg of muscle tissue was collected from each biopsy using manual suction. Following collection of the sample, the muscle was dissected free of adipose and connective tissue and flash-frozen in liquid nitrogen, and stored at -80 °C for later analysis. For immunohistochemistry, a subset of subjects (n = 4) had approximately 10 mg of fresh muscle dissected from the biopsies before the biopsies were flash frozen. This smaller piece of tissue was oriented and mounted in OCT compound (Tissue-Tek, Sakura Finetek, USA) and frozen in isopentane cooled with liquid nitrogen. The mounted samples were stored at -80 °C and then sectioned (7 µm) at -20 °C. The crosssections were mounted on slides and stored at -80 °C for immunohistochemical analysis.
Immunohistochemistry / Immunofluorescence:

Immunohistochemistry / immunofluorescent staining procedures were modified from previously published methods [22,23]. Briefly, 7 µm sections were fixed with 2% paraformaldehyde (PFA, Sigma, USA) for 10 min followed by several washes in 1x PBS. Sections were then covered for 30 min in a blocking solution containing, 2% BSA, 5% FBS, 0.2% Triton-X 100, 0.1% sodium azide followed by incubation in the Pax7 antibody (DSHB, USA) overnight at 4°C. Sections were washed in 1xPBS and incubated in the secondary antibody (Alexafluor 594 goat anti-mouse; 1:200, Invitrogen, Molecular Probes Inc., USA) for 2.5 hr at room temperature. After washing, sections were re-fixed with 2% PFA to prevent migration of the secondary antibody. Sections were then incubated for 30 min in a blocking solution containing 10% goat serum (Sigma, USA) and 0.2% Triton-X 100. Sections were then incubated with an anti-human IGF-1 antibody (1:50, Chemicon, Millipore, USA) for 1.5 hr at room temperature. After washing in PBS, sections were incubated for 1 hr with a polyclonal goat anti-mouse immunoglobulin biotinylated secondary antibody (Dako Canada, Inc., Canada). After washing in PBS, sections were then incubated for 1 hr with a streptavidin-FITC fluorochrome (Biosource. USA) Sections were then washed with PBS and DAPI for nuclear staining. Sections were mounted with a fluorescent mounting medium (Dako Canada, Inc., Canada) to preserve the fluorescent signal. Stained slides were viewed with the Nikon Eclipse 90*i* Microscope (Nikon Instruments, Inc., USA) and images were captured using the Nikon NIS Elements 3.0 software

(Nikon Instruments, Inc., USA). Stains were compared to secondary-only negative controls to ensure the staining patterns observed were not due to no-specific binding of the secondary antibodies. Additionally it is important to note that Pax7 and IGF-1 antibodies shows high specificity of staining. In all sections there were Pax7+ cells that were not IGF-1 positive, as well as IGF-1 positive regions including myonuclei that were not Pax7 positive. Co-localization of IGF-1 and Pax7 did occur at varying degrees at the different time-points, further indicating the specificity of these antibodies.

Blood Measures:

A resting blood sample was obtained from the antecubital vein immediately prior to the intervention. Blood was also drawn at T4, T24, T72 and T120. Approximately 20 mL of blood was collected and separated into one heparinized and one non-heparinized vacutainer tube at each time-point. Samples were separated into 50 µL aliquots, flash-frozen in liquid nitrogen, and stored at -80 °C for analysis at a later date. Serum samples were analyzed for IGF-1 protein using commercially available Enzyme-Linked ImmunoSorbant Assay (ELISA) kits following the manufactures instructions (R&D Systems, Inc., USA).

RNA isolation:

RNA was isolated from homogenized muscle samples using the TRIzol method. Briefly, a total of 1.0 mL of TRIzol Reagent (Invitrogen Corporation, Canada) was added to the homogenizer. Approximately 25 mg (average weight 25.33 ± 1.67

mg) of each muscle sample was cut individually in liquid nitrogen using an RNase-free razor blade treated with Ambion RNaseZap (Ambion Biosystems, USA). Each muscle sample was ground and the muscle homogenate was centrifuged at 12000 X g and 4 °C for 10 minutes. The clear supernatant was allowed to incubate at room temperature for 15 minutes. Chloroform (200 μ L) was mixed with the supernatant and incubated at room temperature for 2-3 minutes, then centrifuged at 12000 X g for 15 minutes at 4 °C. The aqueous phase was transferred to a fresh tube, allowed to incubate with 500 μ L isopropyl alcohol for 10 minutes. The supernatant was removed and the resulting RNA pellet was washed in 1.0 mL of 75% ethanol, resuspended, and centrifuged at 7500 X g for 5 minutes. The pellet was air-dried and dissolved in 10 μ L of sterile double-distilled water (ddH₂O) treated with diethylpyrocarbonate (DEPC).

The resulting RNA solution was quantified for RNA purity and concentration using a spectrophotometer (Ultrospec® 3000 pro UV, Amersham Biosciences, USA). RNA quality was assessed on random samples using denaturing gel electrophoresis. After quantification samples were stored at -80°C for later analysis.

Reverse Transcription (RT):

In 0.2 mL Eppendorf tubes, 20 µL RT reactions were set up on ice for each individual sample using a commercially available kit (Applied Biosystems High

Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, 10 μ L of RNA pre-diluted to 100 ng/ μ L was added to 10 μ L of a master mixture containing 2.0 μ L of 10x RT buffer, 0.8 μ L of 25x dNTP, 2.0 μ L of 10x RT random primers, 1 μ L of MulitScribe reverse transcriptase and 4.2 μ L of nuclease-free H₂O. The cDNA synthesis reaction was carried out using an Eppendorf Mastercycle ep*gradient* thermal cycler (Eppendorf Canada). Following RT, samples were stored at -20 °C until further analysis.

Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR):

25 μL reactions were set up in 0.2 mL Stratagene PCR tubes (Stratagene, USA) for each individual reaction. Reactions were run in duplicate for each time-point. Primers were custom-made using published sequences (Table 1) and were resuspended in 1X TE buffer (10 mM Tris-HCl, 0.11 mM EDTA) and stored at -20[°]C prior to use. In each PCR tube, 1.0 μL of cDNA and 7.5 μL of ddH₂O were added to 16.5 μL of a master mix containing 12.5 μL of RT² Real-Time SYBR Green / Rox PCR master mix (SuperArray Bioscience Corp. USA), 2 μL of the specific forward primer and 2 μL of the specific reverse primer. qRT-PCR reactions were carried out using a Stratagene Mx3000P real-time PCR System (Stratagene, USA). Reactions were set-up using Stratagene MxPro QPCR Software Version 3.00 (Stratagene, USA). Fold changes in gene expression were calculated using the delta-delta Ct method [24]. Briefly, Ct values were first normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). Ct values normalized to GAPDH are expressed as delta-Cts (ΔCt).

 Δ Ct values were then normalized to PRE values, expressed as delta-delta Cts ($\Delta\Delta$ Ct). Values were then transformed out of the logarithmic scale using the formula: fold change = 2^{- $\Delta\Delta$ Ct} [24]. Thus, mRNA values are expressed as a fold change from PRE (mean ± SEM). GAPDH expression was not different from PRE at any of the post-intervention time-points.

Statistical Analysis:

Statistical analysis was performed using SigmaStat 3.0 analysis software (Systat, SPSS Inc., USA). Serum IGF-1 concentrations, MRF mRNA, and IGF-1 splice variant mRNA were analyzed using a 1-way repeated-measures analysis of variance (ANOVA). Correlations were analyzed using the Pearson Product Moment correlation. Statistical significance was accepted at P < 0.05. Differences between the expressions of the different IGF-1 splice variants at a given time point was analyzed with a t-test. Significant interactions and main effects were analyzed using the Tukey's HSD post hoc test. All results are presented as means ± SEM.

<u>Results:</u>

Serum IGF-1:

Serum IGF-1 concentration was not different from rest at any time-point following the exercise intervention (Fig. 1a).

MRF mRNA:

Following muscle lengthening contractions, gene expression of MyoD and Myf5 increased early following exercise, while MRF4 and myogenin increased at later time points following the intervention. MyoD mRNA expression increased approximately 2-fold by T4 (p = 0.001) (Fig. 2a) with a trend to remain elevated at T24 (p = 0.07). There were no significant differences between MyoD mRNA expression from PRE at T72 or T120. Myf5 mRNA expression peaked at T24 (p < 0.05), followed by a gradual return to PRE expression levels by T120 (Fig 2b). MRF4 mRNA expression increased ~2.3-fold at T72 (p = 0.039) and remained ~1.5-fold higher than PRE at T120 (Fig. 3a). Myogenin mRNA expression increased at T4 and T24 (p < 0.05) and peaked at T72 (~2.8-fold, p = 0.009). Myogenin expression remained ~2.5-fold higher than PRE (p = 0.015) at T120 (Fig. 3b).

IGF-1 mRNA:

IGF-1Ea mRNA expression increased ~4-fold at T72 (p = 0.004) and began to decrease by T120 (Fig 4a). Interestingly, the change in IGF-1Eb mRNA expression from baseline was greater than the change in IGF-1Ea expression from baseline at T72 and T120 (p < 0.05), increasing ~5.8-fold at T72 (p < 0.001), and remained elevated (~3.5-fold above PRE, p = 0.035) at T120 (Fig. 4b). MGF mRNA expression increased 48 hours before increases in IGF-1Ea or IGF-1Eb were detected, increasing ~3.3-fold at T24 (p = 0.005), which was also significantly greater than T4 (p = 0.009). MGF expression decreased by T72,

however there was a trend towards MGF remaining elevated above PRE levels at both T72 and T120 (Fig. 4c).

Correlations:

In an attempt to determine the potential temporal relation between the MRF and the IGF-1 isoforms, linear Pearson correlations were performed between variables. The significant correlations are described below.

MGF expression and the MRF: MGF expression showed a positive correlation (r = 0.91, r² = 0.83, p = 0.03) to the expression of Myf5 following muscle damage (Fig. 5a). MGF expression occurred earlier than the other IGF-1 splice variants suggesting that MGF may have a distinct role in muscle repair following contraction-induced damage.

IGF-1Ea and IGF-1Eb expression and the MRF: Muscle IGF-1Ea and Eb expression positively correlated with the expression of MRF4, (r = 0.95, $r^2 = 0.90$, p = 0.015 vs. r = 0.90, $r^2 = 0.81$, p = 0.037; IGF-1Ea and IGF-1Eb respectively; Fig. 5b and 5c).

IGF-1/Pax7 Immunofluorescence:

Baseline muscle sections (T0) and sections from T4 co-stained for IGF-1 and Pax7 had no detectable IGF-1 present in the fiber, myonuclei or the satellite cell (as evaluated by Pax7/DAPI co-staining). Figure 6a shows a representative cross-section at T0 lacking IGF-1 expression. At T24, IGF-1 was detected in the

majority of Pax7 positive cells (fig. 6b) however; IGF-1 was undetectable in the myofiber. By T72 (fig. 6c) and T120 (not shown) IGF-1 was detected in the majority of Pax7 positive cells examined and also diffusely present in the myofiber and more concentrated around the nuclei.

Discussion:

This study examined the temporal relation of all three IGF-1 splice variants and the expression of the MRF, *in vivo*, following an intense muscle lengthening protocol designed specifically to elicit damage in human skeletal muscle. Although IGF-1Ea and MGF mRNA expression have been examined following exercise in humans [5,6,19], no study has yet to examine all of the individual splice variants of IGF-1 in the context of muscle stem cell regulation over such an extended time-course. Furthermore, this is the first study to our knowledge to directly show the expression of IGF-1 in Pax7 positive cells in human muscle *in vivo*.

MRF expression following exercise:

The differential expression of the myogenic regulatory factors following an acute muscle lengthening protocol in the present study was similar to the expression profiles observed in animal and cell culture studies [7,9,25], with both MyoD and Myf5 were upregulated early (~2-fold) following exercise (4-24h)

followed by the induction of MRF4 (72h) and the peak expression of myogenin (72h). Although the timeline of MRF expression in the present study is similar to observations made in animal and cell culture studies [7,9,25], MRF data from human exercise studies are equivocal. In addition, little data exists with regards to Myf5 expression and the post exercise response in humans. Yang et al., (2005) measured Myf5 following heavy resistance exercise, however, they did not observe any changes in Myf5 mRNA expression. Previous studies measuring MRF expression following exercise have reported increases in MyoD immediately following exercise; returning to baseline at 2 h post-exercise, to an increase at 12 h which is sustained after 24 h [5,6]. Furthermore, other studies have reported increased MRF4 and myogenin expression as early as 2-6 h following exercise, returning to baseline levels by 48 h post-exercise [5,6]. In agreement with rodent and in vitro data, our data show MRF4 mRNA expression increased ~72 h after exercise [25]. MRF4 has been shown to act upstream of myogenin, with an increase in MRF4 mRNA leading to an increased myogenin expression [26]. Our data support the temporal association of the expression of MRF4 and myogenin. with myogenin being significantly elevated 72-120 h post-exercise.

Although we reported an increase in myogenin expression 4-24 h following the intervention it is important to note that unlike data from in vitro studies, myogenin may be expressed in post-mitotic fibres, in vivo, following exercise [12]. Our data may differ from previous findings due to the nature of the intervention. This study employed a muscle lengthening model, which has been previously

shown to induce significant muscle damage [21]. Thus the stimulus for inducing satellite cell activation and MRF expression may have been much greater in this study compared to the lower-volume mixed concentric-eccentric protocols performed in other studies [5,6,19]. In addition our intervention employed a dynamometer which allowed us to isolate the *quadriceps* group and limit the contribution from other muscles, as opposed to the free-movement that occurs during regular resistance exercise.

IGF-1 splice variants following exercise:

In the present study the IGF-1 splice variants were differentially expressed following the intervention. MGF mRNA increased significantly 24 h after the intervention, while the expression of both IGF-1Ea and IGF-1Eb mRNA were not elevated until 72 h post intervention. In addition, IGF-1Eb was elevated 120 h following the intervention, while IGF-1Ea was not, suggesting that IGF-1Ea and IGF-1Eb may have specific roles following muscle injury. Immunofluorescent staining of muscle cross-sections with a pan-IGF-1 antibody showed IGF-1 detectable in the satellite cell compartment at 24 h, with IGF-1 becoming detectable in the myofiber (diffuse staining) and satellite cell at 72 and 120 h, the timing of which is in-line with the IGF-1 splice variant mRNA expression. Unfortunately, there is no specific antibody for MGF, thus we are unable to discern the different splice variant protein expression from our immunofluorescent staining. Our IGF-1Ea and MGF data is in agreement with the expression of IGF-1Ea and MGF following exercise in a mouse model, where MGF increased ~24 h

after exercise and IGF-1Ea was increased 5 days after exercise [17,18]. The authors suggested that MGF was related to the activation of satellite cells, while IGF-1Ea was related to the increased need for protein synthesis in late differentiation [17]. Our results agree with this notion since MGF expression was positively correlated with the expression of Myf5, which is robustly expressed in proliferating myoblasts [7]. Furthermore, the time-line of IGF-1Ea expression correlated strongly with the expression of MRF4 which marks the commitment of proliferating myoblasts to terminal differentiation [25,26]. This suggests that the expression of MRF4 and the IGF-1 isoforms Ea and Eb follow similar temporal profiles following muscle damage, the time-line of which is similar to the beginning of terminal differentiation of satellite cells after activation [4]. Although our results agree with animal studies, they differ significantly from other work published on exercise and IGF-1 in human skeletal muscle [6,19,20].

MGF expression has been reported to have a high degree of variability in expression following various contraction protocols in humans [5,6,17,19]. The conflicting results within the literature may be attributed to the differences in the intensity of the exercise stimuli as well as the post-exercise time course examined. In the present study, we chose an intense muscle lengthening protocol that has been previously shown to induce significant muscle damage [21]. Furthermore, we chose an extensive post-contraction time-course aimed at capturing satellite cell activation, expansion and differentiation [7,9,25,26].

IGF-1Eb expression was upregulated 72 h after damaging exercise and remained elevated at 120 h post-exercise. The correlation with MRF4 was robust suggesting a temporal relationship between IGF-1Eb expression and myoblast differentiation. Furthermore, the change in expression of IGF-1Eb was greater compared to IGF-1Ea at T72 (~5.8-fold vs. ~4-fold, p < 0.05) and remained significantly elevated longer (i.e., up to 120 h post exercise). Immunofluorescent analysis shows a visible increase in IGF-1 protein in the myofiber and the satellite cell compartment at T72 and T120 compared to pre-exercise staining. At T0 and T4 there was no overt or diffuse IGF-1 staining within the fiber or the satellite cell. There was discernible increase in diffuse IGF-1 staining in the myofiber at T72 and T120. These findings are of particular interest as they suggest that IGF-1Eb may play an important roll in late differentiation, in which the preferential splicing of IGF-1 is directed from Ea to Eb. The physiological significance of IGF-1Eb is unknown at this time; however, it may be induced due to the increased need for protein and mitochondrial biosynthesis required by terminally differentiating cells. IGF-1 has been shown to regulate differentiation in L6A1 myoblasts through the activation of the phosphotidylinositol 3-kinase (PI3-K) signaling [27]. Although the temporal expression of IGF-1Ea and IGF-1Eb was not correlated to the increase in myogenin expression, the IGF-1 activation of PI3-K/Akt signaling has been shown to stimulate the expression of myogenin in vitro [28,29]. The elevated expression of IGF-1Ea and Eb at 72 h post-intervention coincides with the peak myogenin expression (T72). It is possible that IGF-1Ea and Eb may

influence myogenin expression in the proliferating myoblasts during the postintervention time-course; however, we are not able to discern this from our wholemuscle data. Since myogenin is expressed in post-mitotic muscle following acute exercise [12], it is possible that the unexpected expression profile of myogenin and lack of a significant correlation with IGF-1 was due to the expression of myogenin in the post-mitotic tissue which may have a different expression profile as compared to myogenin the myoblasts. At this time, more work is needed to examine the effect of IGF-1Eb expression of the activation of the PI3-K/Akt pathway and myoblast differentiation in isolated myoblasts.

IGF-1 has also been shown to stimulate the proliferation of satellite cells *in vitro* through the activation of mitogen-activated protein kinase (MAPK) kinase [27]. The results of the present study suggest that the IGF-1Ea and Eb isoforms were not related to proliferation as the expression of both isoforms were not elevated before T72. Furthermore, circulating IGF-1 was not elevated at any time-point following exercise, suggesting that activation/proliferation of satellite cells was not due to an increased blood IGF-1 concentration. IGF-1 has been shown to increase differentiation in primary human myoblasts in culture [30]. In that investigation, the expression of MGF was shown to block myoblast differentiation, thus enhancing proliferation [30]. Furthermore, the ectopic addition of MGF-ct24E (a 24 amino acid peptide corresponding to the c-terminal part of the E-domain of MGF) enhanced proliferation of transplanted human myoblasts *in vitro* [31]. The authors suggest that the enhanced proliferation was

due to a different mechanism than IGF-1 receptor binding. Although it has been shown that MGF does not enhance proliferation through the IGF-1 receptor [31,32], it is possible that MGF may activate the MAP kinase pathway via another mechanism yet to be determined. The expression of MGF following resistance exercise has been shown to increase concomitantly with an increase in cyclin D1 mRNA expression [33]. Cyclin D1 is essential to cell cycle initiation and is upregulated in response to MAP kinase signaling [27]. Further work must be done to understand the mechanism of MGF stimulated myoblast activation/proliferation, specifically identifying the cellular signaling machinery in isolated human satellite cells in order to understand the role of MGF in regulating the myogenic program.

From histological analysis of muscle cross-sections, IGF-1 protein was undetectable in the myofiber and satellite cell compartment at baseline. IGF-1 protein began to be co-expressed with Pax7 at T24 with no IGF-1 detected in the myofiber, suggesting the initial appearance of IGF-1 may be confined to the satellite cell compartment. The timing of the initial IGF-1 expression coincides with the peak in MGF mRNA expression and may be related to the proposed roll of MGF in increasing myoblast proliferation [17]. Interestingly, IGF-1 protein was detectable in both the satellite cell compartment and in the myofiber (diffusely) at T72 and T120 which would agree with the idea that IGF-1 is partially responsible for preparing and directing proliferating satellite cells into terminal differentiation [17,30]. The co-expression of IGF-1 with Pax7 shows that IGF-1 is present in

satellite cells following contraction-induced muscle injury and the correlation of IGF-1 isoforms with MRF expression further confirms the important roll of IGF-1 in regulating the satellite cell response to muscle injury.

In conclusion, these results suggest that the temporal response of MGF expression is related to the activation and proliferative phase of the myogenic program as marked by a significant increase in MyoD and Myf5 mRNA which is strongly correlated with MGF expression and the timing of which is in line with the co-expression of IGF-1 protein with Pax7 in muscle cross sections. Additionally, the expression of IGF-1Ea and IGF-1Eb are temporally related to myogenic differentiation as marked by increased myogenin and MRF4 expression which is strongly correlated with increased IGF-1Ea and Eb expression. The exact mechanisms of MGF and IGF-1Eb signaling are currently unknown. By employing *ex vivo* methods to study the actions of IGF-1 splice variants in isolated human primary myoblasts after damaging muscle contractions, we may be able to provide key insight into the role of these factors and the regenerative response following muscle damage.

Reference List

- Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA (2006) Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol 172: 103-113.
- McKinnell IW, Ishibashi J, Le GF, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ, Rudnicki MA (2008) Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol 10: 77-84.
- Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75: 1351-1359.
- 4. Holterman CE, Rudnicki MA (2005) Molecular regulation of satellite cell function. Semin Cell Dev Biol 16: 575-584.
- Bickel CS, Slade J, Mahoney E, Haddad F, Dudley GA, Adams GR (2005) Time course of molecular responses of human skeletal muscle to acute bouts of resistance exercise. J Appl Physiol 98: 482-488.
- Psilander N, Damsgaard R, Pilegaard H (2003) Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. J Appl Physiol 95: 1038-1044.
- Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17): 2895-2901.
- Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA (1999) Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol 144: 631-643.
- Yablonka-Reuveni Z, Rudnicki MA, Rivera AJ, Primig M, Anderson JE, Natanson P (1999) The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol 210: 440-455.

- Yang Y, Creer A, Jemiolo B, Trappe S (2005) Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. J Appl Physiol 98: 1745-1752.
- Crameri RM, Aagaard P, Qvortrup K, Langberg H, Olesen J, Kjaer M (2007) Myofibre damage in human skeletal muscle: effects of electrical stimulation versus voluntary contraction. J Physiol 583: 365-380.
- Kadi F, Johansson F, Johansson R, Sjostrom M, Henriksson J (2004) Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle. Histochem Cell Biol 121: 329-334.
- Chakravarthy MV, Abraha TW, Schwartz RJ, Fiorotto ML, Booth FW (2000) Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway. J Biol Chem 275: 35942-35952.
- Mourkioti F, Rosenthal N (2005) IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
- Rotwein P, Pollock KM, Didier DK, Krivi GG (1986) Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. J Biol Chem 261: 4828-4832.
- Yang S, Alnaqeeb M, Simpson H, Goldspink G (1996) Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. J Muscle Res Cell Motil 17: 487-495.
- Hill M, Goldspink G (2003) Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J Physiol 549: 409-418.
- 18. Hill M, Wernig A, Goldspink G (2003) Muscle satellite (stem) cell activation during local tissue injury and repair. J Anat 203: 89-99.

- 19. Greig CA, Hameed M, Young A, Goldspink G, Noble B (2006) Skeletal muscle IGF-I isoform expression in healthy women after isometric exercise. Growth Horm IGF Res 16: 373-376.
- 20. Hameed M, Orrell RW, Cobbold M, Goldspink G, Harridge SD (2003) Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. J Physiol 547: 247-254.
- 21. Beaton LJ, Tarnopolsky MA, Phillips SM (2002) Contraction-induced muscle damage in humans following calcium channel blocker administration. J Physiol 544: 849-859.
- Singh MA, Ding W, Manfredi TJ, Solares GS, O'Neill EF, Clements KM, Ryan ND, Kehayias JJ, Fielding RA, Evans WJ (1999) Insulin-like growth factor I in skeletal muscle after weight-lifting exercise in frail elders. Am J Physiol 277: E135-E143.
- Reimann J, Brimah K, Schroder R, Wernig A, Beauchamp JR, Partridge TA (2004) Pax7 distribution in human skeletal muscle biopsies and myogenic tissue cultures. Cell Tissue Res 315: 233-242.
- 24. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
- 25. Smith CK, Janney MJ, Allen RE (1994) Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol 159: 379-385.
- 26. Rhodes SJ, Konieczny SF (1989) Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev 3: 2050-2061.
- 27. Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR (1997) The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272: 6653-6662.
- Xu Q, Wu Z (2000) The insulin-like growth factor-phosphatidylinositol 3kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells. J Biol Chem 275: 36750-36757.

- 29. Wilson EM, Tureckova J, Rotwein P (2004) Permissive roles of phosphatidyl inositol 3-kinase and Akt in skeletal myocyte maturation. Mol Biol Cell 15: 497-505.
- Ates K, Yang SY, Orrell RW, Sinanan AC, Simons P, Solomon A, Beech S, Goldspink G, Lewis MP (2007) The IGF-I splice variant MGF increases progenitor cells in ALS, dystrophic, and normal muscle. FEBS Lett 581: 2727-2732.
- Mills P, Dominique JC, Lafreniere JF, Bouchentouf M, Tremblay JP (2007) A synthetic mechano growth factor E Peptide enhances myogenic precursor cell transplantation success. Am J Transplant 7: 2247-2259.
- Yang SY, Goldspink G (2002) Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett 522: 156-160.
- Kim JS, Cross JM, Bamman MM (2005) Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women. Am J Physiol Endocrinol Metab 288: E1110-E1119.

Table 1:

qRT-PCR Primer Sequences:

Gene Name	Forward Sequence	Reverse Sequence
IGF-1Ea	5'-GACATGCCCAAGACCCAGAAGGA- 3'	5'-CGGTGGCATGTCACTCTTCACTC- 3'
IGF-1Eb	5'- GCCCCCATCTACCAACAAGAACAC-3'	5'- CAGACTTGCTTCTGTCCCCTCCTTC- 3'
MGF	5'- GCCCCCATCTACCAACAAGAACAC-3'	5'-CGGTGGCATGTCACTCTTCACTC- 3'
MyoD	5'-GGTCCCTCGCGCCCAAAAGAT-3'	5'-CAGTTCTCCCGCCTCTCCTAC-3'
Myf5	5'-ATGGACGTGATGGATGGCTG-3'	5'-GCGGCACAAACTCGTCCCCAA-3'
MRF4	5'-CCCCTTCAGCTACAGACCCAA-3'	5'-CCCCTGGAATGATCGGAAAC-3'
Myogenin	5'-CAGTGCACTGGAGTTCAGCG-3'	5'-TTCATCTGGGAAGGCCACAGA-3'
GAPDH	5'-CCTCCTGCACCACCACTGCTT-3'	5'-GAGGGGCCATCCACAGTCTTCT-3'

IGF-1, insulin-like growth factor-1; MGF, mechano growth factor (IGF-1Ec); MyoD, myogenic determination factor; Myf5, myogenic factor-5; MRF4, myogenic regulatory factor-4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Figure Descriptions:

Figure 1. Average serum insulin-like growth factor-1 (IGF-1) concentration. Time 0hr corresponds to pre-exercise serum concentrations; all other time-points correspond to post-exercise time (hr). Values are reported as mean \pm S.E.M. *p < 0.05 vs. 0hr; \pm p < 0.05 vs. 120hr

Figure 2. Relative expression of MyoD mRNA (a) and Myf5 mRNA (b) expression following exercise, expressed as fold-change from 0hr (pre-exercise). Data is normalized to GAPDH and reported as mean \pm S.E.M. *p < 0.05 vs. 0hr

Figure 3. Relative expression of MRF4 mRNA (a) and Myogenin mRNA (b) expression following exercise, expressed as fold-change from 0hr (pre-exercise). Data is normalized to GAPDH and reported as mean \pm S.E.M. *p < 0.05 vs. 0hr

Figure 4. Relative expression of IGF-1Ea mRNA (a) and IGF-1Eb mRNA (b), and mechano growth factor (MGF) mRNA (c) expression following exercise, expressed as fold-change from 0hr (pre-exercise). Data is normalized to GAPDH and reported as mean ± S.E.M.

*p < 0.05 vs. 0hr †p < 0.05 vs. 4hr ‡p < 0.05 vs. IGF-1Ea at T72 & T120

Figure 5. a) Pearson correlation of the time-course of MGF mRNA expression (fold-change) versus the time-course of Myf5 mRNA expression (fold-change) ($r^2 = 0.83$; p = 0.03). b) Pearson correlation of the time-course of IGF-1Ea mRNA expression (fold-change) versus the time-course of MRF4 mRNA expression (fold-change) ($r^2 = 0.90$; p = 0.015). c) Pearson correlation of the time-course of IGF-1Eb mRNA expression (fold-change) versus the time-course of MRF4 mRNA expression (fold-change) ($r^2 = 0.81$; p = 0.037). Correlations are representative of the individual data points and are presented as mean values (\bullet) ± SD (error bars).

Figure 6. Immunofluorescent staining of muscle cross sections triple-stained for nuclei (DAPI), satellite cells (Pax7; TRITC), and IGF-1 protein expression (FITC)

at T0 (6a; 100x objective, arrows denote Pax7+/DAPI+ satellite cells), T24 (6b; 100x objective), T72 (6c; 100x objective). For Fig. 6a-c, arrows denote satellite cells (Pax7+/DAPI+) (6b-c) expressing IGF-1 protein. Note: No IGF-1 present at T0 (and T4, not shown) and diffuse IGF-1 staining in the myofiber at T72 and T120 (not shown).





















Chapter 3: Manuscript 2

Public Library of Science: ONE (PLoS One): 2009 Jun 24;4(6):e6027

Association of Interleukin-6 Signalling with the Muscle Stem Cell Response Following Muscle-Lengthening Contractions in Humans

Bryon R. McKay¹, Michael De Lisio¹, Adam P. W. Johnston¹, Ciara E. O'Reilly¹, Stuart M. Phillips¹, Mark A. Tarnopolsky² and Gianni Parise^{1, 3}

Departments of Kinesiology¹, Pediatrics² and Medical Physics & Applied Radiation Sciences³, McMaster University, Hamilton Ontario, L8S 4L8

Correspondence: Gianni Parise, PhD Departments of Kinesiology, and Medical Physics & Applied Radiation Sciences McMaster University Hamilton, Ontario, Canada L8S 4L8

 Telephone:
 (905) 525-9140 x27353

 Fax:
 (905) 523-6011

 E-mail:
 pariseg@mcmaster.ca

Abstract:

Background:

The regulation of muscle stem cells in humans in response to muscle injury remains largely undefined. Recently, interleukin-6 (IL-6) has been implicated in muscle stem cell (satellite cell)-mediated muscle hypertrophy in animals; however, the role of IL-6 in the satellite cell (SC) response following muscle-lengthening contractions in humans has not been studied.

Methodology/Principal Findings:

Eight subjects (age 22 ± 1 y; 79 ± 8 kg) performed 300 maximal unilateral lengthening contractions (3.14 rad.s⁻¹) of the knee extensors. Blood and muscle samples were collected before and at 4, 24, 72, and 120 hours post intervention. *IL*-6, IL-6 receptor (*IL*-6*Ra*), *cyclin D1*, *suppressor of cytokine signling-3* (*SOCS3*) mRNA were measured using quantitative RT-PCR and serum IL-6 protein was measured using an ELISA kit. JAK2 and STAT3 phosphorylated and total protein was measured using western blotting techniques. Immunohistochemical analysis of muscle cross-sections was performed for the quantification of SCs (Pax7⁺ cells) as well as the expression of phosphorylated STAT3, IL-6, IL-6R α , and PCNA across all time-points. The SC response, as defined by an amplification of Pax7⁺ cells, was rapid, increasing by 24 h and peaking 72 h following the intervention. Muscle *IL*-6 mRNA increased following the intervention, which correlated strongly (R² = 0.89, p < 0.002) with an increase in serum IL-6 concentration. SC IL-6R α protein was expressed on the fiber, but was also

localized to the SC, and IL-6⁺ SC increased rapidly following muscle-lengthening contractions and returned to basal levels by 72 h post-intervention, demonstrating an acute temporal expression of IL-6 with SC. Phosphorylated STAT3 was evident in SCs 4 h after lengthening contraction, and the downstream genes, *cyclin D1* and *SOCS3* were significantly elevated 24 hours after the intervention.

Conclusions/Significance:

The increased expression of STAT3 responsive genes and expression of IL-6 within SCs demonstrate that IL-6/STAT3 signaling occurred in SCs, correlating with an increase in SC proliferation, evidenced by increased Pax7⁺/PCNA⁺ cell number in the early stages of the time-course. Collectively, these data illustrate that IL-6 is an important signaling molecule associated with the SC response to acute muscle-lengthening contractions in humans.

Introduction:

Muscle specific stem cells, named satellite cells (SCs), are necessary for muscle repair and regeneration and are known to reside in skeletal muscle between the basal lamina and the sarcolemma. Successful repair of muscle damage is dependent on SCs to activate, proliferate and terminally differentiate [1,2]. The contribution of new myonuclei to muscle fibers, from SCs, is necessary to promote postnatal muscle growth and to prevent the loss of functional capacity and increased morbidity and mortality associated with muscle loss in advanced age and disease (bedrest, cachexia, etc.) [3–5]. The paired box transcription factor, Pax7, is necessary for the induction of the myogenic response following

muscle injury by promoting the transcription of the myogenic regulatory factors (MRFs) as well as the maintenance of the SC compartment through self-renewal [6–11]. It has been previously shown that the SC pool is responsive to exercise-induced muscle damage [12–14]; however, the regulation of this response remains poorly understood. Many growth factors and cytokines such as, hepatocyte growth factor (HGF), insulin-like growth factor-one (IGF-1), IL-4 and leukemia inhibitory factor (LIF), among many others, have been implicated in playing some role in regulating the SC response [15–20]. However, the contribution of many of these potential SC regulators to hypertrophy and muscle repair, *in vivo*, is not known.

IL-6 is a multifunctional cytokine primarily involved in immune function [21]; however, the chronic systemic elevation of IL-6 in disease states has been associated with promoting a catabolic state leading to muscle atrophy [22–24]. It has been previously demonstrated that exercise can induce increases in muscle derived *IL-6* mRNA and plasma IL-6 concentrations following exercise [25,26]. However, whether changes in IL-6 concentration can influence human muscle SC function has yet to be examined.

Several recent studies provide key evidence that IL-6 signaling may be important in myogenisis. It has been shown that skeletal muscle possesses the IL-6 receptor (IL-6R α) on the sarcolemma and that the IL-6R α is responsive to exercise [27]. It is well established that IL-6 is produced by muscle in response to inflammation and exercise [28–31] (for review see ref. [26]). However, it was

only recently that IL-6 was shown to play a significant role in SC-mediated muscle hypertrophy [17]. IL-6 knock-out (IL-6^{-/-}) mice demonstrated a blunted hypertrophic response and less SC-related myonuclear accretion compared to wild-type mice following compensatory hypertrophy [17]. Furthermore, SCs from IL-6^{-/-} mice demonstrated an impaired proliferative capacity, both *in vivo* and *in vitro*, which was shown to be related to a lack of IL-6 mediated signal transducer and activator of transcription-3 (STAT3) signaling [17]. Although IL-6 signaling has been shown to play an important role in the hypertrophic response to overload in mice, the role of IL-6 in muscle repair following injury in humans has not been elucidated.

Using a model of repeated muscle-lengthening contractions, which we have previously shown to induce significant muscle damage [32–34], we sought to induce a SC response and examine the association of IL-6 with the SC response. We hypothesized that IL-6 would play a key role in the human SC-mediated response to contraction-induced muscle damage. Furthermore, based on the work of Serrrano et al., [17] we hypothesized that SC-mediated regulation by IL-6 would act through STAT3 signaling.

Results:

The IL-6 Response to Muscle-Lengthening Contractions (MLC):

In response to 300 maximal muscle-lengthening contractions, serum IL-6 protein increased by approximately 2-fold (0.75 pg/mL PRE to 2.25 pg/mL at T4, p < 0.05) by T4 with respect to baseline (PRE) levels. However, this response was

transient, returning to PRE levels by T120 (Fig.1a). Interestingly, muscle *IL-6* gene expression demonstrated a parallel response. *IL-6* mRNA increased 4.5-fold from PRE at T4 (p = 0.019) and returned to baseline levels by T120 (Fig.1b). Serum IL-6 was strongly correlated to the increase in muscle *IL-6* mRNA ($r^2 = 0.89$, p = 0.016) (Fig.1c).

In order to test whether *IL-6Ra* gene expression was responsive to our MLC protocol, we measured muscle *IL-6Ra* mRNA. *IL-6Ra* gene expression was significantly increased in whole muscle at T4 (p < 0.001); however, these changes were transient as expression levels returned to PRE values by T24 (Fig. 1d). In addition, it has been recently shown that primary myoblasts isolated from mice possess the IL-6Ra, however it remains unknown whether human satellite cells possess the IL-6Ra [17]. Our observational immunofluorescent analysis revealed that Pax7⁺ cells co-localized with IL-6Ra, verifying that SCs in humans possess the IL-6Ra (Fig. 1e).

Satellite Cell Response:

The response of Pax7⁺ cells to contraction-induced injury was quantified across the post intervention time-course (see supplemental Fig. S1). The number of Pax7⁺ cells (expressed as a percentage of total myonuclei) increased 155% at T24 ($3.32 \pm 3.37\%$ PRE to $8.48 \pm 0.66\%$ T24), peaking at T72 with a 184% increase from PRE ($3.32 \pm 3.37\%$ PRE to $9.45 \pm 0.83\%$ T72; p < 0.005). The number of Pax7⁺ cells began to decrease at T120, but remained elevated as compared to PRE (p = 0.007) (Fig. 2a, b).
To determine the percentage of Pax7⁺ cells that were proliferating, muscle cross-sections were co-stained with an antibody against proliferating cell nuclear antigen (PCNA), a marker for cells in early G1 and S phase of the cell cycle (see Fig. S3). Pax7/PCNA co-staining revealed an increase in Pax7⁺/PCNA⁺ cells as early as T4 (26% of Pax7⁺ cells were PCNA⁺), peaking at T72 (58% of Pax7⁺ cells were PCNA⁺) and remaining elevated at T120 (Fig. 2c, d). In order to test whether IL-6 was present in SCs, we conducted triple immunofluorescent staining for Pax7, IL-6, and DAPI.

Immunofluorescent triple staining of Pax7, IL-6, and DAPI, revealed a substantial increase in the localization of IL-6 protein within SCs (see Fig. S2). Figure 3a and 3b demonstrate the time-course of IL-6 protein expression in Pax7⁺ cells. IL-6⁺ SCs (expressed as a percentage of all Pax7⁺ cells) increased from ~2.16 \pm 1.47 % of satellite cells PRE, to 58.94 \pm 7.2% at T4 (Fig. 3c; p < 0.001) and peaked at 80.7 \pm 5.9% of satellite cells at T24 (p < 0.001). By T72 the number of IL-6⁺ SCs dropped 83% from T24 (Fig. 3d) and returned to baseline levels by T120. The increase in Pax7⁺/ IL-6⁺ cells positively correlated with the increase in Pax7⁺/PCNA⁺ cells observed from PRE to T24 (R² = 0.52, p < 0.0001); however, after T24 there was no correlation as SCs no longer expressed IL-6, whereas PCNA⁺ SC number was still increasing.

JAK2/STAT3 signaling:

It has been shown that IL-6-mediated muscle hypertrophy acts via the Janus kinase/signal transducers and activators of transcription (JAK/STAT3) signaling

pathway [17]. To test if IL-6 was associated with JAK/STAT3 signaling in response to our MLC protocol, we analyzed JAK2 and STAT3 protein. Total and phosphorylated JAK2 and STAT3 were unchanged across all time-points following muscle damage (Fig.4a, b). However, immunofluorescent staining revealed phosphorylated STAT3 (p-STAT3) co-localized with Pax7⁺ cells only at T4 in all subjects (Fig.4c), with no detectable p-STAT3 at PRE, T24, T72 and T120 indicating that STAT3 signaling was transiently active within SCs at T4 (see Fig. S4).

In support of the observed p-STAT3 protein in SCs at T4, we observed increases in both *cyclin D1* and suppressor of cytokine signaling-3 (*SOCS3*) mRNA in whole muscle, which are both downstream genes regulated by IL-6mediated JAK2/STAT3 signaling [35,36]. *Cyclin D1* increased 4-fold at T4, peaked at T24 with a 6-fold increase in expression, and returned to baseline by T72 as compared to PRE (p < 0.05; Fig.5a). *SOCS3* mRNA expression increased 6-fold at T24 (P < 0.05) and showed a trend to remain elevated at T72 (p = 0.07) returning to baseline by T120 (Fig.5b). Furthermore, *SOCS3* mRNA was positively correlated with the number of IL-6⁺ SCs (R² = 0.50, p = 0.02; Fig. 5c).

Discussion:

The release of growth factors and cytokines in response to muscle damage are an essential component of the muscle repair process [12,16,31,37–39]. We have discovered that the pleiotropic effects of IL-6 extend to the human satellite cell

(SC) compartment in response to eccentrically biased contraction-induced muscle damage. Importantly, we have shown that human muscle SCs express IL-6R α in response to acute muscle injury suggesting that IL-6 may play a role in regulating SCs. In addition, we have shown that IL-6 is intimately associated with the SC response by a reporting a significant increase in IL-6 protein content within SCs at T4 and T24, coinciding with an increase in Pax7⁺ cell number at T24 and an increase in the percentage of $Pax7^+$ cells actively proliferating (evidenced by an increased percentage Pax7⁺ cells expressing PCNA). We observed p-STAT3 within the SCs at T4, with the downstream genes cyclin D1 and SOCS3 increasing from T4-T24, suggesting that the IL-6/STAT3 signaling pathway was active within SCs early, during early proliferation, prior to the expansion of the SC population. These findings establish evidence that, in humans, IL-6 is fundamentally important to the contribution of SCs to muscle repair and may, at least in part, contribute to the proliferative component of the myogenic program [17,29,40].

We observed a transient increase in *IL-6* mRNA and IL-6 protein which agrees with previous findings where IL-6 peaked early after intense resistance exercise in humans or compensatory hypertrophy in animals [17,41]. It is well established that IL-6 can be derived from skeletal muscle and released into circulation following acute exercise [25,26,29]. Thus, it is not surprising that our MLC protocol induced an increase in *IL-6* mRNA and circulating IL-6. In the present study, the increase in serum IL-6 was similar to previous studies which

employed knee extensor or short duration exercise [42–45]. However, since the MLC protocol used in the present investigation only required approximately 90 seconds of time under tension, coupled with the relatively small muscle mass required by our protocol (*quadriceps* group only), the contribution of circulating IL-6 from the muscle was not as large as observed in studies involving strenuous whole-body exercise (i.e. 2-fold vs. 128-fold increase) [46]. In the present study a positive correlation between circulating IL-6 and muscle *IL-6* mRNA expression was observed (Fig. 1c), suggesting that muscle-derived IL-6 protein may be released into circulation accounting for the rise in plasma IL-6.

There are several mechanisms responsible for contraction-induced muscle IL-6 production. For example, an increase in intracellular calcium concentration in C2C12 myoblasts has been shown to be a potent activator of *IL-6* expression [29]. Although calcium concentrations were not measured in this study, it is possible that changes in intracellular calcium concentration as a result of repeated muscle contractions may have contributed to the increase in *IL-6* expression [27,47]. In support of this notion it has previously been reported that calcium channel blockers administered prior to the same protocol used in the present investigation resulted in reduced skeletal muscle damage, suggesting a role for calcium-induced cellular damage [32]. Importantly, contraction-induced calcineurin-NFAT, IL-1, AP-1, NF_KB, and nitric oxide signaling have been shown to induce *IL-6* gene expression in muscle [48,49] (for an extensive review see ref. [26]), suggesting a potential role for calcium-induced IL-6 expression. Muscle

produced IL-6 can act in an autocrine fashion, binding to the IL-6Rα on the sarcolemma, initiating the activation of the JAK/STAT3 signaling pathway [17,26,50]. Although it has been established that muscle can produce IL-6, SC-mediated IL-6 production has never been reported following injury in human tissue.

Satellite cells contained virtually no IL-6 protein prior to the MLC protocol (Fig.3c). However, by T24, approximately 80% of all Pax7⁺ SCs were positive for IL-6. Interestingly, there was a positive correlation between the increase in IL-6 and the increase in PCNA co-localization with Pax7 early in the post-intervention time-course. Furthermore, the number of SCs was not significantly increased until T24, which suggests a potential role for IL-6 in newly proliferating SCs. The number of SCs peaked at T72, at which time IL-6⁺ SCs had decreased close to baseline levels (<10% IL-6⁺ cells). Interestingly, we have previously reported that *Myogenin* and *MRF4* mRNA peak at the same time-point that SC number peaks [39]. Since IL-6 was rapidly down-regulated prior to this time it is worth suggesting that IL-6 may be intimately involved in the proliferative process and the absence if IL-6 may be necessary for differentiation to occur. Additionally, the timing of increased IL-6 levels in the SCs in the current study is in agreement with previous *in vitro* findings from animal studies [51] that demonstrate a role for IL-6 in myoblast proliferation. In order to investigate this notion, *in vivo*, we examined JAK2/STAT3 signaling in muscle.

Upon IL-6 binding to the IL-6R α , JAK2 is phosphorylated and initiates the phosphorylation of STAT3. STAT3 then forms a dimer and translocates to the nucleus where it can act as a transcription factor for several target genes [50]. JAK2/STAT3 signaling has been shown to induce the transcription of genes that regulate cell cycle progression and proliferation such as cyclin D1 and c-myc [52-54]. In this study, there was no increase in whole muscle p-JAK2 or p-STAT3 protein at the time-points in which the muscle biopsies were obtained; however, the phosphorylation of STAT3 is very rapid and transient and it is possible that any increase in p-STAT3 detectable at the whole-muscle level occurred before T4 [41,55]. Trenerry et al. (2007), observed an increase in p-STAT3 protein in whole muscle 2 h after intense resistance exercise that returned to baseline by 4h. Alternatively, it is possible that changes in protein phosphorylation events within the SCs were too subtle to detect at the whole muscle level using standard western blotting techniques. Indeed, using immunofluorescent staining we showed p-STAT3 protein in the nuclei of SCs at T4 but no other time-point. The co-localization of p-STAT3 with Pax7 may be due to the phosphorylation of STAT3 protein within the SCs induced by the increase in IL-6 protein observed in the SCs at T4. IL-6 may act in an autocrine manner, binding to the IL-6R α on the SC membrane, inducing the phosphorylation of STAT3 via GP130/JAK2 activation within the SC [17,50,55]. The timing of the appearance of p-STAT3 in the SCs is in agreement with observations made in rats demonstrating that SCs expressed phosphorylated STAT3 3 h following injury [55]. The authors of that

study concluded that STAT3 signaling in SCs following muscle injury was important for successful muscle regeneration [55]. It is not surprising that p-STAT3 was found in the nuclei of SCs following muscle injury, as SCs represent the only resident mitotic cells within skeletal muscle and thus STAT3 signaling within these cells is inherently important for promoting the activation of genes such as *cyclin D1* which may help direct cell cycle progression and proliferation of the SC pool.

Our data demonstrate the timing of *cyclin D1* mRNA expression not only mirrors the protein expression of IL-6 in the satellite cells, but also coincides with the increasing proliferation of Pax7⁺ cells. Interestingly, IL-6^{-/-} mice demonstrated a significantly blunted number of p-STAT3⁺ SCs accompanied by a significantly lower level of *cyclin D1* expression after 3 d of compensatory hypertrophy as compared to wild-type animals [17]. In that study, the IL-6^{-/-} animals had less muscle hypertrophy and less SC-mediated myonuclear addition following compensatory overload, as a result of impaired SC proliferation [17]. These findings illustrate the importance of IL-6 mediated SC proliferation to the process of skeletal muscle growth following overload or injury.

In addition, the rapid down-regulation of *cyclin D1* at T72 illustrates the tight regulation of SC proliferation, and that rapid down-regulation of cell-cycle genes may be necessary for the induction of differentiation. In addition to inducing entry into the cell cycle, cyclin D1 may also act in a negative feedback manner to repress STAT3 activity by reducing nuclear p-STAT3 [56]. The reduction of

nuclear p-STAT3 may be necessary for the induction of differentiation [57] and the timing of withdrawal of SC p-STAT3 observed in the present study (i.e. no p-STAT3 evident at T24 or later) supports this idea. Thus, the withdrawal of IL-6 mediated STAT3 signaling may be necessary for differentiation to occur. This notion is strengthened by our observations that IL-6 protein is evident in 80% of satellite cells at T24 and less than 10% of satellite cells at T72 corresponding to increases in *MRF4* and *Myogenin* (myogenic regulatory factors responsible for the initiation of differentiation) observed at T72 previously [39]. This provides further evidence that IL-6 signaling is withdrawn as differentiation begins.

STAT3 phosphorylation is regulated by SOCS3 as part of a negative feedback loop controlling the activation status of STAT3 proteins [58,59]. SOCS3 can bind phosphotyrosines on the JAK2 receptors, physically blocking STAT3 binding, as well as biding to JAK2, directly blocking JAK kinase activity [50]. In addition, SOCS3 can also recruit ubiquitin-transferases, facilitating the ubiquitination of JAK2, targeting the JAK protein for proteosomal degradation [26,50]. The expression of SOCS3 positively correlated with the increase in IL-6⁺ SC, such that the subjects with the highest number of IL-6⁺ satellite cells had the highest expression of SOCS3 mRNA (Fig 5c). This correlation provides further support that IL-6 is signaling through the STAT3 pathway. Moreover, the timing of peak SOCS3 expression coincides with the peak in satellite cell number and the down-regulation of *cyclin D1* expression. Trenerry et al. (2007), reported similar findings indicating that SOCS3 mRNA expression peaked 2 h after intense

resistance exercise, coinciding with the peak in *c-myc* mRNA and p-STAT3 protein, after which p-STAT3 signaling was no longer evident. The expression of *SOCS3* and the absence of other components of the IL-6/STAT3 signaling network beyond T24 illustrate the role of this network in promoting SC proliferation. In addition, the transient nature of IL-6 expression may be of critical importance in distinguishing IL-6 as a promoter of SC-mediated muscle repair.

Classically, elevated IL-6 has been implicated in muscle wasting diseases such as cachexia [23]. The pleiotropic roles of IL-6 appear to be dependent on the stimuli, nature of the immune and local tissue responses, and the temporal expression of IL-6 in inducing different phenotypic traits [17,21,23]. This study examined IL-6 signaling in the context of muscle stem cell proliferation induced by MLC. Our findings compliment recent data that implicate IL-6 as an essential regulator of SC-mediated hypertrophy in mice and provide insight into the control of the SC response to muscle injury and repair in humans. In summary, these findings suggest that IL-6 may play a key role in SC proliferation in humans by inducing genes such as *cyclin D1* through the activation of JAK2/STAT3 signaling and that IL-6 is a factor mediating the repair response to acute muscle damage.

Materials and Methods:

Subjects:

Eight healthy males (age 22 ± 1 y, height 185 ± 2 cm, weight 79 ± 8 kg) were recruited from the McMaster University community. Subjects underwent a routine medical screening, completed a health questionnaire, and were required to not

have been involved in a lower-body resistance exercise training program for at least 6 months prior to participating in the study. Subjects were told to refrain from exercising throughout the time-course of the study. All subjects were informed of the procedures and potential risks associated with the study and gave their written informed consent to participate. This study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to all declarations on the use of human subjects as research participants.

Muscle Damage Protocol:

We employed a protocol involving maximal isokinetic unilateral muscle lengthening contractions of the *quadriceps femoris* performed on a Biodex dynamometer (Biodex-System 3, Biodex Medical Systems, Inc., USA) at 3.14 rad.s⁻¹. Briefly, for each subject, one leg was selected randomly to complete the protocol described below. Movement at the shoulders, hips, and thigh were restrained with straps in order to isolate the knee extensors during the protocol.

Immediately prior to the intervention, subjects underwent a brief familiarization trial involving 5 to 10 submaximal lengthening contractions of the leg under investigation. Subjects were required to perform 30 sets of 10 maximal muscle lengthening contractions with one minute rest between sets, for a total of 300 lengthening contractions. During each set, investigators provided verbal encouragement in an attempt to elicit a maximal effort. It has been well documented that our protocol induces a significant level of skeletal muscle damage and substantial cellular disruption evidenced by extensive z-band

streaming, desmin disruption, a significant increase in plasma creatine kinase (3.9-fold increase 48 h after exercise), a significant infiltration of macrophages and neutrophils (4-fold and 14- fold increase 48 h after exercise respectively) in addition to a significant myogenic regulatory factor and satellite cell response (based on NCAM staining) [12,32–34,39].

Muscle Biopsies:

Five percutaneous needle biopsies were obtained from the mid-portion of the vastus lateralis under local anesthetic (1% lidocaine) using manual suction [60,61]. One muscle biopsy was obtained from the non-working leg prior to the intervention. Baseline measures were generated from the pre-intervention biopsy (PRE) taken prior to beginning the damage protocol. Four biopsies were obtained from the working leg at different time points following the intervention. Muscle biopsies and blood draws were taken concurrently, at 4 h (T4), 24 h (T24), 72 h (T72), and 120 h (T120) post-intervention each from a new incision 3-5 cm proximal to the last biopsy site. Approximately 150 mg of muscle tissue was collected from each biopsy. Following collection of the sample, the muscle was dissected free of adipose and connective tissue, divided into separate pieces for RNA and protein analysis, flash-frozen in liquid nitrogen, and stored at -80 °C for later analysis. Approximately 25 mg of each sample was mounted in Optimum Cutting Temperature (OCT) compound and frozen in isopentane cooled in liquid nitrogen. For each subject, 4, 24, 72 and 120 h biopsies were conducted

at approximately the same time of day (±1 hour) in order to minimize variability between subjects.

Immunofluorescence:

7 µm sections were cryosectioned and stained with antibodies against Pax7 (neat; DSHB, USA); IL-6 (500 ng/mL, MAB 2061, R&D Systems, USA); p-STAT3 (p-STAT3 Y705 1:100, Cell Signaling Technologies Inc., USA); IL-6Rα (1:50, MCA822, Serotec, UK); PCNA (1:200, ab15497, Abcam Inc., USA); and Laminin (1:1000, L8271, Sigma-Aldrich, Canada). Secondary antibodies used were: Pax7 (AlexaFluor 488 or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA); IL-6, IL-6Rα (immunoglobulin biotinylated secondary antibody, 1:200, Dako Canada, Inc.; followed by a streptavidin-FITC fluorochrome, 1:100. Biosource. USA); p-STAT3 (R-Phycoerythrin; 1:200; Pierce, USA); PCNA (Texas Red, 1:500, Invitrogen, Molecular Probes Inc., USA) and Laminin (AlexaFluor 488, 1:500, Invitrogen, Molecular Probes Inc., USA). For co-immunofluorescent staining for Pax7 and IL-6, Pax7 and IL-6R α , Pax7 and Laminin, and Pax7 and p-STAT3 sections were fixed with 2% paraformaldehyde (PFA, Sigma, USA) for 10 min followed by several washes in PBS. Sections were then covered for 30 min in a blocking solution containing, 2% BSA, 5% FBS, 0.2% Triton-X 100, 0.1% sodium azide; whereas for Pax7 and PCNA co-staining, fixing was done with icecold acetone, and blocked with 10% goat serum (GS) in 0.01% Triton-X 100 (Sigma, USA). Following blocking, sections were incubated in the primary antibody at 4°C overnight. After several washes, sections were then incubated in

the appropriate secondary antibodies. Sections were then re-fixed in 2% PFA (Sigma, USA) to prevent migration of the secondary antibodies and re-blocked in 10% GS in 0.01% Triton-X 100 (Sigma, USA). The sections were then incubated in the second primary antibody, followed by incubation in the appropriate secondary antibody. Sections were then washed with PBS and 4',6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for nuclear staining. Staining was verified using the appropriate positive and negative controls to ensure specificity of staining (see Fig. S5). Stained slides were viewed with the Nikon Eclipse 90*i* Microscope (Nikon Instruments, Inc., USA) and images were captured and analyzed using the Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., USA).

Immunofluorescent Analysis:

All immunostaining quantification and enumerations were conducted on all subjects at all time points (n = 8, 5 time-points, 40 biopsies total). For each time-point, images were taken at 40x to ensure a high resolution image to accurately determine nuclei which were associated with myofibers and displayed co-localization of immunolabeling. For each time-point measurement at least 15 different images per subject, per time-point were taken corresponding to at least 1000 myonuclei.

Satellite cell enumeration: Satellite cell enumeration was conducted with an anti-Pax7 antibody staining satellite cells and DAPI staining all nuclei in the muscle cross sections. Only nuclei which stained positive for Pax7 and DAPI were

counted as satellite cells. In order to minimize enumeration error only nuclei associated with myofibers were counted and interstitial nuclei (based on their location relative to the basal lamina) were excluded from enumeration. Data are presented with Pax7 positive (Pax7⁺) cells as a percentage of total myonuclei.

IL-6 positive satellite cells enumeration: Enumeration was conducted using tripleimmunolabeling with antibodies against IL-6 and Pax7 and using DAPI to stain nuclei. Only nuclei associated with myofibers were used to enumerate the percentage of Pax7⁺ cells which stained positive for IL-6 (Pax7⁺/IL-6⁺). Data are presented with IL-6+ satellite cells (Pax7⁺/IL-6⁺) as a percentage of total Pax7⁺ cells as well as a percentage of total myonuclei.

Proliferating cell nuclear antigen (PCNA) positive satellite cells: Enumeration was conducted using triple-immunolabeling with antibodies against PCNA and Pax7 and using DAPI to stain nuclei. Only nuclei associated with myofibers were used to enumerate the percentage of Pax7⁺ cells which stained positive for PCNA (Pax7⁺/PCNA⁺). Data are presented with PCNA⁺ satellite cells (Pax7⁺/PCNA⁺) as a percentage of total Pax7⁺ cells.

Blood Measures:

A resting blood sample was obtained from the antecubital vein immediately prior to the intervention. Blood was also drawn at T4, T24, T72 and T120. Approximately 5 mL of blood was collected and separated into one heparinized and one non-heparinized vacutainer tube at each time-point. Samples were

separated into 50 µL aliquots and stored at -80 °C for analysis at a later date. Serum samples were thawed on ice and analyzed for IL-6 protein using a commercially available Enzyme-Linked ImmunoSorbant Assay (ELISA) kit according to manufactures instructions (R&D Systems, Inc., USA). Samples were run in duplicate with an average intra-assay CV of less than 2% with all subjects run on the same plate.

RNA isolation:

RNA was isolated from homogenized muscle samples using the TRIzol/RNeasy method. Briefly, approximately 25 mg of each muscle sample was homogenized in a total of 1.0 mL of TRIzol Reagent (Invitrogen Corporation, Canada) using a rotary homogenizer. Homogenized samples were incubated at room temperature for 5 min followed by the addition of 0.2 mL of chloroform then shaken vigorously for 15 s. After another 5 min incubation at room temperature, samples were centrifuged at 12 000 g at 4°C for 10 min. The aqueous phase was then transferred to a new tube and the volume was measured. 1 volume of 70% ethanol was added to the aqueous phase and mixed. Multiple 700 µL aliguots were then transferred into a Qiagen RNeasy mini spin column and RNA was purified by using the RNeasy mini kit (Cat. # 74106), following the manufacturer's instructions (Qiagen Sciences, USA). The RNA was guantified and purity was assessed using a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Random samples were also tested for RNA quality using denaturing gel electrophoresis.

Reverse Transcription (RT):

Individual samples were reverse transcribed in 20 µL reactions using a commercially available kit (Applied Biosystems High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA) according to the manufacturer's instructions. The cDNA synthesis reaction was carried out using an Eppendorf Mastercycle ep*gradient* thermal cycler (Eppendorf, Canada).

Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR):

Individual 25 µL reactions were prepared in 0.2 mL Stratagene PCR tubes (Stratagene, USA) and run in duplicate for each time-point. Primers were custom-made using published sequences (Table 1) and were re-suspended in 1X TE buffer (10 mM Tris-HCl, 0.11 mM EDTA) and stored at -20 C prior to use. In each reaction tube, 1.0 μ L of cDNA and 7.5 μ L of ddH₂O were added to 16.5 μ L of a master mix containing 12.5 µL of RT² Real-Time SYBR Green / Rox PCR master mix (SuperArray Bioscience Corp., USA) along with 2 µL of the specific forward and reverse primers. gRT-PCR reactions were carried out using a Stratagene Mx3000P real-time PCR System (Stratagene, USA) using Stratagene MxPro QPCR Software Version 3.00 (Stratagene, USA). Fold changes in gene expression were calculated using the delta-delta Ct method [62] normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). Thus, mRNA values were expressed as a fold change from PRE (mean ± SEM). GAPDH expression was not different from PRE at any of the post-intervention time-points.

Western Blot Analysis:

Muscle homogenates (7% wt/v) were prepared in ice-cold homogenization buffer (20 mM Tris-HCl, 1 mM Na₃VO₄, 50 mM NaF, 40 mM β-glycerolphosphate, 20 mM NaPyrphosphate, 0.5 % Triton-X-100, 2 complete mini Roche protease inhibitor tabs, pH 7.2). Equal amounts (50 µg) of protein, as determined by Bradford Assay (Thermo Fisher Scientific, USA), were loaded into lanes of a 7.5% gel, separated at 100 V for 90 min then transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore, Canada) for 60 min at 70 V. After blocking for 1 h at 4°C in 5% bovine serum albumin (BSA, Santa Cruz Biotechnology, USA) blots were probed with phospho-specific primary antibody overnight at 4°C then relevant secondary antibody. After phosphorylated protein detection by ECL (SuperSignal West Dura; Thermo Fisher Scientific, USA) and Alpha Innotech FluorChem SP (Alpha Innotech Corporation, USA) membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, USA) for 20 min at room temperature and detection of total-specific antibodies was conducted. Bands were quantified using AlphaEase FC Software, Version 5.0.2 (Alpha Innotech Corporation, USA). Levels of phosphorylated protein were expressed relative to total.

The following primary antibodies were used; phospho-Stat3 (Tyr705) 1:1000, phospho-Jak2 (Tyr1007/1008) 1:500, Stat3 1:1000, and Jak2 (D2E12)1:500. All primary antibodies were raised in rabbit and purchased from

Cell Signaling Technology, USA. A goat polyclonal to Rabbit IgG (HRP) secondary antibody (1:50,000; Abcam Inc., USA) was used.

Statistical Analysis:

Statistical analysis was performed using SigmaStat 3.1.0 analysis software

(Systat, SPSS Inc., USA). Serum IL-6 concentrations, mRNA, protein, Pax7⁺/ IL-

6⁺, and Pax7⁺/ PCNA⁺ enumeration were analyzed using a 1-way repeated-

measures analysis of variance (ANOVA). Correlations were analyzed using the

Pearson Product Moment correlation. Statistical significance was accepted at P

< 0.05. Significant interactions and main effects were analyzed using the

Tukey's HSD post hoc test. All results are presented as means ± SEM.

Reference List

- 1. Charge SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84: 209-238.
- 2. Le GF, Rudnicki MA (2007) Skeletal muscle satellite cells and adult myogenesis. Curr Opin Cell Biol 19: 628-633.
- 3. Rando TA (2006) Stem cells, ageing and the quest for immortality. Nature 441: 1080-1086.
- 4. Gopinath SD, Rando TA (2008) Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell 7: 590-598.
- Zammit PS, Partridge TA, Yablonka-Reuveni Z (2006) The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem 54: 1177-1191.
- Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA (2006) Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol 172: 103-113.

- Olguin HC, Yang Z, Tapscott SJ, Olwin BB (2007) Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. J Cell Biol 177: 769-779.
- Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, et al. (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102: 777-786.
- McKinnell IW, Ishibashi J, Le Grand F, Punch VG, Addicks GC, et al. (2008) Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol 10: 77-84.
- Olguin HC, Olwin BB (2004) Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 275: 375-388.
- 11. Oustanina S, Hause G, Braun T (2004) Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J 23: 3430-3439.
- O'Reilly C, McKay B, Phillips S, Tarnopolsky M, Parise G (2008) Hepatocyte growth factor (HGF) and the satellite cell response following muscle lengthening contractions in humans. Muscle Nerve 38: 1434-1442.
- Crameri RM, Langberg H, Magnusson P, Jensen CH, Schroder HD, et al. (2004) Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. J Physiol 558: 333-340.
- Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA (2006) Satellite cell numbers in young and older men 24 hours after eccentric exercise. Muscle Nerve 33: 242-253.
- Chakravarthy MV, Abraha TW, Schwartz RJ, Fiorotto ML, Booth FW (2000) Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway. J Biol Chem 275: 35942-35952.
- Mourkioti F, Rosenthal N (2005) IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
- Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P (2008) Interleukin-6 is an essential regulator of satellite cellmediated skeletal muscle hypertrophy. Cell Metab 7: 33-44.

- Spangenburg EE, Booth FW (2002) Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation. Am J Physiol Cell Physiol 283: C204-C211.
- Allen RE, Sheehan SM, Taylor RG, Kendall TL, Rice GM (1995) Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J Cell Physiol 165: 307-312.
- Horsley V, Jansen KM, Mills ST, Pavlath GK (2003) IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. Cell 113: 483-494.
- 21. Kishimoto T (2005) Interleukin-6: from basic science to medicine--40 years in immunology. Annu Rev Immunol 23: 1-21.
- 22. Ershler WB, Keller ET (2000) Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med 51: 245-270.
- 23. Baltgalvis KA, Berger FG, Pena MM, Davis JM, Muga SJ, et al. (2008) Interleukin-6 and cachexia in ApcMin/+ mice. Am J Physiol Regul Integr Comp Physiol 294: R393-R401.
- 24. Haddad F, Zaldivar F, Cooper DM, Adams GR (2005) IL-6-induced skeletal muscle atrophy. J Appl Physiol 98: 911-917.
- 25. Steensberg A, van HG, Osada T, Sacchetti M, Saltin B, et al. (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. J Physiol 529 Pt 1: 237-242.
- 26. Pedersen BK, Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev 88: 1379-1406.
- 27. Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, et al. (2005) Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6. FASEB J 19: 1181-1183.
- Gallucci S, Provenzano C, Mazzarelli P, Scuderi F, Bartoccioni E (1998) Myoblasts produce IL-6 in response to inflammatory stimuli. Int Immunol 10: 267-273.
- Weigert C, Dufer M, Simon P, Debre E, Runge H, et al, (2007) Upregulation of IL-6 mRNA by IL-6 in skeletal muscle cells: role of IL-6 mRNA stabilization and Ca2+-dependent mechanisms. Am J Physiol Cell Physiol 293: C1139-C1147.
- 30. Keller C, Steensberg A, Pilegaard H, Osada T, Saltin B, et al. (2001) Transcriptional activation of the IL-6 gene in human contracting

skeletal muscle: influence of muscle glycogen content. FASEB J 15: 2748-2750.

- Tomiya A, Aizawa T, Nagatomi R, Sensui H, Kokubun S (2004) Myofibers express IL-6 after eccentric exercise. Am J Sports Med 32: 503-508.
- Beaton LJ, Tarnopolsky MA, Phillips SM (2002) Contraction-induced muscle damage in humans following calcium channel blocker administration. J Physiol 544: 849-859.
- Beaton LJ, Allan DA, Tarnopolsky MA, Tiidus PM, Phillips SM (2002) Contraction-induced muscle damage is unaffected by vitamin E supplementation. Med Sci Sports Exerc 34: 798-805.
- Mahoney DJ, Safdar A, Parise G, Melov S, Fu M, et al. (2008) Gene expression profiling in human skeletal muscle during recovery from eccentric exercise. Am J Physiol Regul Integr Comp Physiol 294: R1901-R1910.
- 35. Leslie K, Lang C, Devgan G, Azare J, Berishaj M, et al. (2006) Cyclin D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3. Cancer Res 66: 2544-2552.
- 36. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, et al. (1999) Stat3 as an oncogene. Cell 98: 295-303.
- Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, et al. (2001) Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. Am J Physiol Endocrinol Metab 280: E383-E390.
- Yang SY, Goldspink G (2002) Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett 522: 156-160.
- McKay BR, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G (2008) Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans. J Physiol 586: 5549-5560.
- Cantini M, Massimino ML, Rapizzi E, Rossini K, Catani C, et al. (1995) Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes. Biochem Biophys Res Commun 216: 49-53.

- 41. Trenerry MK, Carey KA, Ward AC, Cameron-Smith D (2007) STAT3 signaling is activated in human skeletal muscle following acute resistance exercise. J Appl Physiol 102: 1483-1489.
- Helge JW, Stallknecht B, Pedersen BK, Galbo H, Kiens B, et al. (2003) The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. J Physiol 546: 299-305.
- Ullum H, Haahr PM, Diamant M, Palmo J, Halkjaer-Kristensen J, et al. (1994) Bicycle exercise enhances plasma IL-6 but does not change IL-1 alpha, IL-1 beta, IL-6, or TNF-alpha pre-mRNA in BMNC. J Appl Physiol 77: 93-97.
- 44. Hiscock N, Fischer CP, Sacchetti M, van HG, Febbraio MA, et al. (2005) Recombinant human interleukin-6 infusion during low-intensity exercise does not enhance whole body lipolysis or fat oxidation in humans. Am J Physiol Endocrinol Metab 289: E2-E7.
- Bruunsgaard H, Galbo H, Halkjaer-Kristensen J, Johansen TL, MacLean DA, et al. (1997) Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. J Physiol 499 (Pt 3): 833-841.
- Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK (1999) Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol 515 (Pt 1): 287-291.
- Keller C, Steensberg A, Hansen AK, Fischer CP, Plomgaard P, et al. (2005) Effect of exercise, training, and glycogen availability on IL-6 receptor expression in human skeletal muscle. J Appl Physiol 99: 2075-2079.
- Cahill CM, Rogers JT (2008) Interleukin (IL) 1beta induction of IL-6 is mediated by a novel phosphatidylinositol 3-kinase-dependent AKT/IkappaB kinase alpha pathway targeting activator protein-1. J Biol Chem 283: 25900-25912.
- 49. Yu Y, Ge N, Xie M, Sun W, Burlingame S, et al. (2008) Phosphorylation of Thr-178 and Thr-184 in the TAK1 T-loop is required for interleukin (IL)-1-mediated optimal NFkappaB and AP-1 activation as well as IL-6 gene expression. J Biol Chem 283: 24497-24505.
- 50. Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signaling pathway. J Cell Sci 117: 1281-1283.
- 51. Wang X, Wu H, Zhang Z, Liu S, Yang J, et al. (2008) Effects of interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor on the

proliferation and differentiation of adult human myoblasts. Cell Mol Neurobiol 28: 113-124.

- 52. Masuda M, Suzui M, Yasumatu R, Nakashima T, Kuratomi Y, et al. (2002) Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. Cancer Res 62: 3351-3355.
- 53. Wei Q, Paterson BM (2001) Regulation of MyoD function in the dividing myoblast. FEBS Lett 490: 171-178.
- 54. Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, et al. (1999) STAT3 is required for the gp130-mediated full activation of the cmyc gene. J Exp Med 189: 63-73.
- 55. Kami K, Senba E (2002) In vivo activation of STAT3 signaling in satellite cells and myofibers in regenerating rat skeletal muscles. J Histochem Cytochem 50: 1579-1589.
- 56. Bienvenu F, Gascan H, Coqueret O (2001) Cyclin D1 represses STAT3 activation through a Cdk4-independent mechanism. J Biol Chem 276: 16840-16847.
- 57. Snyder M, Huang XY, Zhang JJ (2008) Identification of novel direct Stat3 target genes for control of growth and differentiation. J Biol Chem 283: 3791-3798.
- Naka T, Fujimoto M, Kishimoto T (1999) Negative regulation of cytokine signaling: STAT-induced STAT inhibitor. Trends Biochem Sci 24: 394-398.
- 59. Chen XP, Losman JA, Rothman P (2000) SOCS proteins, regulators of intracellular signaling. Immunity 13: 287-290.
- 60. Hennessey JV, Chromiak JA, Della VS, Guertin J, MacLean DB (1997) Increase in percutaneous muscle biopsy yield with a suctionenhancement technique. J Appl Physiol 82: 1739-1742.
- 61. Bergstrom J (1975) Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. Scand J Clin Lab Invest 35: 609-616.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Figure Descriptions:

Figure 1. Circulating IL-6 and muscle IL-6 mRNA and IL-6 receptor response muscle lengthening contractions (MLC). (1a) Average serum interleukin-6 (IL-6) concentration. Time 0hr corresponds to pre-intervention values; all other timepoints correspond to post-intervention time (hr). (1b) Relative IL-6 mRNA expression, expressed as fold-change from 0hr. (1c) Pearson correlation of serum IL-6 concentration versus muscle IL-6 mRNA (fold-change), correlation is representative of the individual data points and presented as mean values $(\bullet) \pm$ SD (error bars). (1d) Relative IL-6 receptor (*IL-6Ra*) mRNA expression, expressed as fold-change from 0hr. (1e) Immunofluorescent image (40x) of a muscle cross-section triple-stained for Pax7 (red), IL-6Rα (green) and nuclei (DAPI = blue): IL-6R α staining is apparent on the sarcolemma and satellite cell membrane. White arrow denotes one of the $Pax7^{+}$ nuclei which co-localized with IL-6R α (scale bar = 100µm). Values are reported as mean ± S.E.M. Mean values represent the mean for all 8 subjects per time-point (8 samples per timepoint, 40 samples total) *p < 0.05 vs. 0hr

Figure 2. **Muscle satellite cell response to muscle lengthening contractions** (**MLC**). (2a) Triple-immunofluorescent staining of 7µm muscle cross-section for satellite cells (red = Pax7⁺), laminin (green) and nuclei (DAPI = blue). Thin arrow denotes the Pax7⁺ cell located beneath the basal lamina in the satellite cell niche (scale bar = 20µm). (2b) Pax7⁺ nuclei expressed as a percentage to total myonuclei following MLC. (2c) PCNA⁺ cells expressed as a percentage of all Pax7⁺ satellite cells following MLC. (2d) Triple-immunofluorescent staining of 7µm muscle cross-section for satellite cells (green = Pax7⁺), PCNA (red) and nuclei (DAPI = blue). Thin arrow denotes the PCNA⁺/Pax7⁺ cell, thick arrow denotes a Pax7⁺ nuclei with no PCNA staining (scale bar = 20µm). Values are reported as mean ± S.E.M. Mean values represent the mean for all 8 subjects per time-point (8 samples per time-point, 40 samples total) *p < 0.05 vs. 0hr; †p < 0.05 vs. 4hr; ‡p < 0.05 vs. 120hr

Figure 3. Satellite cell IL-6 protein expression following muscle lengthening contractions (MLC). (3a) II-6⁺ satellite cells expressed as a percentage of all Pax7⁺ satellite cells following MLC. (3b) II-6⁺ satellite cells expressed as a

percentage of total myonuclei following MLC. Values are reported as mean \pm S.E.M. Mean values represent the mean for all 8 subjects per time-point (8 samples per time-point, 40 samples total)

*p < 0.05 vs. 0hr; †p < 0.05 vs. 4hr

(3c)IL-6/Pax7 co-localization following muscle lengthening contractions (MLC). Triple-immunofluorescent staining of 7µm muscle cross-sections for satellite cells (red = Pax7⁺), IL-6 protein (green) and nuclei (DAPI = blue). The thin arrow denotes a Pax7⁺/IL-6⁺ cell and the thick arrows denote Pax7⁺ cells with no IL-6 staining (scale bar = 10µm). (3d) Representative image of 72hr (T72) where white arrows denotes a Pax7⁺ cells with no IL-6 staining (scale bar = 20μ m).

Figure 4. JAK2/STAT3 activity following muscle lengthening contractions (MLC). Representative western blot of phospho-Jak2 and Jak2 (4a) and phospho-Stat3 and Stat3 (4b). Graphs depict the ratio of phosphorylated to total protein. Data are presented as mean \pm SEM. Mean values represent the mean for all 8 subjects per time-point (8 samples per time-point, 40 samples total). (4c) Triple-immunofluorescent staining of 7µm muscle cross-sections for satellite cells (green = Pax7⁺), phosphorylated STAT3 protein (red) and nuclei (DAPI = blue). White arrows denotes Pax7⁺/p-STAT3⁺ cell at 4hr (T4), scale bar = 10 µm. Note: No p-STAT3 staining found at T0, T24, T72 or T120 (not shown)

Figure 5. The response of downstream genes of STAT3 signaling following muscle lengthening contractions (MLC). (5a) Relative *cyclin D1* mRNA expression (fold-change from 0hr). (5b) Relative *SOCS3* mRNA expression (fold-change from 0hr). Values are reported as mean \pm S.E.M. (5c) Pearson correlation of IL-6⁺ satellite cells (number of cells) versus muscle *SOCS3* mRNA (fold-change), correlation is representative of the individual data points and presented as mean values (\bullet) \pm SD (error bars). Mean values represent the mean for all 8 subjects per time-point (8 samples per time-point, 40 samples total)

*p < 0.05 vs. 0hr; †p < 0.05 vs. 4hr

Table 1:

qRT-PCR Primer Sequences:

Gene Name	Forward Sequence	Reverse Sequence	Entrez Gene Accession Number
IL-6	5'-GAAAGCAGCAAAGAGGCACT-3'	5'-AGCTCTGGCTTGTTCCTCAC-3'	3569
IL-6Rα	5'-GACAATGCCACTGTTCACTG-3'	5'GCTAACTGGCAGGAGAACTT-3'	3570
CyclinD1	5'-CACGATTTCATTGAACACTTCC- 3'	5'-TGAACTTCACATCTGTGGCAC-3'	595
SOCS3	5'-GACCAGCGCCACTTCTTCA-3'	5'-CTGGATGCGCAGGTTCTTG-3'	9021
GAPDH	5'-CCTCCTGCACCACCAACTGCTT- 3'	5'-GAGGGGGCCATCCACAGTCTTCT- 3'	2597

IL-6, interleukin-6; *IL-6R* α , interleukin-6 receptor; *SOCS3*, suppressor of cytokine signaling 3; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase

Supplemental Figure Legend:

Note: All images are representative of the individual stains at each time-point. All enumeration and quantification was conducted with sections from all subjects (n = 8) at all time-points. Images for enumeration and/or quantification were acquired using the 40x objective (see Materials and Methods).

Figure S1:

Muscle satellite (stem) cell response to muscle-lengthening contractions (MLC):

Triple-immunofluorescent staining of 7µm muscle cross-section for satellite cells (red = $Pax7^+$), laminin (green) and nuclei (DAPI = blue). Thin arrows denote the $Pax7^+$ cells located beneath the basal lamina in the satellite cell niche (20x objective). (**S1a**): Pre-intervention; (**S1b**): 4 hours (T4); (**S1c**): 24 hours (T24); (**S1d**): 72 hours (T72); (**S1e**): 120 hours (T120) post-intervention.

Figure S2:

Satellite cell IL-6 protein expression following muscle lengthening contractions (MLC):

Triple-immunofluorescent staining of 7µm muscle cross-sections for satellite cells (red = Pax7⁺), IL-6 protein (green) and nuclei (DAPI = blue). (Figures S2a-c: 20x objective; Figures S2d-f: 40x objective). (**S2a**): Pre-intervention (T0), note an absence of IL-6 co-localization (20x); (**S2b**): Higher magnification of T0 (40x) arrows denote Pax7⁺ cells with no IL-6 positivity; (**S2c**): 4 hours (T4), note some IL-6 positivity (20x) see manuscript for higher magnification image of T4; (**S2d**): 24 hours (T24), note increased IL-6 expression and Pax7/IL-6 co-localization (20x); (**S2e**): Higher magnification of T24 (40x) showing satellite cells staining positive for IL-6. (**S2f**): 120 hours (T120) post-intervention (40x), note an absence of IL-6 co-localization. Note for T72 see manuscript.

Figure S3:

Satellite cell co-localization with proliferating cell nuclear antigen (PCNA) following muscle lengthening contractions (MLC):

Triple-immunofluorescent staining of 7 μ m muscle cross-section for satellite cells (green = Pax7⁺), PCNA (red) and nuclei (DAPI = blue). (**S3a**): Pre-intervention (T0), note absence of PCNA; (**S3b**): 4 hours (T4); (**S3c**): 24 hours (T24); (**S3d**): 72 hours (T72); (**S3e**): 120 hours (T120) post-intervention. All images acquired with 40x objective.

Figure S4:

Satellite cell co-localization with phosphorylated STAT3 protein following muscle lengthening contractions (MLC):

Triple-immunofluorescent staining of 7 μ m muscle cross-sections for satellite cells (green = Pax7⁺), phosphorylated STAT3 protein (red) and nuclei (DAPI = blue). (**S4a**): Pre-intervention note absence of satellite cell associated p-STAT3; (**S4b**): 4 hours (T4); (**S4c**): 24 hours (T24), note absence of satellite cell associated p-STAT3. Images acquired with 40x objective.

Figure S5:

Control Images:

(**S5a**): Immunofluorescent (IF) stain of 7μ m muscle cross-sections for IL-6R α (green – streptavidin-FITC), nuclei (DAPI) and the secondary antibody used for

Pax7 (secondary only – Alexa 594) acquired using the 20x objective. Arrows denote nuclei that co-localize with IL-6R α and do not show any non-specific secondary binding of the Alexa 594, or any Alexa 594 interaction with the IL-6R α antibody.

(**S5b**): Immunofluorescent (IF) stain of 7µm muscle cross-sections for Pax7 (red – Alexa 594), nuclei (DAPI) and the secondary antibody used for IL-6 (secondary – tertiary only: immunoglobulin biotinylated secondary antibody + streptavidin-FITC) acquired using the 20x objective. Arrows denote nuclei that co-localize with Pax7 and do not show any non-specific secondary binding of the secondary antibodies (FITC), or any FITC interaction with the Pax7 antibody.

(**S5c**): Immunofluorescent (IF) stain of 7µm muscle cross-sections for IL-6 (green – streptavidin FITC), nuclei (DAPI) and the secondary antibody used for Pax7 (secondary only – Alexa 594) acquired using the 20x objective. Arrows denote nuclei that co-localize with IL-6 and do not show any non-specific secondary binding of the Alexa 594, or any Alexa 594 interaction with the IL-6 antibody.

(**S5d**): Immunofluorescent (IF) stain of 7µm muscle cross-sections for PCNA (red – Texas Red), nuclei (DAPI) and the secondary antibody used for Pax7 (secondary only – Alexa 488) acquired using the 20x objective. Arrows denote nuclei that co-localize with PCNA and do not show any non-specific secondary binding of the Alexa 488, or any Alexa 488 interaction with the PCNA antibody.

(**S5e**): Immunofluorescent (IF) stain of 7µm muscle cross-sections for Pax7 (green – Alexa 488), nuclei (DAPI) and the secondary antibody used for PCNA (secondary only: Texas Red) acquired using the 20x objective. Arrows denote nuclei that co-localize with Pax7 and do not show any non-specific secondary binding of the secondary antibody (Texas Red), or any Texas Red interaction with the Pax7 antibody.

(**S5f**): Immunofluorescent (IF) stain of 7µm muscle cross-sections for phosphorylated STAT3 (p-STAT3) (red – Texas Red), nuclei (DAPI) and the secondary antibody used for Pax7 (secondary only – Alexa 488) acquired using the 20x objective. Arrows denote nuclei that co-localize with p-STAT3 and do not show any non-specific secondary binding of the Alexa 488, or any Alexa 488 interaction with the PCNA antibody.













Supplemental Figures:
























Chapter 4: Manuscript 3

Aging Cell. In Review – July 20, 2011

Myostatin is Associated with the Inhibition of Muscle Stem Cell Activity in Aging Humans

Bryon R. McKay¹, Daniel I. Ogborn², Leeann Bellamy¹, Mark A. Tarnopolsky³ and Gianni Parise^{1, 4}

Departments of Kinesiology¹, Health Sciences², Pediatrics³ and Medical Physics & Applied Radiation Sciences⁴, McMaster University, Hamilton Ontario, L8S 4L8

Running Title: Dysfunction of satellite cells in aging

Keywords: Pax7, Myostatin, Aging, Cell-Cycle, Fiber-type Specific

Correspondence: Gianni Parise, PhD Departments of Kinesiology, and Medical Physics & Applied Radiation Sciences McMaster University Hamilton, Ontario, Canada L8S 4L8

Telephone:	(905) 525-9140 x27353
Fax:	(905) 523-6011
E-mail:	pariseg@mcmaster.ca

<u>Summary:</u>

Normal human aging is accompanied by a progressive loss of muscle mass (sarcopenia). We tested the hypothesis that elderly men (OM) would have a blunted myogenic response to a physiological stimulus, which would be associated with a higher proportion of muscle stem cells (SCs) co-localized with MSTN compared to young controls (YM). OM had 35% fewer SCs at baseline vs. YM and a type-II fiber specific impairment in SC content and proliferation. OM demonstrated an impaired entry into S-phase of the cell-cycle at 24h and 48h with an accumulation of SCs in G_0/G_1 . MyoD⁺ SCs were also significantly reduced in OM confirming blunted cell proliferation. Furthermore, MyoD⁺/Pax7⁻ cells were lower in OM at 48h illustrating a severely blunted progression through the myogenic program. Myostatin (MSTN; GDF-8) is a member of the TGF- β superfamily and has been shown to be involved in muscle wasting disorders. Whole muscle MSTN protein and mRNA were higher in OM vs. YM (p<0.05). MSTN mRNA was ~140% higher at 24h and 48h in OM. SC-specific MSTN levels were not different at baseline however there were significantly more $MSTN^{\dagger}$ SCs in OM vs. YM at both 24h (YM: 42±2% vs. OM: 56±3%, p<0.05) and 48h (YM: 38±2% vs. OM: 49±3%, p<0.01). Consistent with an impaired type-II SC response, there were 67% more MSTN⁺ type-II SCs in OM at 24h with no differences for age in the type-I response. These data illustrate an age-related impairment of SC function in a fiber-type specific manner. The SC-associated impairment may be a function of a significantly greater proportion of SCs

associated with MSTN in OM, suggesting the co-localization of MSTN with SCs may impair the myogenic capacity of aged muscle.

Introduction:

There is an abundance of descriptive clinical information characterizing the loss of skeletal muscle with aging, and long-term studies have demonstrated a progressive decline in muscle mass and strength (sarcopenia) starting in the fifth decade of life [1,2]. The progressive nature of age-related muscle wasting leads to an increased incidence of injury and loss of autonomy, resulting in a significant loss in quality of life and increased risk of all-cause mortality [3,4]. Unfortunately there is a paucity of information on the specific mechanisms of human muscle aging. Therefore determining the biological processes underlying age-related muscle loss is imperative to design effective treatment strategies for sarcopenia.

The regulation of muscle growth and maintenance of muscle mass is governed by a unique population of muscle stem cells referred to as satellite cells (SCs) [5]. Data from animal studies indicate that depletion or dysfunction of this cell population leads to a complete loss in the capacity for muscle to undergo hypertrophy [6]. Furthermore, an age-related decline in the SC pool size as well as an impaired response to muscle damage has been documented as a consequence of aging in mice [7,8]. This age-related impairment may be due to a dysfunction in the intrinsic properties of aged SCs, hindering propagation through the myogenic program and increasing their susceptibility to undergo

apoptosis [9] as well as decreasing their capacity to repopulate the reserve pool [7,10]. The aged systemic milieu as well as the SC niche have also been implicated in the dysfunction of aged SCs to respond to physiological stimuli [11,12] – for a review see Gopinath and Rando [13].

In humans, the literature on whether the SC pool size is maintained with age is equivocal, with some studies showing a reduced SC pool in the elderly [14–16] and others illustrating no change with age [17,18]. However, recent data from Verdijk et al. [16] illustrated a significant reduction in the SC pool in elderly men and more specifically that the reduction appeared to be more pronounced in type-II fibers [16]. Research in human aging has primarily focused on quantifying the SC response to physiological stimuli [17,19]; however, few studies have focused on the mechanisms of SC dysfunction in humans. In recent years, research using animal and cell culture models has implicated myostatin as a potential negative regulator of muscle mass and importantly SC activity [20,21].

Myostatin (MSTN, also called GDF-8) is a transforming growth factor- β (TGF- β) family member that acts as a negative regulator of skeletal muscle growth [21–23]. The function of myostatin and the biologically active region of the myostatin protein appears to be highly conserved across species [24] and the loss or mutation of the portion of the gene encoding the c-terminal region of myostatin results in a 'double muscled' phenotype [21,24]. Muscle from myostatin null mice is typically 2-3 times larger than wild-type mice, which appears to be a consequence of both hypertrophy and hyperplasia of muscle

fibers [21]. In humans there is little work demonstrating a direct relationship between myostatin and muscle wasting; however there is some evidence to support the role of myostatin in muscle atrophy in disease states. In patients with HIV-associated muscle wasting, there was an elevation of both serum and intramuscular concentrations of myostatin, suggesting that the upregulation of myostatin contributes to the overall loss of skeletal muscle [25]. In addition, agerelated elevations in serum myostatin have been shown to inversely correlate with muscle mass, suggesting that increased serum myostatin may be a contributing factor to sarcopenia [26]. Myostatin inhibition of aged mice produced an increase in muscle fiber cross-sectional area and maximal force generation compared to control animals [27]. Furthermore, the lifelong lack of myostatin (*Mstn^{-/-}* mouse model) appears to reduce the aged phenotype compared to agematched wild-type controls [28] suggesting myostatin is a key contributor to sarcopenia in rodents. Although, total muscle mass is still reduced as a consequence of aging in *Mstn^{-/-}* animals, indicating elevated systemic myostatin is not the sole mediator of sarcopenia [20]. Importantly, however, the precise role of MSTN in regulating muscle mass remains unknown. Whether MSTN exerts its control of growth on the muscle fiber or the SC (or both) is unclear.

The majority of human research on aging and myostatin has focused on myostatin gene expression between young and older adults, illustrating that myostatin expression is upregulated when compared to young muscle [29,30]. However, few aging studies have directly examined myostatin protein levels in

aged muscle [30] and to date, no in vivo human studies have assessed the role of myostatin in SC function in the context of aging. Using animal and cell culture models it has been demonstrated that myostatin is capable of upregulating p21, a cyclin-dependant kinase (Cdk) inhibitor which inhibits the cell-cycle [31]. Myostatin also suppresses Cdk2 expression and Rb phosphorylation, which are critical for cell-cycle progression, thus negatively regulating G₀/G₁ to S-phase progression, impairing SC proliferation [31]. In addition myostatin may directly inhibit myogenic regulatory factor expression (e.g. MyoD), preventing SC proliferation and differentiation [32]. Thus evidence from animal studies and preliminary work in humans illustrates that myostatin is upregulated in aged muscle and may be responsible for some degree of muscle wasting. Therefore to determine the relevance of myostatin in age-related function of SCs in humans, we used a model of acute resistance exercise to induce a myogenic response in young and older men. Blood and muscle biopsies were collected before, 3h, 24h and 48h after the exercise to ascertain the early temporal changes in SC cell-cycle kinetics. For the first time, we have examined MSTN in the context of human aging and how it relates to SC activity *in vivo*. We hypothesized that elevated muscle and SC MSTN levels would be associated with a blunted SC response in the older men compared to young men.

<u>Results:</u>

Subject Characteristics:

Characteristics of both groups are shown in Table 1. There were no differences in height, weight, lean body mass or fat mass between young (YM) and older men (OM) however 1-repetition maximum (1RM) of single-leg leg press, knee extension and maximal voluntary contraction (MVC) of the quadriceps were all 30% lower in OM vs. YM (p<0.05).

Muscle Response to Acute Exercise:

To assess the effectiveness of the same relative load (75% of 1RM) during an acute exercise bout, blood creatine kinase (CK) was assayed. Both YM and OM had a significant increase in blood CK at 24 and 48h post exercise with YM increasing 76% and OM increasing 184% from PRE at 48h (p<0.0001). The change in CK in OM was significantly greater at 48h vs. YM (p<0.05).

Muscle Stem Cell Response:

Pax7⁺ cell response:

Muscle SC number was determined via flow cytometry and immunofluorescent techniques. Basal SC number was 35% lower in OM (YM: 13345.30 \pm 1708.93 Pax7⁺ cells·mg⁻¹ vs. OM: 8665.78 \pm 1849.77 Pax7⁺ cells·mg⁻¹, p<0.05) which represented 4.43 \pm 0.78% (YM) and 3.38 \pm 0.68% (OM) of total myonuclei, p=0.043 (Fig. 1a). SC number tended to increase at 24h but was not significantly elevated until 48h in both groups (p<0.008, Fig. 1a). SC number remained 32% lower in OM at 24h (p=0.047) and tended (p=0.07) to remain lower 48h post-

exercise with the SC pool being 24% smaller in OM vs. YM (YM: 32342.01 \pm 6172.02 Pax7⁺ cells·mg⁻¹ vs. OM: 24657.98 \pm 5052.48 Pax7⁺ cells·mg⁻¹; Fig.1a).

Fiber-type Specific Response:

Previous data from Verdijk et al. [16] demonstrate a basal fiber-type specific decrement in SC number in elderly men (44% decrease in type II associated SCs). Therefore, to further characterize the SC response, the fiber-type specific SC response was determined via immunohistochemical analysis. Figure 1b is a representative image of the fiber-type specific stain with myosin heavy chain slow isoform (MHCs; type I) stained green, the basal lamina stained orange, SCs stained red (Pax7) and nuclei stained blue (DAPI). The closed arrow indicates a type I associated SC. Basal type I associated SCs were not different in OM vs. YM (YM: 0.059 ± 0.008 vs. OM: 0.060 ± 0.014 Pax7⁺ cells/type I myofiber (I-MF), Fig. 1c). In YM, type I SCs increased 44% at 24h (0.059 ± 0.008 to $0.085 \pm$ 0.014 Pax7⁺ cells/I-MF, trend, p=0.06) while type I SCs in OM did not demonstrate any increase (0.060 \pm 0.014 to 0.066 \pm 0.010 Pax7⁺ cells/I-MF, Fig. 1c). In both YM and OM type I SCs were similarly elevated ~46% above baseline levels 48h post exercise (YM: 0.086 \pm 0.016 and OM: 0.087 \pm 0.014 Pax7⁺ cells/MF; p=0.018).

Basal type II associated SCs however, were 47% lower in OM vs. YM (YM: 0.095 ± 0.011 vs. OM: 0.050 ± 0.011 Pax7⁺ cells/II-MF, p=0.04; Fig. 1d) and were unresponsive to the exercise stimulus in OM over the time-course

measured (PRE: 0.050 ± 0.011 vs. 48h: 0.053 ± 0.008 Pax7⁺ cells/II-MF; Fig 1d). In YM, type II associated SCs increased 46% 48h after exercise (PRE: 0.095 ± 0.011 vs. 0.137 ± 0.017 Pax7⁺ cells/II-MF, p=0.049) which resulted in a 61% lower type II SC pool in OM 48h after exercise.

Myonuclear domain, muscle cross-sectional area (CSA) and SC per fiber area data are shown in Table 2. Type I fiber CSA and myonuclear domain was not different between YM and OM, however Type II CSA was 21% lower and myonuclear domain was 19% lower in OM vs. YM (p<0.05). This was also the case for SC/mm² where there was no difference in SC/mm² on type I fibers but there were 30% fewer SC/mm² on type II fibers in OM vs. YM in the basal condition (p<0.05). Muscle CSA and myonuclear domain were not changed in the 48h post exercise time-course (data not shown). Type I SC/mm² increased equally (44%, p<0.05) in both YM and OM 48h post exercise. Type II SC/mm² increased 62% (p<0.05) in the YM only, further confirming a lack of type IIassociated SC response in the OM.

Cell-cycle Kinetics:

To further investigate the age-related differences in the SC response to acute resistance exercise, SC specific cell-cycle kinetics were assessed via propidium iodide and flow cytometry of mononuclear cells freshly isolated from muscle biopsies. This technique has been validated in our lab to give an estimate of *in vivo* cell-cycle kinetics of $Pax7^+$ cells [33]. In the basal state (where the majority

of SCs exist in quiescence (G₀), OM had 37% fewer SCs in the G₀/G₁ phase of the cell-cycle (p=0.04, Fig. 2a). YM demonstrated a trend for more SCs in G₀/G₁ 24h after exercise (p=0.067) while SCs in OM remained 34% lower than YM (p=0.027). Both YM and OM demonstrated a significant increase in Pax7⁺ cells in G₀/G₁ 48h after exercise with 21% fewer SCs in G₀/G₁ in OM (trend, p=0.085).

The percentage of SCs in S-phase of the cell-cycle at baseline represented approximately only 2% of the total SC pool with no differences between groups. Although there was a trend for an increase in total Pax7⁺ cells in S-phase at 24h in YM, statistical significance was not achieved until 48h post exercise (107% increase, PRE: 1599.45 ± 239.56 vs. 48h: 5100.19 ± 1089.89 Pax7⁺ cells·mg⁻¹, p=0.003 YM only). In OM however, there was no exerciseinduced increase in Pax7⁺ cells in S-phase (Fig. 2b). There were no differences in Pax7⁺ cells in S-phase at PRE or 24h between YM and OM, however at 48h there were 182% more SCs in S-phase in YM vs. OM (p=0.026).

The proportion of Pax7⁺ cells in G₂/M at baseline was 50% lower in OM vs. YM (YM: 335.01 ± 135.69 vs. OM: 166.57 ± 68.98 Pax7⁺ cells·mg⁻¹; p=0.04, Fig. 2c). The number of cells in G₂/M increased significantly by 48h (p=0.002) in both YM and OM with no difference between the age groups (Fig. 2c).

MRF Cell-Cycle Genes:

Gene analysis from whole muscle homogenates revealed that basal *Myf5* expression was not different between age groups (Fig. 2d). Following exercise

Myf5 mRNA increased 1.5 fold from baseline in YM at 48h (p=0.044 vs. PRE) and was unchanged from PRE in OM (Fig. 2d). Although there was no baseline difference in *Myf5* expression between YM and OM, *Myf5* was 2.8 fold higher in YM 48h after exercise vs. OM (p=0.002, Fig. 2d). *MRF4* mRNA expression was also not different between YM and OM pre-exercise, however in the post exercise time-course *MRF4* expression increased 159% at 3h in OM only and remained elevated ~130% above PRE levels at 24 and 48h (Fig. S8; p<0.05). *MRF4* expression in the post exercise showed a trend to be elevated in OM vs. YM (p=0.091 at 24h) however this did not reach significance over the time-course.

In agreement with *Myf5* expression, the cell-cycle genes *cyclin B1* and *cyclin B2* were elevated 2-fold and 2.6-fold (*cyclin B1* and *cyclin B2* respectively) in YM at 24 and 48h (Fig. 2e-f). *Cyclin B1* was also elevated 24 and 48h after exercise in OM (2-fold increase from PRE, Fig. 2e, p<0.05). There were no effects of age on *cyclin B1* or *cyclin B2* expression; however, *cyclin B2* was not significantly elevated in the post exercise time-course in OM (trend at 48h, p=0.065 Fig. 2f). *Cyclin D1* and *CDK1* were not significantly different between YM and OM and were not significantly affected by exercise (Fig. S8). Although *HDAC4* was not significantly different at baseline, *HDAC4* expression was 79% higher in YM vs. OM 48h post exercise (Fig. S8; p=0.048).

Together these data illustrate a reduced basal SC number in OM and a blunted increase in SC transition into S-phase of the cell-cycle. The impaired SC

response appears to be restricted to SCs associated with type II fibers. The delay in SC activation and proliferation was also confirmed with a lack of induction of *Myf5, cyclin B2* and *HDAC4* mRNA in OM.

MyoD1 and Pax7 Populations:

In order to further characterize the SC response between YM and OM, we assessed MyoD1⁺ cell populations via immunofluorescent analysis. Figure 3a is a representative image of an active MyoD1⁺(red)/Pax7⁺(green) cell beneath the basal lamina (orange – open arrow) and a quiescent MyoD1⁻/Pax7⁺ cell (closed arrow). A higher magnification of a MyoD1⁺ SC illustrating the anatomical position below the basal lamina is shown in figure S4. MyoD1⁺ cells (total MyoD1⁺ cells) were not different between YM and OM at baseline however the total number of MyoD1⁺ cells per 100 myofibers (MF) were significantly higher in YM at 24 and 48h (Fig. 3b). MyoD1⁺ cells increased linearly (107% at 3h, 215% at 24h and 365% at 48h vs. PRE, p<0.01) in YM, while in OM, MyoD1+ cells were not increased until 48h (170% increase, p<0.01).

This population was further characterized as $MyoD^+/Pax7^+$ (proliferating), MyoD1⁺/Pax7⁻ (committed to differentiation) and MyoD1⁻/Pax7⁺ (quiescent). MyoD⁺/Pax7⁺cells/100MF followed a similar trend as $MyoD^+$ cells, increased 267% in YM (PRE: 3.82 ± 0.97 vs. 48h: 14.02 ± 2.72 cells/100MF) and 151% in OM at 48h (PRE: 2.65 ± 0.40 vs. 48h: 6.65 ± 0.67 cells/100MF) with significantly fewer $MyoD^+/Pax7^+$ cells in OM at 3h, 24h and 48h (p<0.02, Fig. 3c).

 $MyoD1^{+}/Pax7^{-}$ cells represented only 0.64 ± 0.24 cells/100MF in YM at baseline, however this cell population increased significantly at 24 and 48h with 942% more MyoD1⁺/Pax7⁻ cells/100MF at 48h vs. PRE in YM (48h: 6.67 \pm 1.12 cells/100MF, p<0.001, Fig. 3d). There was a trend for more MyoD1⁺/Pax7⁻ cells/100MF at PRE and 3h in OM vs. YM (p=0.09) with some of these cells being centrally located myonuclei in a subset of the oldest OM (Fig. S5). Although there tended to be more MyoD1⁺/Pax7⁻ cells at PRE in OM, there was no increase in this population until 48h post exercise. This translated into a 212% increase (vs. 942% in YM) in MyoD1⁺/Pax7⁻ cells/100MF in OM 48h post exercise (PRE: 1.20 ± 0.56 vs. 48h: 3.75 ± 0.58 cells/100MF, p<0.05). Therefore at 24h and 48h there were significantly more $MyoD^{+}/Pax7^{-}$ cells in YM (p<0.02, Fig. 3d). The Pax7⁺/MyoD1⁻ pool was significantly larger at PRE and 3h in YM vs. OM (PRE: YM: 10.53 ± 1.23 vs. OM: 4.87 ± 0.54 cells/100MF, p<0.05). This population of SCs was significantly lower at 24 and 48h in YM and at 48h only in OM vs. PRE however, this pool of cells was still significantly lower in OM vs. YM (48h: YM: 5.16 ± 0.81 vs. OM: 2.04 ± 0.36 cells/100MF, p<0.05; Fig. 3e). Together these data illustrate a reduced induction of MyoD in the Pax7 population of cells and a reduced or delayed progression through the myogenic program in OM following acute exercise.

Whole Muscle Myostatin:

To determine whether MSTN was involved in altered SC cell-cycle kinetics we investigated the response of muscle MSTN as a function of exercise and age.

Whole muscle MSTN protein levels were assessed via western blotting. OM had over 2-fold more MSTN protein at baseline compared to YM (Fig. 4a). These higher levels of MSTN protein persisted until 48h where there was a 30% reduction in MSTN in OM (Fig. 5a, p=0.006 vs. PRE). OM had significantly higher MSTN levels at 3h and 24h but the reduction in MSTN in OM brought the protein levels of MSTN in OM closer to that of YM by 48h (Fig. 4a).

Whole muscle gene expression of *MSTN* was 1.7-fold higher at baseline in OM vs. YM (Trend, p=0.067, Fig. 4b-c). Although *MSTN* mRNA expression decreased similarly in both groups over the post exercise time-course, relative *MSTN* expression of OM compared to YM was 3-fold and 2.4-fold higher at 24 and 48h respectively (p<0.001, Fig. 4c). *Follistatin* expression was 149% higher in OM at baseline (p=0.037) but this normalized to the same expression of YM post-exercise and was not elevated in response to exercise (Fig. S7). Interestingly, baseline *follistatin like-1* (*FSTL1*) expression was 115% lower in OM vs. YM (Fig. S7). *FSTL1* expression was not different over time in YM but at 48h the expression in OM increased (p=0.01) to the same relative level as YM. Therefore, at the whole muscle level there was a significant elevation of whole muscle myostatin protein and gene expression in OM, which persisted at the mRNA level throughout the time course.

SC Specific Myostatin levels:

In order to assess the MSTN response in relation to the SC compartment we first wanted to confirm that human SCs expressed the main receptor responsible for canonical MSTN signaling – activin IIb (ActRIIb). Figure S1a shows a representative image of ActRIIb (brown) on the sarcolemma and co-localized with Pax7 (black) in a 7 μ m muscle cross-section. In order to verify that ActRIIb was indeed localized on the cell membrane of human SCs and not just localized with the sarcolemma below the SC *in situ*, we isolated human primary myoblasts from a sub-set of subjects. Figure S1b shows the cellular localization of ActRIIb (green) with a Pax7⁺ (red) human myoblast confirming that these cells do possess the MSTN receptor.

To assess the relation of MSTN with the SC response to acute resistance exercise, we conducted immunofluorescent analysis of MSTN with Pax7 and the slow isoform of myosin heavy chain. A representative image of the immunofluorescent staining of MSTN and Pax7 is shown in Figure 5a. The boxed area is broken down below into various overlays of Pax7 (red), nuclei (blue), MSTN (orange), MHCs (green) and laminin (green). To further confirm the presence of MSTN in human SCs, we isolated primary myoblasts from a subset of subjects. Figure 5b shows the localization of MSTN protein (green) with Pax7⁺ cells (red), confirming the presence of MSTN in human SCs. At baseline, MSTN co-localized with 70 ± 2% of SCs in YM and 76 ± 4% of SCs in OM. By 3h there was a reduction of MSTN⁺ SCs in both groups (YM: 60 ± 4% vs. OM: 67 ± 5% of total Pax7⁺ cells) which further decreased at 24 and 48h in both groups

(p<0.001; Fig. 6a). There were significantly more MSTN+ SCs in OM vs. YM at both 24h (YM: 42 \pm 2% vs. OM: 56 \pm 3%, p<0.05) and 48h (YM: 38 \pm 2% vs. OM: 49 \pm 3%, p<0.01).

To determine the fiber-type specificity of this response, type I and type II associated SCs were analyzed for MSTN. At baseline, there were no differences in the proportion of SCs co-localized with MSTN between groups (Fig. 6b). At 3 and 24h there was a significant reduction in percentage of SCs that were positive for MSTN in YM only (PRE: $75 \pm 7\%$ vs. 24h: $50 \pm 9\%$), however there were no differences due to age. On type II fibers, MSTN⁺ SCs were reduced at 3h (p<0.012) in both groups and were further reduced at 24 and 48h (p<0.004, Fig. 6c) in YM only. OM had a reduction in $MSTN^+$ SCs at 48h (p=0.02), however there were 67% more MSTN⁺ SCs in OM at 24h (p=0.004) and 21% more in OM at 48h (trend, p=0.07) vs. YM. These data illustrate a fiber-type specific response in myostatin signaling such that the same relative stimulus (between YM and OM) resulted in significantly fewer MSTN⁺ SCs in YM at 24h which was further reduced in YM only at 48h. OM displayed significantly more SCs co-localized with MSTN in the type-II fibers specifically compared to YM post exercise (67% -24h, p<0.05; 21% - 48h, p=0.07 more MSTN⁺/Pax7⁺ cells vs. YM).

Discussion:

Understanding the cellular mechanisms responsible for age-related muscle loss is essential for the development of treatment strategies to promote the

maintenance of healthy muscle mass. Although in vitro and animal research is fundamentally important for the understanding of biological and physiological processes, confirming these processes in vivo in humans is of the utmost importance especially when dealing with clinically relevant phenotypes such as sarcopenia. In agreement with previous work in humans [16], we confirm that elderly individuals (65-75yr) have a lower SC pool size and that this is mainly due to a significant reduction in type-II fiber associated SCs. In response to the same relative acute physiological stimulus (lower leg resistance exercise at 75% 1RM), elderly men demonstrated a markedly blunted myogenic response which was isolated to the type-II fibers compared with young healthy controls. Importantly, this coincided with a significantly higher percentage of type-II SCs expressing MSTN protein in the elderly throughout the post-exercise time course. MyoD1⁺ SCs were also significantly lower following exercise in the elderly, suggesting a blunted entry of SCs into the myogenic program which was further verified by a lack of SC progression into S-phase of the cell cycle and a lack of Myf5. cyclin B2 and HDAC4 mRNA response in the older men.

In the present study we report that men aged 65-75yrs demonstrate a 30% reduction in muscle strength, 21% reduction of type-II muscle fiber cross sectional area (CSA) and a 19% lower type-II fiber myonuclear domain compared to young lean body mass matched controls. These data confirm work from Verdijk et al, [16] who reported almost identical reductions in the 1RM strength, CSA of type-II fibers, and type-II myonuclear domain in men aged 76±1 vs.

20±1yrs [16]. The age-related preferential loss in type-II fibers has been well characterized previously [34], however Verdijk et al. [16] recently reported that the type-II fibers were also associated with a reduced SC content, suggesting that the observed decline in type-II SC content in the elderly may be an important factor in the etiology of type-II fiber atrophy [16].

In the present study we definitively show a 35% reduction in the basal SC number in the elderly using both flow cytometry and immunohistochemical/immunofluorescent detection methods. Although an agerelated decline in SC number has been reported before [14–16], this finding is not consistent across the literature [17,18]. The discrepancy may be strongly influenced by differences in age, activity status and health of the elderly subjects. In the present study we excluded subjects who were actively participating in regular resistance exercise or subjects that were completely sedentary in an attempt to get an accurate cross-section of the average 70 year old. Furthermore, differences in anti-bodies or methods used to enumerate SCs in human muscle (i.e. m-cadherin, Pax7, N-Cam, C-Met) may also contribute somewhat to the equivocal findings. We have previously reported that Pax7, N-Cam and C-Met are differentially expressed in muscle cross-sections and in cell isolates, suggesting heterogeneity in the expression of these markers [33]. The use of Pax7 or N-Cam (or both) appears to yield the most consistent numbers in SC content across studies and have been shown to be co-expressed on ~95% of SCs suggesting these are the most appropriate markers for detection of human

SCs to date [16,33,35]. In agreement with Verdijk et al. [16] who reported 44% fewer SC on type-II fibers in the elderly, we report 47% fewer type II-associated SC in the elderly compared to young. There were no differences in the basal number of SCs on type I fibers, supporting the hypothesis that a reduction in type-II SC content in the elderly may be a contributing factor to the age-related decline in type-II muscle CSA [16]. Although a fiber-type specific decline in SCs has been noted previously, no data exists on the acute response of these cells to a physiological perturbation. Previous data suggests that older men have a reduced SC response 24 h following a bout of eccentrically biased exercise (51%) increase in OM vs. 141% increase in YM) suggesting an age-related dysfunction in SC recruitment, however the fiber-type specificity of this response was not measured [17]. In the present study we illustrate a complete lack of type-II fiber SC response to resistance exercise in the elderly (46% increase in YM) over a 48h time-course. While SCs associated with type-I fibers did illustrate a delayed response at 24h in the elderly compared to the 44% increase observed in young. by 48h the total number of type-I SCs were the same in both groups. Taken together these data describe an age-related dysfunction in the activation of SCs as a whole and specifically the inability to activate or induce a proliferative response in SCs associated with type-II fibers. This finding is supported by the cell-cycle data, which clearly demonstrates a delay or absence of SCs shifting from G_0/G_1 into S-phase of the cell-cycle following exercise in the elderly. SCs from young muscle illustrated a significant linear increase in to S-phase

(increasing 107% and 219% from PRE at 24h and 48h respectively). There were 182 % more SCs in S-phase at 48h compared with the elderly, which is further supported by a significant increase in *Myf5* mRNA in the young (2.8-fold higher expression vs. old at 48h). Additionally, MyoD1⁺ cells were not increased in the elderly until 48h suggesting an impaired ability for SCs to progress through the myogenic program. Notably, gene expression of *Myf5* was not upregulated in the elderly following exercise, nor were cell-cycle related genes *cyclin B2* or *HDAC4* upregulated in the elderly, which were both upregulated in the young.

In an attempt to understand the observed age-related impairment in SC function, we chose to examine MSTN signaling as a potential mediator of the impairment. MSTN has been shown to be elevated as a consequence of aging and has been implicated in cell-cycle inhibition of SCs *in vitro*. In humans, the role of MSTN, as it pertains to SC function has never been evaluated *in vivo* or in the context of aging. MSTN is believed to bind to the serine/threonine kinase receptor ActRIIb which phosphorylates receptor-regulated Smad2 and Smad3 which form a complex with Smad4 and translocate to the nucleus where they regulate the transcription of target genes [36]. Although MSTN has been shown to have alternate signaling pathways such as Ras/Erk 1/2 and TAK1-MKK4 [36], the MSTN/ActRIIb/Smad pathway is considered especially important for the inhibition of myogenesis. We demonstrate via immunohistochemistry that ActRIIb was co-localized with human SCs *in vivo*, which was further confirmed to be concentrated on the plasma membrane of primary myoblasts *in vitro* using 3-D

deconvolution from multiple automated z-stacked images. We also confirmed the presence of MSTN in the cytosol of isolated human primary myoblasts using 3-D deconvolution, verifying the immunofluorescent co-localization of MSTN with Pax7 *in vivo*. Interestingly, we also noted the presence of MSTN in a subset of myonuclei which was verified using nuclear and cytoplasmic fractions of muscle protein (Figure S8). This was also observed *in vitro*, localizing to the nucleus of some human primary cells in addition to the cytosol (confirmed with 3-D deconvolution, not shown). Although no clear evidence supports a role for MSTN in the nucleus, others report nuclear localized MSTN in myoblast cultures, rat and human muscle [37–39]. Clearly addition work is needed to clarify the role of nuclear associated MSTN.

In mice, normal aging is accompanied by approximately double the concentration of MSTN in muscle lysates of old animals compared to young [40]. This was accompanied by an increase in p21 protein and muscle cell apoptosis (<1% in young, vs. 8% apoptosis in old) detected using a TUNEL assay [40]. Furthermore, excess MSTN in circulation induced a significant reduction in muscle mass in mice [41]. This finding is consistent between species where men with HIV-related muscle wasting have higher circulating and intramuscular MSTN levels as compared to healthy controls [25]. Not surprisingly we observed that elderly men had approximately 2-fold higher levels of MSTN protein in whole-muscle homogenates, with higher basal *MSTN* gene expression. Interestingly, across the post-exercise time course, *MSTN* mRNA expression remains over

145% higher compared to young despite showing a time-dependant downregulation which was similar to the young. *Follistatin* mRNA was higher in the elderly at baseline which may have been upregulated as a compensatory mechanism to counteract the high levels of MSTN observed in the elderly. Although MSTN protein content was not altered by exercise, mRNA levels were reduced in both groups, suggesting either the time-course was not adequate to detect changes in whole muscle protein content or that the stimulus was not sufficient to alter MSTN protein content at the whole-muscle level. It may be that a more robust stimulus is needed to induce a rapid decrease in MSTN protein (i.e. repeated bouts of resistance exercise) however more work is needed to understand the whole-muscle *in vivo* response of MSTN to a physiological stimulus.

Importantly, for the first time, we describe the localization of MSTN to the SC *in vivo* and have demonstrated that MSTN localization can be manipulated through physical activity. To investigate the role of MSTN and age-related SC dysfunction, we conducted immunofluorescent analysis of MSTN with fiber-type specific SC staining. Although elderly muscle had over 2-fold higher MSTN whole-muscle protein levels than the young, SC-specific MSTN levels were not different between groups at baseline. The response to exercise however revealed an age-related increase in the proportion of SCs which were positive for MSTN at 24h and 48h compared to young. This response could almost be completely explained by the MSTN response in the type-II SCs. There were

similar reductions in the proportion of type-I associated SCs that expressed MSTN over time between groups although the decrease in $MSTN^{+}$ SCs was only significant in the young at 24h and 48h. Elderly type-II SCs did not respond to the same magnitude as young SCs, maintaining a higher proportion that were copositive for MSTN at 24h (67% higher in OM, p<0.05) and 48h (21% higher in OM, p=0.07). The higher level of MSTN in the type-II SCs specifically may be a key factor underlying the inability of the type-II SCs to respond to exercise in the elderly muscle. Previous work in animals has shown quiescent SCs on single myofiber cultures have a high level of MSTN expression and once activated, MSTN expression is significantly down-regulated [42]. MSTN was shown to be capable of maintaining quiescence by inhibiting cell-cycle progression [31]. Specifically MSTN induces the upregulation of Cdk inhibitor p21, which hypophosphorylates pRb and prevents progression from G_1 to S-phase [43]. SCs in culture treated with MSTN, illustrate a significant increase in p21 protein, a down regulation of Cdk2 and a down-regulation in hyperphosphorylated Rb with a concomitant increase in hypophosphorylated pRb [31]. This occurred in a dosedependent manner with the highest levels of MSTN causing a severe reduction in proliferation [44]. Further, human myoblasts treated with recombinant human MSTN upregulate p21 which causes a reduction of SCs in S-phase and a significant accumulation of cells in G_1 [44]. In the present study we observed an inability for SC from elderly muscle to progress from G₁ into S-phase of the cellcycle at 24h. At this time point, there was a significantly higher level of $MSTN^+$

SCs (67% higher vs. YM), greater whole-muscle MSTN protein content and a 3fold higher level of *MSTN* mRNA in the elderly. This finding suggests that the maintenance of PRE levels of MSTN in the SCs of elderly men may be preventing the activation and progression of the SCs through the cell-cycle. This notion is further supported by the fact that the number of MSTN⁺ SCs on type-I fibers were not different between young and elderly men, and the SCs associated with type-I fibers did not show an impairment in proliferation. In addition type-II associated SCs in the elderly had a significantly higher proportion of SCs colocalized with MSTN as compared to young and were unresponsive to the exercise stimulus. These data agree with previous reports of high MSTN levels inhibiting proliferation, suggesting that in elderly muscle SCs (especially type-II associated SCs) do not down-regulate MSTN as rapidly or to the same extent as young muscle. This could result in a higher proportion of SCs in the quiescent state, ultimately blunting the myogenic response.

In addition to the upregulation of p21, MSTN may also inhibit SC proliferation and differentiation by the inhibition of the myogenic regulatory factor MyoD [32]. In order for SCs to progress through the myogenic program MyoD must be upregulated in order to direct activated SCs through proliferation and into differentiation [45,46]. A recent study by McFarlane at al. [44] reported that MSTN inhibited MyoD in human primary myoblasts through the upregulation of Smad3 signaling which is consistent with previous data in murine myoblasts [32]. In the present study, there was a robust increase in MyoD⁺ cells in young muscle

over time, however there was little to no induction of MyoD in Pax7⁺ cells in the elderly until 48h. Although there was a delayed induction of MyoD in the $Pax7^+$ cells of older adults there were still 110% fewer $MyoD^{+}$ SCs in the elderly at 48h. Upregulation of Smad3 via canonical MSTN signaling observed in both human and murine cultured myoblasts inhibits MyoD activity and MyoD expression which impairs myotube formation and inhibits differentiation of myoblasts in low-serum culture conditions [32,44]. We observed a 942% increase in MyoD⁺/Pax7⁻ cells 48h post-exercise in young, suggesting these cells were progressing through the myogenic program, down-regulating Pax7 in preparation to differentiate [47]. At baseline and at 3h the number of MyoD⁺/Pax7⁻ cells were not different between groups, however at 24h these cells were significantly increased in the young but remained unchanged in the elderly. By 48h these cells were increased 9-fold in the young but were only beginning to accumulate in the elderly. The blunted induction of MyoD in SCs (Pax7⁺/MyoD⁺) of older adults, which is further supported by the lack of *Myf5* gene expression in the older adults, resulted in fewer cells progressing towards differentiation (MyoD⁺/Pax7⁻) and may have been a consequence of or further contributed to the lack of cell-cycle progression observed via flow-cytometry.

The greater proportion of SCs co-localized with MSTN in elderly men was associated with a reduction of SC activation/proliferation as verified by a lack of progression of SCs from G_0/G_1 into S-phase of the cell cycle. Furthermore, the expression of MSTN was significantly higher in SCs of type-II fibers in the elderly

which was associated with a lack of a type-II SC response following exercise. These findings suggest that MSTN may inhibit SC activation/proliferation to a greater degree in elderly muscle, blunting or delaying the myogenic response to acute exercise, which is further evidenced by a suppressed induction of MyoD in the elderly. The data from this study suggest that increased expression of MSTN at the protein and mRNA level as well as increased MSTN co-localized with SCs (specifically type-II SCs) negatively regulates the myogenic response to an acute bout of heavy resistance exercise. These findings help to explain the reduced myogenic capacity of aged human skeletal muscle. Furthermore, the inability to promote myonuclear turnover in skeletal muscle may increase the susceptibility for skeletal muscle to undergo apoptosis [40] and may contribute to the etiology of sarcopenia. Future studies should examine the role of therapeutic strategies such as myostatin inhibition and the in vivo response to exercise to ascertain if myostatin based therapies can promote the preservation of skeletal muscle mass with aging.

Experimental Procedures:

Subjects:

Nine healthy young males (YM) aged 21±3y and nine healthy older males (OM) aged 70±4y were recruited from the McMaster University community. Subjects underwent a routine screening, completed a health questionnaire, and were not involved in a lower-body resistance exercise training program for at least six

months prior to participating in the study. Exclusion criteria included evidence of coronary heart disease, respiratory disease (i.e. COPD), uncontrolled hypertension, renal disease, diabetes, major orthopedic disability, and smoking. Subjects were told to refrain from exercising throughout the time-course of the study. All subjects were informed of the procedures and potential risks associated with the study and gave their written informed consent to participate. This study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to all declarations on the use of human subjects as research participants.

Baseline Measures and Familiarization:

A few days prior to the acute exercise protocol, subjects visited the lab for a baseline blood draw, body composition assessment via dual-energy x-ray absorptiometry (DEXA; GE Medical, Canada), and familiarization of the baseline strength measurement procedure (maximal voluntary contractions; MVC) using a Biodex Dynamometer (Biodex-System 3, Biodex Medical Systems, Inc., USA) and the exercise protocol. Subjects were instructed to refrain from caffeine and alcohol prior to and throughout the time course of the study, and to consume the same breakfast prior to each visit to minimize any potential effect of nutrition on the outcome variables.

Acute Exercise Protocol:

Subjects arrived at the clinic at 6am and underwent a series of 3 MVC tests separated with 3 min rest. After completion of the baseline strength test, subjects conducted a unilateral incremental 1 repetition maximum (1-RM) test for both leg press and knee extension. Following 1-RM assessment, subjects completed 4 sets of 10 repetitions of unilateral leg press and unilateral knee extension at 75% of their 1-RM separated by 2 min of rest between sets. All exercise was conducted under the supervision of 2 trained investigators. After 3hrs of supervised rest, subjects underwent 2 muscle biopsies (one in each leg) with the unexercised leg acting as the non-exercise control biopsy for baseline measurements (PRE). A 3hr blood draw was completed at this time. 24 and 48hrs after the exercise, subjects reported back to the clinic and underwent a subsequent muscle biopsy from the exercised leg and a blood draw. Subject characteristics and strength measures are listed in Table 1.

Muscle Biopsies:

A total of 4 percutaneous needle biopsies were obtained from the mid-portion of the *vastus lateralis* under local anesthetic (1% lidocaine) using manual suction [48,49]. One muscle biopsy was obtained from the non-working leg for baseline analysis (PRE). The other biopsies were obtained from the working leg 3, 24, and 48hrs post-intervention. Incisions and biopsies on the exercise leg were spaced by approximately 3cm and the order of distal, mid and proximal incision was randomized to minimize any effect of the previous biopsy. From each biopsy, approximately 25 mg was mounted in Optimum Cutting Temperature

(OCT) compound and frozen in isopentane cooled in liquid nitrogen for histological analysis. The remaining portion was split into 2 pieces for mRNA and protein analysis. From the PRE, 24 and 48hr biopsies, approximately 50 mg (50.7 ± 3.9 mg) of fresh muscle tissue was placed in ice-cold Ham's (F-10) culture media (Gibco, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) for immediate flow cytometry preparation.

Flow Cytometry for Determination of Myogenic Satellite Cell Number and Cell Cycle Kinetics using Pax7 and propidium iodide (PI):

PRE, 24 and 48h muscle biopsy tissue was prepared for flow cytometry analysis using standard methods and criteria [33,45,50,51]. Briefly, after weighing, muscle samples were mulched using sterile surgical scissors in 3.5 cm tissue culture plates in a tissue culture hood. After tissue was adequately mulched, 0.5 mL of a collagenase/dispase solution (10mg/mL Collagenase B, Roche Diagnostics, Germany; 2.4U/mL Dispase, Gibco USA; with 5µL/mL of 0.5M CaCl₂.) was added to the plate and placed in a tissue culture incubator for 10 minutes (5% CO₂, 37°C). Another 0.5 mL of collagenase/dispase was added to the plate and the mixture was manually triturated through a 5 mL and 1 mL plastic serological pipette to further disrupt the tissue. The mixture was then incubated for 5-10 minutes. Following the incubation, 5 mL of ice-cold Ham's (F-10 with 20% FBS and 1% penicillin/streptomycin) culture media was added and the mixture was triturated several times and filtered through a 50 µm mesh filter

(BD biosciences, BD Canada) into a 50 mL conical bottom tube. The mixture was then centrifuged at 800 g for 5 min to obtain a pellet containing mononuclear cells. The pellet was re-suspended in 1 mL of 1x phosphate-buffered saline (10mM PBS, pH 7.4) and a 10 µL sample was taken for analysis of total mononuclear cell concentration using a hemacytometer (Hausser Scientific, USA). The cell suspension was added drop-wise to ice-cold 70% ethanol under gentle agitation for fixation. After fixation, cells were pelleted and prepared for flow cytometry for determination of the Pax7⁺ cell population and cell-cycle kinetics via propidium iodide stained as previously described [33]. Satellite cells were identified with an anti-human Pax7 primary antibody (cell supernatant from hybridoma cells obtained from DSHB, USA; $50 \pm 2 \mu g/mL$ mAb) using Alexafluor 488 (Invitrogen, USA) for detection. The specificity on Pax7 in our mononuclear preparation was confirmed via immunocytochemistry for Pax7 (DSHB and Alexafluor 594) and DAPI (Fig.S2b). Cell-cycle was determined using Propidium iodide (PI: 0.1mg/mL PI (Calbiochem, USA), 0.2mg/mL RNase A (Roche Diagnostics, Germany) and 0.1% Triton-X 100 (Sigma-Aldrich, Canada) in 1x PBS). Flow cytometry was conducted using a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, USA). Optical alignment and fluidics of the cytometer were verified before each experiment using Flow-Check fluorospheres (Beckman-Coulter, USA) and optimum instrument settings were verified using a Flow-Set kit (Beckman-Coulter, USA) by a trained technician. Gating strategies were optimized through multiple experiments which included various unstained,

secondary only, and P.I. only samples as described previously [33]. Repeated control experiments were conducted periodically throughout the study to ensure consistency of our gating strategies. Specific fluorochrome details are as follows: P.I. excitation = 520 nm, emission = 610 nm; Pax7 (Alexa 488) excitation = 494 nm, emission = 519 nm. Representative cell-cycle histograms are illustrated in figure S2a.

RNA isolation:

RNA was isolated from homogenized muscle samples using the TRIzol/RNeasy method [52]. Briefly, approximately 25 mg of each muscle sample was homogenized in a total of 1.0 mL of TRIzol Reagent (Invitrogen Corporation, Canada) using a glass homogenizer (2mL Kontes, Kimble Kontes, Germany). Homogenized samples were incubated at room temperature for 5 min followed by the addition of 0.2 mL of chloroform then shaken vigorously for 15 s. After another 5 min incubation at room temperature, samples were centrifuged at 12 000 g at 4°C for 10 min. The aqueous phase was then transferred to a new tube and the volume was measured. 1 volume of 70% ethanol was added to the aqueous phase and mixed. Multiple 700 µL aliquots were then transferred into a Qiagen RNeasy mini spin column and RNA was purified by using the RNeasy mini kit (Cat. # 74106), following the manufacturer's instructions (Qiagen Sciences, USA). The RNA was quantified using a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA) and RNA integrity was assessed using a

bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies Canada Inc.). Average RIN values were 9.36 ± 0.14 for YM and 9.1 ± 0.16 for OM.

Reverse Transcription (RT):

Individual samples were reverse transcribed in 20 µL reactions using a commercially available kit (Applied Biosystems High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA) according to the manufacturer's instructions. The cDNA synthesis reaction was carried out using an Eppendorf Mastercycle ep*gradient* thermal cycler (Eppendorf, Canada).

Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR):

Individual 25 µL reactions were prepared in 0.2 mL Stratagene PCR tubes (Stratagene, USA) and run in duplicate for each time-point. Primers were custom-made using published sequences (Table 2) and were re-suspended in 1X TE buffer (10 mM Tris-HCl, 0.11 mM EDTA) and stored at -20[°]C prior to use. In each reaction tube, 1.0 µL of cDNA and 7.5 µL of ddH₂O were added to 16.5 µL of a master mix containing 12.5 µL of RT² Real-Time SYBR Green / Rox PCR master mix (SuperArray Bioscience Corp., USA) along with 2 µL of the specific forward and reverse primers. qRT-PCR reactions were carried out using a Stratagene Mx3000P real-time PCR System (Stratagene, USA) using Stratagene MxPro QPCR Software Version 3.00 (Stratagene, USA). mRNA expression was calculated using the delta Ct method (2^{-Δct}) and fold changes from baseline were calculated using the delta Ct method [53]. Gene expression was normalized

to the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* (Table S1). Thus, mRNA values were expressed as either total mRNA expression (for between groups comparisons) and/or fold change from PRE for within group comparisons (mean ± SEM). GAPDH expression was not different from PRE at any of the post-intervention time-points or between age groups at any time point.

Immunofluorescence:

7 µm muscle cross-sections were stained with antibodies against Pax7 (neat; cell supernatant from cells obtained from the DSHB, USA); myostatin (MSTN, GDF-8, near C-terminus; 1:100, AB3239, Millipore, Canada. Previously validated [25,54]. with no bands observed via western blot in the myostatin knock-out mouse muscle [55]); Activin IIb (ActRIIb;1:100; AP7105a, Abgent, USA); A4.951 (Myosin Heavy Chain Slow (MHC-I) isoform; neat; cell supernatant from cells obtained from the DSHB, USA); Laminin (1:1000, L8271, Sigma-Aldrich, Canada and Abcam ab11575, Abcam, USA); MyoD1 (Anti-MyoD1, Clone 5.8A, Dako Canada Inc., Canada). Secondary antibodies used were: Pax7 (Dylight 488, 1:500, Thermo Scientific, Canada or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA); MyoD1(immunoglobulin biotinylated secondary antibody, 1:200, Vector Canada, Inc., Canada; followed by a streptavidin-594 fluorochrome, 1:500, Invitrogen, Molecular Probes Inc., USA); A4.951(AlexaFluor 488 or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA); Laminin (AlexaFluor 647, 1:500 or AlexaFluor 350, 1:20, or Alexafluor 488, 1:500,

Invitrogen, Molecular Probes Inc., USA); Myostatin (AlexaFluor 647, 1:500, Invitrogen, Molecular Probes Inc., USA) and Activin IIb (immunoglobulin biotinylated secondary antibody, 1:200, Vector Canada, Inc., Canada; followed by the Vectastain Elite ABC and DAB kits according to the manufacturers' instructions (Vector Canada, Inc., Canada). Histochemical methods were adapted from previously published methods from our lab [33]. Briefly, for coimmunofluorescent staining, sections were fixed with 2% paraformaldehyde (PFA, Sigma-Aldrich, Canada) for 10 min followed by several washes in PBS. Sections were then covered for 60 min in a blocking solution containing, 2% bovine serum albumin (BSA; Santa Cruz Biotechnology, USA), 5% fetal bovine serum (FBS: Gibco, USA), 0.2% Triton-X 100 (Sigma-Aldrich, Canada), 0.1% sodium azide (Sigma-Aldrich, Canada), 5% goat serum (GS, Sigma-Aldrich, Canada). Following blocking, sections were incubated in the primary antibody cocktail (i.e. MHC-I and Laminin diluted in 1% BSA) at 4°C overnight. After several washes, sections were then incubated in the appropriate secondary antibodies. Sections were then re-fixed in 2% PFA to prevent migration of the secondary antibodies and re-blocked in 10% GS in 0.01% Triton-X 100. The sections were then incubated in the second primary antibody cocktail, followed by incubation in the appropriate secondary antibodies. Sections were then washed with PBS and 4',6-diamidino-2-phenylindole (DAPI, 1:20000) (Sigma-Aldrich, Canada) for nuclear staining. Staining was verified using the appropriate positive and negative controls to ensure specificity of staining. Multiple secondary only
controls were used for stains where multiple antigens were probed on the same slide. Controls for MSTN and MyoD1 are shown in figure S3 and S6. Stained slides were viewed with the Nikon Eclipse 90*i* Microscope outfitted with a high resolution QImaging camera for fluorescence detection and 5 fluorescent filter cubes (DAPI, 488/FITC, TRITC, Cy5, Triple Cube; Nikon Instruments, Inc., USA) and images were captured and analyzed using the Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., USA).

Primary Myoblast Isolation/Culture and Immunocytochemistry:

For verification of myostatin and ActRIIb staining of satellite cells primary myoblasts were isolated from a subset of subjects (22±3y, N=4). Mononuclear cells were isolated as described above and myoblasts were purified to 95% via fluorescence activated cell sorting (CyFlow Space, Partec GmbH, Germany) using an anti-NCAM antibody (5.1H11 DSHB; Secondary: Alexa 594, Invitrogen, Molecular Probes Inc., USA). Cells were then plated in 4-well collagen coated chamber slides (Lab-Tek, Nalge Nunc International, USA) and incubated overnight to allow cells to adhere. The slides were then stained for myostatin (MSTN, 1:200, AB3239, Millipore, Canada) and Pax7 (DSHB) or ActRIIb (1:200; AP7105a, Abgent, USA) and Pax7 (DSHB). Secondary antibodies were (Pax7) goat anti-mouse Alexafluor 549 and (ActRIIb, MSTN) goat anti-rabbit Alexafluor 488, nuclei were stained with DAPI (Sigma-Aldrich, Canada). Myoblasts were positively identified with Pax7 and MSTN and ActRIIb staining was confirmed against a secondary only control (Fig. S7). Cellular localization of the staining

was conducted from 30-45 automated z-stacked images which were digitized into a 3-D image. Deconvolution of the 3-D images was conducted, producing a pseudo-confocal 3-D image of the localization of the staining. Automated zstacking and deconvolution was conducted using NIS Elements Advance Research software and the Nikon Eclipse 90i microscope with a fully automated stage (Nikon NIS Elements 5, Nikon Instruments, Inc., USA)

Protein Analysis:

25mg of muscle tissue was minced with scissors and subjected to three, fivesecond bouts of electric homogenization on ice. Extraction of nuclear and cytosolic fractions was performed as per the manufacturers' instructions (Pierce, Rockford, IL, USA). Protein concentration was determined on each fraction using the bichinconic acid method as per the manufacturer's recommendations (Pierce, Rockford, IL, USA) with a spectrophotometer (Benchmark Plus, Biorad, Hercules, CA, USA). For myostatin, 20ug of total cytosolic proteins were run on a 12.5% gel for two hours and transferred to nitrocellulose membranes at 100V for one hour (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked in 3% fat-free milk in TBST at 4°C for one hour at room temperature and incubated overnight at 4°C in anti-myostatin primary antibody in 3% milk (1:1000, AB3239, Millipore, Canada). The following day the blots were incubated in secondary anti-rabbit antibody (1:10000; GE Healthcare, Piscataway, NJ, USA) in 3% milk TBST for one hour at room temperature, developed with ECL plus (GE Healthcare, Piscataway, NJ, USA) and exposed to x-ray film (GE Healthcare, Piscataway, NJ,

USA). All films were digitized and band density was determined with ImageJ (NIH, Bethesda, Maryland, USA). Ponceau staining was performed on all blots to confirm equivalent loading between samples.

Blood Measures:

A resting blood sample was obtained from the antecubital vein immediately prior to the intervention and at 3, 24 and 48h post. Approximately 20 mL of blood was collected and separated into one serum and one plasma vacutainer tube (BD, Canada) at each time-point. Samples were separated into 50 µL aliquots and stored at -80 °C for analysis at a later date. Serum samples were thawed on ice and analyzed for creatine kinase (CK) protein concentration using a commercially available kit according to manufacturers' instructions (Pointe Scientific Inc., USA).

Statistical Analysis:

Statistical analysis was performed using SigmaStat 3.1.0 analysis software (Systat Software Inc., USA). A 2-way repeated-measures analysis of variance (ANOVA) with one factor for time and one for age was conducted for all measures except PRE subject characteristics where a t-test was used for differences between age groups. Statistical significance was accepted at P < 0.05. Significant interactions and main effects were analyzed using the Tukey's HSD post hoc test. All results are presented as means ± SEM except subject characteristics which are reported as mean±SD.

Acknowledgements:

We would like to acknowledge Nicole MacFarlane and Dr. Doug Boreham for their technical assistance with the flow cytometry and FACS analysis and Todd Prior for his technical assistance.

The Pax7 hybridoma cells developed by Dr. A. Kawakami, the A4.951 hybridoma cells and 5.1H11 hybridoma cells developed by Dr. H. Blau were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242 USA

Author Contributions:

BRM and GP contributed to the conception of this project. BRM, DIO, MAT and GP contributed to the experimental design, subject recruitment, exercise testing and tissue collection. BRM was responsible for immunohistochemical, immunofluorescent, RNA and primary myoblast experiments. DIO and LB were responsible for the RNA isolation and reverse transcription reactions. DIO was responsible for the protein extraction and western blotting. The manuscript was largely written by BRM and GP however all authors were involved with the writing and final approval of the manuscript.

References

- Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnohr P, Saltin B (1990) Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds. Acta Physiol Scand 140: 41-54.
- 2. Roubenoff R (1999) The pathophysiology of wasting in the elderly. J Nutr 129: 256S-259S.
- 3. Roubenoff R, Hughes VA (2000) Sarcopenia: current concepts. J Gerontol A Biol Sci Med Sci 55: M716-M724.
- Melton LJ, III, Khosla S, Crowson CS, O'Connor MK, O'Fallon WM, Riggs BL (2000) Epidemiology of sarcopenia. J Am Geriatr Soc 48: 625-630.
- 5. MAURO A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9: 493-495.
- Adams GR, Caiozzo VJ, Haddad F, Baldwin KM (2002) Cellular and molecular responses to increased skeletal muscle loading after irradiation. Am J Physiol Cell Physiol 283: C1182-C1195.
- Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z (2006) Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294: 50-66.
- Conboy IM, Conboy MJ, Smythe GM, Rando TA (2003) Notch-mediated restoration of regenerative potential to aged muscle. Science 302: 1575-1577.
- Jejurikar SS, Henkelman EA, Cederna PS, Marcelo CL, Urbanchek MG, Kuzon WM, Jr. (2006) Aging increases the susceptibility of skeletal muscle derived satellite cells to apoptosis. Exp Gerontol 41: 828-836.
- Day K, Shefer G, Shearer A, Yablonka-Reuveni Z (2010) The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev Biol 340: 330-343.
- Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433: 760-764.
- Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317: 807-810.
- 13. Gopinath SD, Rando TA (2008) Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell 7: 590-598.

- Sajko S, Kubinova L, Cvetko E, Kreft M, Wernig A, Erzen I (2004) Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. J Histochem Cytochem 52: 179-185.
- 15. Kadi F, Charifi N, Denis C, Lexell J (2004) Satellite cells and myonuclei in young and elderly women and men. Muscle Nerve 29: 120-127.
- Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, van Loon LJ (2007) Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. Am J Physiol Endocrinol Metab 292: E151-E157.
- Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA (2006) Satellite cell numbers in young and older men 24 hours after eccentric exercise. Muscle Nerve 33: 242-253.
- Roth SM, Martel GF, Ivey FM, Lemmer JT, Metter EJ, Hurley BF, Rogers MA (2000) Skeletal muscle satellite cell populations in healthy young and older men and women. Anat Rec 260: 351-358.
- 19. Verdijk LB, Gleeson BG, Jonkers RA, Meijer K, Savelberg HH, Dendale P, van Loon LJ (2009) Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type-specific increase in satellite cell content in elderly men. J Gerontol A Biol Sci Med Sci 64: 332-339.
- 20. Morissette MR, Stricker JC, Rosenberg MA, Buranasombati C, Levitan EB, Mittleman MA, Rosenzweig A (2009) Effects of myostatin deletion in aging mice. Aging Cell 8: 573-583.
- McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387: 83-90.
- 22. Lee SJ, McPherron AC (2001) Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A 98: 9306-9311.
- 23. Lee SJ (2004) Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol 20: 61-86.
- 24. McPherron AC, Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94: 12457-12461.
- 25. Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S (1998) Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci U S A 95: 14938-14943.
- 26. Yarasheski KĚ, Bhasin S, Sinha-Hikim I, Pak-Loduca J, Gonzalez-Cadavid NF (2002) Serum myostatin-immunoreactive protein is increased in 60-92 year old women and men with muscle wasting. J Nutr Health Aging 6: 343-348.
- 27. Murphy KT, Koopman R, Naim T, Leger B, Trieu J, Ibebunjo C, Lynch GS (2010) Antibody-directed myostatin inhibition in 21-mo-old mice

reveals novel roles for myostatin signaling in skeletal muscle structure and function. FASEB J

- Siriett V, Platt L, Salerno MS, Ling N, Kambadur R, Sharma M (2006) Prolonged absence of myostatin reduces sarcopenia. J Cell Physiol 209: 866-873.
- 29. Raue U, Slivka D, Jemiolo B, Hollon C, Trappe S (2006) Myogenic gene expression at rest and after a bout of resistance exercise in young (18-30 yr) and old (80-89 yr) women. J Appl Physiol 101: 53-59.
- Leger B, Derave W, De BK, Hespel P, Russell AP (2008) Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res 11: 163-175B.
- McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (2003) Myostatin negatively regulates satellite cell activation and selfrenewal. J Cell Biol 162: 1135-1147.
- 32. Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R (2002) Myostatin inhibits myoblast differentiation by downregulating MyoD expression. J Biol Chem 277: 49831-49840.
- McKay BR, Toth KG, Tarnopolsky MA, Parise G (2010) Satellite cell number and cell cycle kinetics in response to acute myotrauma in humans: immunohistochemistry versus flow cytometry. J Physiol 588: 3307-3320.
- 34. Lexell J, Henriksson-Larsen K, Winblad B, Sjostrom M (1983) Distribution of different fiber types in human skeletal muscles: effects of aging studied in whole muscle cross sections. Muscle Nerve 6: 588-595.
- Lindstrom M, Thornell LE (2009) New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. Histochem Cell Biol 132: 141-157.
- Huang Z, Chen X, Chen D (2011) Myostatin: A novel insight into its role in metabolism, signal pathways, and expression regulation. Cell Signal 23: 1441-1446.
- Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K, Gonzalez-Cadavid NF (2002) Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in C2C12 skeletal muscle cells. J Cell Physiol 190: 170-179.
- Mendler L, Zador E, Ver HM, Dux L, Wuytack F (2000) Myostatin levels in regenerating rat muscles and in myogenic cell cultures. J Muscle Res Cell Motil 21: 551-563.
- Testelmans D, Crul T, Maes K, Agten A, Crombach M, Decramer M, Gayan-Ramirez G (2010) Atrophy and hypertrophy signalling in the diaphragm of patients with COPD. Eur Respir J 35: 549-556.
- 40. Kovacheva EL, Hikim AP, Shen R, Sinha I, Sinha-Hikim I (2010) Testosterone supplementation reverses sarcopenia in aging

through regulation of myostatin, c-Jun NH2-terminal kinase, Notch, and Akt signaling pathways. Endocrinology 151: 628-638.

- 41. Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ (2002) Induction of cachexia in mice by systemically administered myostatin. Science 296: 1486-1488.
- 42. Cornelison DD, Olwin BB, Rudnicki MA, Wold BJ (2000) MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Dev Biol 224: 122-137.
- 43. Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R (2000) Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 275: 40235-40243.
- 44. McFarlane C, Gu ZH, Wong ZW, Lau HY, Lokireddy S, Ge X, Mouly V, Butler-Browne G, Gluckman PD, Sharma M, Kambadur R (2011) Human myostatin negatively regulates human myoblast growth and differentiation. Am J Physiol Cell Physiol
- 45. Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10: 1173-1183.
- 46. Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17): 2895-2901.
- Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y (1998) Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J Cell Sci 111 (Pt 6): 769-779.
- 48. Hennessey JV, Chromiak JA, Della VS, Guertin J, MacLean DB (1997) Increase in percutaneous muscle biopsy yield with a suctionenhancement technique. J Appl Physiol 82: 1739-1742.
- 49. Bergstrom J (1975) Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. Scand J Clin Lab Invest 35: 609-616.
- 50. Alexander CM, Puchalski J, Klos KS, Badders N, Ailles L, Kim CF, Dirks P, Smalley MJ (2009) Separating stem cells by flow cytometry: reducing variability for solid tissues. Cell Stem Cell 5: 579-583.
- 51. Parise G, McKinnell IW, Rudnicki MA (2008) Muscle satellite cell and atypical myogenic progenitor response following exercise. Muscle Nerve 37: 611-619.
- 52. McKay BR, De LM, Johnston AP, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G (2009) Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. PLoS One 4: e6027.

- 53. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
- 54. Tee JM, van RC, Boonen R, Zivkovic D (2009) Regulation of slow and fast muscle myofibrillogenesis by Wnt/beta-catenin and myostatin signaling. PLoS One 4: e5880.
- 55. Artaza JN, Reisz-Porszasz S, Dow JS, Kloner RA, Tsao J, Bhasin S, Gonzalez-Cadavid NF (2007) Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. J Endocrinol 194: 63-76.

Figure Legends:

Figure 1:

Muscle stem cell response to acute resistance exercise: 1a) Analysis of SC number (Pax7⁺ cells·mg⁻¹) via flow cytometry at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect. p<0.05 vs. young, **time effect, p<0.05 vs. PRE, bar denotes effect of time for both young and old. **1b)** Representative immunofluorescent image of a muscle cross-section stained with myosin heavy chain slow isoform (MCH1, green), Pax7 (red), Laminin (orange) and Nuclei (DAPI, blue). Boxed area is magnified below, illustrating a Pax7+ myonuclei (SC) localized to a type-I fiber. i) DAPI/Laminin overlay showing the location of myonuclei beneath the basal lamina; ii) Pax7/Laminin overlay showing the location of the SC below the basal lamina; iii) Pax7/MHC1 overlay showing the localization of the SC to the type-I fiber; iv) MHC1/Laminin overlay showing the space where the SC lies beneath the basal lamina on the type-I fiber. 1c) Quantification of the type-I fiber associated SCs at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. **time effect, p<0.05 vs. PRE, bar denotes effect of time for both young and old. 1d) Quantification of the type-II fiber associated SCs at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE, bar denotes effect of time for both young and old. All graphs are mean±SEM.

Figure 2:

Cell-cycle analysis and muscle gene expression following acute resistance exercise: Analysis of the number of SCs ($Pax7^+$ cells·mg⁻¹) in G₀/G₁ phase (2a),

S-phase (2b), and G₂/M phase (2c) of the cell-cycle analyzed via flow cytometry at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE, bar denotes effect of time for both young and old. Whole muscle mRNA expression of the myogenic regulatory factor *Myf5* (2d), and the cell-cycle genes *cyclin B1* (2e) and *cyclin B2* (2f) at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE, bar denotes effect of time for both young and old. All graphs are mean±SEM.

Figure3:

Characterization of the MyoD1 response of the muscle stem cells: **3a**) Representative immunofluorescent image of a muscle cross-section stained for Pax7 (green), MyoD1 (red), Laminin (orange) and Nuclei (DAPI, blue). Boxed area is magnified below (i-iv) and broken down into DAPI/Laminin (i), Pax7 (ii), MyoD1 (iii) and merged (iv), illustrating a Pax7⁺/MyoD1⁻ (quiescent) SC (closed arrowhead) and a Pax7⁺/MyoD1⁺ (active) SC (open arrowhead). The quantification of these cell populations are broken down into (**3b**) the number of total MyoD1⁺ cells, (**3c**) the number of MyoD1+/Pax7+ cells (active SCs), (**3d**) the number of MyoD+/Pax7- cells (SCs committed to differentiation), and (**3e**) the number of Pax7+MyoD1- cells (quiescent SCs). These are reported as the number of cells per 100 myofibers at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE; ***time effect, p<0.05 vs. PRE, 3h and 24h, bar denotes effect of time for both young and old. †time effect, p<0.05 vs. PRE (for old at 48h). All graphs are mean±SEM.

Figure 4:

Whole muscle myostatin protein and gene expression: **4a**) Representative western blot of one old and one young subject detecting the active 26kDa immunoreactive peptide for each time-point normalized to ponceau. The graphical representation of the western blot data is reported at baseline (PRE), 24h and 48h post-exercise. Open bars = young men, closed bars = older men. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE. **4b**) Absolute mRNA expression of *MSTN*, and **(4c)** mRNA expression of *MSTN* relative to

young at baseline (PRE), 24h and 48h post-exercise. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE, ***time effect, p<0.05 vs. PRE and 3h, bar denotes effect of time for both young and old. All graphs are mean±SEM.

Figure 5:

Immunofluorescent detection and localization of myostatin to human muscle stem cells in vivo and in vitro: **5a)** Multi-panel representation of a muscle cross-section stained for Pax7 (red), myosin heavy chain slow isoform (MHC1, green), MSTN (orange), Laminin (green) and Nuclei (DAPI, blue). This image depicts two Pax7+ cells (SCs), one positive for MSTN and located on a type-I fiber (boxed) and one negative for MSTN (also located on a type-I fiber). The boxed area is further depicted at a higher magnification showing the localization of all 5 markers (merge), as well as various 2 or 3 marker overlays which clearly illustrate the localization of MSTN with Pax7. **5b**) *in vitro* localization, **(ii)** 40x magnification which is further separated into images of **(iii)** DAPI, **(iv)** MSTN and **(v)** Pax7. Further magnification (100x) of two human primary myoblasts confirms the localization of MSTN to human SCs **(vi-ix)**. For secondary only controls for the *in vivo* immunostaining see Fig. S6 and for the *in vitro* immunostaining see Fig. S7.

Figure 6:

Fiber-type specific co-localization of MSTN protein with the muscle stem cells: The co-localization of MSTN with all Pax7+ cells (as a percentage of total SC number) is shown in **(6a)**. The fiber-type specific response of MSTN (colocalization of MSTN as a percentage of either type-I or type-II associated SCs) is shown for type-I associated SCs (6b) and type-II associated SCs (6c) at baseline (PRE), 24h and 48h post-exercise. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE, ***time effect, p<0.05 vs. PRE and 3h, ****time effect, p<0.05 vs. PRE, 3h and 24h, bar denotes effect of time for both young and old. All graphs are mean±SEM. NOTE: strong trend for an effect of age in type-II associated MSTN⁺ SCs at 48h (p=0.07).

Supplemental Figure Legends:

Figure S1:

Localization of ActRIIb with human muscle stem cells in vivo and in vitro: **S1a**) Immunohistochemical detection of ActRIIb (brown) with Pax7 (black) in a muscle cross-section (red box). Boxed are is further magnified to illustrate localization of ActRIIb (brown) around the Pax7+ (black) cell. Pax7+ cell was confirmed to be co-localized with a myonuclei via propidium iodide staining visualized with fluorescence microscopy (not shown). Note the localization of ActRIIb on the plasma membrane of the myofibers. **S1b**) *In vitro* confirmation of the localization of ActRIIb with a human primary myoblast (Pax7⁺).

Figure S2:

S2a) Representative cell-cycle histograms of young (YM) and elderly (OM) Pax7+ cells isolated from human muscle collected at PRE and 48h post exercise. Both YM and OM display an increase in G_2/M , however YM display a lager shift into S-phase at 48h. **S2b)** Confirmation of the specificity of our Pax7 antibody (DSHB, red) for the detection of SCs in mononuclear cell isolated from muscle biopsies. Of the 7 cells (see DAPI) in this image, 3 are positive for Pax7.

Figure S3:

Secondary only control for MyoD1, illustrating a Pax7⁺ cell (green) with no colocalization of the MyoD1 red secondary antibody (boxed area). **i)** high magnification of the boxed area showing DAPI (blue)/Laminin (orange), **ii)** showing Pax7 (green), **iii)** showing absence of MyoD1 secondary Ab binding, **iv)** showing the merged image. Secondary uses is a biotinylated GAM visualized with streptavidin 594

Figure S4:

Co-localization of MyoD1 (red) and Pax7 (green). High magnification of the boxed area demonstrates a Pax7⁺/MyoD1⁺ (active) SC, located beneath the basal lamina (laminin, orange).

Figure S5:

Three images of MyoD1⁺ cells (red co-localized with DAPI) which are centrally located within the myofiber (MyoD⁺ centrally located myonuclei), indicating a newly fused SC (fully differentiated and fused) with residual MyoD1 protein in the

nucleus. These were only occasionally seen in the elderly at PRE. Top panel is from a 76 year old, bottom left is from a 72 year old and bottom right is from a 74 year old. No centrally located myonuclei were found in the young.

Figure S6:

Secondary only control for MSTN, illustrating a $Pax7^+$ cell (red) with no colocalization of the MSNT orange secondary antibody (boxed area). **i)** high magnification of the boxed area showing Pax7 (red) and DAPI, **ii)** showing the localization of Pax7 to a type-II fiber (green) beneath the basal lamina (green), **iii)** showing the absence of the MSTN secondary Ab (Cy5 – Alexafluor 647) binding with Pax7, **iv)** showing the merged image.

Figure S7:

Secondary only control of the GAR Alexafluor 488 used for the detection of ActRIIb and MSTN in human primary myoblasts *in vitro*. Pax7 is shown in red, localized to the nuclei (DAPI) of the primary cells. Note very little green (488) background of the secondary only.

Figure S8:

Whole muscle gene analysis of the myogenic regulatory factor *MRF4*, cell-cycle genes *cyclin D1*, *CDK1*, *HDAC4*, and MSTN associated genes *FSTL1* and *Follistatin* at baseline (PRE), 24h and 48h post-exercise. *Age effect, p<0.05 vs. young, **time effect, p<0.05 vs. PRE. All genes were normalized to *GAPDH* using the $2^{-\Delta Ct}$ method. All graphs are mean±SEM.

A representative image of myostatin in nuclear and cytoplasmic fractions of skeletal muscle protein. Six lanes are shown alternating nuclear (N) and cytoplasmic (C) fractions with increasing concentrations of protein loaded from 10µg to 20µg, illustrating the presence of myostatin in the nuclear fraction.

Table 1:

Subject Characteristics:

Variable	Young	Old	% of Young
Age (y)	21±3	70±4*	n/a
Height (cm)	179.6±8.2	176.6±4.9	n/a
Weight (kg)	91.7±21.9	87.6±11.5	96%
Lean Body Mass (kg)	65.9±10.8	62.9±6.4	95%
% Body Fat	26.7±8.6	27.9±5.3	n/a
Fat Mass (kg)	25.7±14.1	24.8±7.1	96%
1-RM Leg Press (lbs)	228.0±93.8	160.8±50.1*	70%
1-RM Knee Extension (lbs)	117.6±47.4	81.9±22.8*	70%
MVC (N•m)	324.6±85.4	228.3±45.1*	70%

*P<0.05 vs. Young; mean±SD

Table 2 :

Myonuclear Domain and Satellite Cell Content:

	Muscle CSA (μm²)	
Fiber Type	Young	Old
Туре І	6566.39 ± 367.85	6781.24 ± 438.16
Type II	8389.87 ± 709.59†	6635.15 ± 596.29*
	Myonuclear Domain (μm²)	
Fiber Type	Young	Old
Туре І	1918.24 ± 111.93	1981.39 ± 94.96
Type II	2440.49 ± 192.29†	1966.74 ± 169.11*
	Type I SC/mm ²	
Time	Young	Old
PRE	8.79 ± 0.94	8.79 ± 1.92
24h	12.88 ± 1.90	10.13 ± 1.80
48h	12.69 ± 2.10**	12.92 ± 2.08**
	Type II SC/mm ²	
Time	Young	Old
PRE	10.46 ± 2.01	7.30 ± 1.27*
24h	14.30 ± 14.30	11.07 ± 1.89
48h	16.93 ± 1.97**	8.31 ± 1.29*

*P<0.05 vs. Young; **P<0.05 vs. PRE; †P<0.05 vs. Type I Fibers; mean±SEM

Table S1:

qRT-PCR Primer Sequences:

Gene	Forward Sequence Reverse Sequence		NCBI
Name			Gene
			Number
Myostatin	5'-TGGTCATGATCTTGCTGTAAC-3'	5'- TGTCTGTTACCTTGACCTCTA-3'	2660
Follistatin	5'-AGTCCAGTACCAAGGCAGATG-3'	5'-GGTCACACAGTAGGCATTATT-3'	10468
FSTL1	5'-AGAGGAGGAGATGACCAGATA-3'	5'-CGCTGAAGTGGAGAAGATGC-3'	11167
Cyclin B1	5'-AAGAGCTTTAAACTTTGGTCT-3'	5'-CTTTGTAAGTCCTTGATTTACC-3'	891
Cyclin B2	5'-AAAGTTGGCTCCAAAGGGTCC-3'	5'-GAAACTGGCTGAACCTGTAAA-3'	9133
Cyclin D1	5'-CACGATTTCATTGAACACTTCC-3'	5'-TGAACTTCACATCTGTGGCAC-3'	595
CDK1	5'-CACATGAGGTAGTAACACTCT-3'	5'-ATGGTGCCTATACTCCA-3'	983
HDAC4	5'-ACGCCTCTGTTCAACTTG-3'	5'-AACTCCCACCAACACATAC-3'	9759
Myf5	5'-ATGGACGTGATGGATGGCTG-3'	5'-GCGGCACAAACTCGTCCCCAA-3'	4617
MRF4	5'-CCCCTTCAGCTACAGACCCAA-3'	5'-CCCCCTGGAATGATCGGAAAC-3'	4618
GAPDH	5'-CCTCCTGCACCACCAACTGCTT-	5'-GAGGGGCCATCCACAGTCTTCT-	2597
	3'	3'	

FSTL1, Follistatin like-1; CDK1, cyclin dependant kinase-1; HDAC4, histone deacetylase-4; Myf5, myogenic factor 5; MRF4, muscle-specific regulatory factor 4 (Myf6); GAPDH, glyceraldehyde 3-phosphate dehydrogenase



































Chapter 5

Integrative Discussion

5.1 INTRODUCTION:

The work outlined in this thesis provides support for the proposed divergent effects of the IGF-1 splice variants on satellite cell function. IGF-1 appears to be preferentially spliced as IGF-1Ec during the proliferative phase of the myogenic program while IGF-1Ea and Eb appear as the predominant splice variants during the initiation of differentiation based on the expression of the MRFs. Furthermore, the localization of IGF-1 with Pax7 in muscle-cross sections in the post-exercise time-course lends support to the importance of IGF-1 in the myogenic response to myotrauma. This thesis also provides novel evidence to support the role of IL-6 in the regulation of satellite cell proliferation in response to acute muscle damage in humans. These data confirm the findings of Serrano et al. [1] that demonstrated IL-6 imparts its action on the satellite cell via the JAK2/STAT3 pathway. As outlined in chapter 4, this thesis demonstrates, for the first time, that myostatin is altered by acute exercise in both young and older adults and this effect is most notable in the satellite cell compartment. In addition, these data implicate myostatin as a contributing factor to age-related satellite cell dysfunction in response to exercise (or myotrauma).

5.2 IGF-1 AND FUTURE WORK:

In 2009 Papasani et al. [2] published a journal club entry in the Journal of *Physiology* critically analyzing the findings of chapter 2 in this thesis. Although their main conclusions were positive and suggested that the work furthered the body of literature with regards to the *in vivo* functions of IGF-1, they criticized the temporal gene expression profiles of the MRFs [2]. Their main criticism was that the expression of myogenin mRNA, which was upregulated 4h post-exercise, preceded that of Myf5 and MyoD, making the results difficult to interpret. Although the increase in myogenin mRNA was early and a potential point of concern, data from Kadi et al. (2004) illustrate an increase in myogenin positive myonuclei following a bout of exercise with no increases in myogenin positive satellite cells [3]. Their data demonstrate that the myofiber upregulates myogenin expression after acute exercise, which may have attributed to the increased myogenin mRNA observed in our study. In agreement with the expected temporal profile of myogenin, the mRNA response in our study peaked at 72h, corresponding with the peak in MRF4 mRNA. Therefore, keeping in mind the potential contribution from myofiber derived myogenin transcripts observed at 4h and 24h, we used the peak of the myogenin mRNA response at 72h to determine the onset of myogenic differentiation. Accordingly, this coincided with an increase in MRF4 mRNA and decrease in Myf5 mRNA at 72h. Although this was described in the discussion, the authors of the journal club article fail to mention this in their review. Interestingly, the temporal expression of the IGF-1 splice variants observed in chapter 2 were recently confirmed at the protein level in

human muscle in response to exercise-induced myotrauma [4]. Philippou et al. (2009) not only replicated our IGF-1 splice variant mRNA findings exactly, illustrating MGF (IGF-1Ec) upregulation preceded that of IGF-1Ea and Eb, they also confirmed the temporal preference of MGF using protein analysis [4]. Using both immunohistochemical detection methods and western blotting, they demonstrated MGF protein (using an antibody raised against the last 24 amino acids of the predicted MGF sequence of the E domain [5]) peaked 48h after damaging exercise while IGF-1Ea protein peaked 120h after exercise [4]. Unfortunately, they did not conduct any satellite cell specific analysis in this study; thus, whether these proteins differentially co-localize with the satellite cells in the post exercise time-course remains to be elucidated. The authors went on to corroborate the proposed differential action of MGF and IGF-1 in C_2C_{12} cultures, confirming that the distinct E domain of MGF blocks myoblast differentiation and promotes proliferation [4,6,7]. MGF appears to act via the Raf-MEK-ERK 1/2 pathway, activating ERK 1/2, inducing cyclin D1 expression [6] and increasing proliferation [4]. Furthermore, IGF-1Ea appears to exert its effect via the PI3 kinase/Akt pathway, increasing p21 and MyoD thus inhibiting proliferation and inducing differentiation [4,6,7]. Supporting the role of IGF-1 in inducing myogenic differentiation, muscle from IGF-1 knock-out mice demonstrated a significant reduction in MRF4 and myogenin mRNA compared to wild-type littermates, with no observed differences in MyoD or Myf5 [8]. The proposed actions of MGF and IGF-1 on satellite cells are shown in Figure 1. In light of

recent data that has confirmed the temporal elevations of the IGF-1 splice variants observed in this thesis and demonstrated a potential mechanism for the specific proliferative actions of MGF [4], more research is warranted to confirm that this mechanism is responsible for the proliferative phase of the myogenic program *in vivo*.





Figure 1: The controversial E-peptide of IGF-1Ec mRNA has been suggested to signal through and alternate unknown receptor, initiating the MEK-ERK1/2 signaling cascade, which upregulates the cell-cycle gene cyclin D1, initiating proliferation. The IGF-1Ea splice variant is thought to signal via canonical IGF-1 signaling, binding to the IGF-1 receptor and initiating the PI3 kinase/Akt pathway inducing the upregulation of p21 and MyoD thus withdrawing the satellite cell from the cell-cycle and inducing myogenic differentiation.
Although the use of antibodies raised to detect the sequence corresponding to the last 24 amino acids in the E domain of the predicted MGF peptide (based on the mRNA sequence – so called MGF-E protein), to date the existence of a mature functional peptide in vivo has never been confirmed and remains a topic of intense debate [9]. Interestingly, all IGF-1 mRNA splice variants contain exons 3 and 4 which encode the 70 amino acid mature IGF-1 peptide [10]. The mature IGF-1 peptide consists of the A, B, C and D domains responsible for its protein-protein and receptor interactions [11]. IGF-1Ea contains exons 3 and 4 spliced to exon 6 (removal of exon 5), while IGF-Ec (MGF) contains exons 3 and 4 spliced to exon 5 and 6. IGF-1Eb contains exons 3 and 4 spliced with exon 5 only and is only found in humans [12,13]. These alternately spliced genes encode different c-terminal E-domain extensions and are suggested to produce the mature IGF-1 peptide plus different E-peptides [9]. Thus, a confounding factor to the individual and divergent actions of the splice variants is the fact that they all encode the full-length IGF-1 protein in addition to their specific E-peptide at a 1:1 ratio. This makes the potential biological action of these E-peptides in competition with canonical IGF-1 signaling. In addition, IGF-1 alone has been demonstrated to induce both proliferation (via ERK) and differentiation (via PI3 kinase) in myoblasts, further confounding the specific actions of IGF-1 in satellite cell function [14]. Figure 2 is a schematic representation of the alternate gene splicing of IGF-1 and the potential peptides produces as a consequence of the splice variants in humans. Investigating what

causes the same molecule to preferentially stimulate one molecular pathway over another and what induces the switch from ERK to PI3 kinase is necessary to delineate these confounding results. The role of IGF1 binding proteins may provide some insight into this conundrum [15]; however, more work to determine how these proteins affect satellite cell function is also warranted. Perhaps the Epeptides themselves serve to augment the IGF-1 response simply by causing IGF-1 to signal in a preferential manner (i.e. MGF E-peptide directing IGF-1 signaling to favour ERK1/2). This theory has never been tested, yet it may help explain why the differential splicing of IGF-1 encodes both the mature IGF-1 and unique smaller E-peptides.





Alternate Gene Splicing of IGF-1

Figure 2: Alternate gene splicing in response to exercise favours IGF-1Ec (MGF) during the proliferative stages of the satellite cell response, while the transition to differentiation favours IGF-1Ea and IGF-1Eb. It remains unknown how the E-peptides function, however they may serve to influence IGF-1/IGF-1R signaling to favour proliferation (ERK) or differentiation (PI3K/Akt) or act via an undescribed receptor to mediate these divergent actions.

There are very few studies investigating the IGF-1 splice variants in the context of human aging. Following acute exercise, MGF mRNA has been shown to increase significantly in young males, however MGF mRNA failed to respond to exercise in elderly men [16]. Although some data exist, suggesting a differential expression of the IGF-1 splice variants in the elderly compared to young controls [16], other studies fail to illustrate an age-related decline in MGF or IGF-1 expression in response to exercise [17]. It is of interest to investigate this potential deficiency in IGF-1/MGF gene expression as a consequence of aging to determine if there is a link between an impaired myogenic response (see chapter 4) and IGF-1 splice variant expression in the elderly. Further research investigating the localization of IGF-1 and MGF-E with Pax7⁺ cells in various stages of the cell-cycle (using P.I. and flow cytometry) would help provide invaluable information about the contribution of IGF-1 to proliferation and differentiation. Furthermore, classifying the co-localization of IGF-1 and the MGF-E peptide with MyoD1 and Pax7 would provide necessary evidence to support the differential role of MGF and IGF-1 with the myogenic program.

5.3 INTERLEUKIN-6: THE PLEIOTROPIC REGULATOR:

In chapter 3, the effect of IL-6 on the satellite cell response to myotrauma was speculated as being regulated by STAT3. Although we demonstrated pSTAT3 was co-localized with Pax7 early in the post-exercise time-course, the response was not quantified. In a follow-up study by Toth et al. [18], the satellite cell-specific pSTAT3 response was quantified at 1h, 3h and 24h after 300

maximal eccentrically-biased muscle contractions. pSTAT3⁺ satellite cells increased from ~20% to ~60% at 24h post-exercise [18], illustrating that the peak in $pSTAT3^+$ satellite cells coincided with the peak in IL-6⁺ satellite cells. This study further confirmed IL-6 was signaling via the JAK2/STAT3 axis in satellite cells by the quantification of cMyc, which is instrumental to the initiation of the cell-cycle and is a downstream target of IL-6/STAT3 [18]. cMyc⁺ satellite cells increased from less than 10% to ~45% 24h after exercise, further confirming the role of IL-6 in mediating satellite cell proliferation in response to exercise-induced myotrauma [18]. Future work regarding IL-6 and the satellite cell response should include whether human aging affects the efficacy of IL-6 to induce proliferation. Age-associated systemic elevations in IL-6 are correlated with a loss in skeletal muscle mass and strength [19,20]. Preliminary data from our lab suggests that in response to acute exercise, elderly men have a larger increase in IL-6 at the mRNA level in skeletal muscle and in circulation (see appendix II). Plasma IL-6 concentrations were approximately 75% higher following exercise (appendix 2, p<0.05). Furthermore, whole muscle SOCS3 mRNA was twice as high in the elderly compared to the young 24h and 48h post-exercise (p<0.05). In addition SOCS1 mRNA was higher in elderly men 48h after exercise while cMyc mRNA was significantly lower 3h post-exercise (appendix2, p<0.05). These preliminary data suggest that although an augmented IL-6 response is observed in elderly muscle, the downstream target gene cMyc (which contributes to cellcycle progression) is significantly lower compared to young. Furthermore both

SOCS3 and SOCS1 are elevated in the elderly [21] which may contribute to the blunted myogenic response demonstrated in chapter 4 of this thesis. Clearly future work is needed to investigate this age-related difference in the IL-6-mediated satellite cell response to exercise.

Although IL-6 appears to exert a positive myogenic effect on the muscle stem cell compartment, elevations in systemic IL-6 are often described as catabolic in nature [22]. How IL-6 can induce two completely divergent responses in the same tissue is of great interest, requiring further research, especially in the context of aging where elevations in systemic IL-6 may to be a normal consequence of aging [23,24].

The role of IL-6 in skeletal muscle metabolism is perhaps best illustrated in the context of exercise, where skeletal muscle has been shown to produce and release IL-6 into circulation [25,26]. During prolonged exercise, skeletal muscle IL-6 mRNA expression can increase up to 100-fold and protein levels determined by immunohistochemical staining can be elevated by ~130% compared to rest [27]. During prolonged exercise, circulating IL-6 levels (measured in venous blood) can increase 2-10-fold and arteriovenous balance studies indicate that skeletal muscle release of IL-6 can account for the vast majority of this increase [26]. In fact, muscle-derived IL-6 is hypothesized to act in a hormone-like fashion to affect tissues such as adipose, liver, and the brain. However, this increase in circulating IL-6 may be the result of a spillover from locally produced IL-6, which acts primarily to regulate metabolism in the exercising muscles themselves.

Muscle interstitial IL-6 concentration is 5-100-fold higher than circulating peripheral IL-6 concentration during exercise [28,29]. This suggests that IL-6 may be compartmentalized between the local muscle environment and the circulation. An area of high local IL-6 concentration may have profoundly different actions on exercising skeletal muscle than lower serum IL-6 concentrations at distant tissues. Locally produced IL-6 may act in an autocrine or paracrine fashion to influence muscle metabolic processes at the site of production, acting primarily through AMPK activation [30,31]. Measurable spillover into circulation does occur during prolonged exercise [25], perhaps having some effects on other organs/tissues. However, the primary function of muscle-derived IL-6 during exercise may be in regulating skeletal muscle metabolism.

This local effect of IL-6 on skeletal muscle may help explain the muscle catabolic effects of chronic IL-6 infusion in rodents [22]. Constant local IL-6 infusion could be interpreted as a signal of chronic energy deficit (i.e., similar to prolonged exercise), activating AMPK and shutting down muscle protein synthesis (MPS) via inhibition of mTOR and downstream targets. This idea is supported by the robust reduction in the phosphorylation status of p70-S6 kinase (S6K1), a key regulator of MPS and a downstream target of mTOR, following IL-6 infusion [22]. This hypothesis could explain the reduction in myofibrillar protein seen following interstitial IL-6 infusion in these studies [22].

Compartmentalization of IL-6 is also supported during muscle damage, repair and growth involving muscle stem cells (see chapter 3). Following contraction induced myotrauma, immunohistochemical analysis of IL-6 shows high levels of IL-6 concentrated to the muscle stem cell compartment compared to the muscle fiber [1,32]. In fact, the punctate localization of IL-6 within the satellite cells may be attributed to the observation that pSTAT3 was evident only in the satellite cells with no pSTAT3 evident in the myofiber in the post-damage time-course [18,32]. Thus the increase in IL-6 in the satellite cell compartment may have lead to the increase in proliferation, while the less robust increase in fiber IL-6 may have lead to the activation of alternate signaling pathways, such as AMPK [33], however this was not tested in this thesis.

Compartmentalization of IL-6 also appears to occur between the blood and cerebral spinal fluid (CSF). CSF concentrations are approximately 2-fold higher than plasma concentrations at rest [34]. Moreover, increases in IL-6 measured in plasma in response to exercise are not accompanied by parallel changes in CSF, indicating compartmentalized regulation [34]. Compartmentalization of IL-6 may also play a fundamental role in the progression of inflammatory bowel disease (IBD) into colon cancer [35–37]. Localization of IL-6 to the intestinal mucosa in IBD and inflammatory colon cancer is derived from T-cells and myeloid cells in close proximity to the tumor, creating an area of high IL-6 concentration around the tumor. This local elevation has profound effects on cancer progression and tumor formation in that local microenvironment. Therefore, the local

concentration of IL-6 in the vicinity of the satellite cell niche may be of more relevance then the systemic concentration of IL-6 on the satellite cell response. However, the systemic elevation of IL-6 may promote increase SOCS3 within the fiber, decreasing the exercise induced production of IL-6, which may negative implications on the satellite cells. Thus the compartmentalization of IL-6 in the context of human aging must be considered when investigating the role of IL-6 in regulating the satellite cell response in young and elderly men.

5.4 MYOSTATIN, AGING AND THE PERSERVATION OF MUSCLE MASS:

The data provided in chapter 4 of this thesis and by recent cell culture work conducted with human primary cells clearly demonstrate that myostatin is a key regulator of human satellite cell function [38]. Elevated myostatin in the elderly appears to prevent the induction of the myogenic program and subsequent proliferation and differentiation of satellite cells in elderly muscle. Thus these data suggest myostatin may in fact be a good candidate for pharmacological intervention for the treatment of sarcopenia. It remains unknown if exercise training can attenuate or abolish the age-related increase in myostatin at either the whole muscle level or in the satellite cell. However, if resistance exercise training in the elderly can restore satellite cell function via reductions in satellite cell-associated myostatin, then that would provide evidence for the use of a myostatin neutralizing antibody to restore normal satellite cell function. Currently great progress has been made on the development of a neutralizing antibody for the treatment of human Duchenne muscular dystrophy.

The use of this antibody is currently being used in human clinical trials in children with muscular dystrophy (Canadian Phase II clinical trials for ACE-031 http://www.clinicaltrials.gov/ct2/show/NCT01099761?term=ACE-031&rank=3). Therefore it is critically important to provide sufficient data to support myostatin as a therapeutic target as clinical trials underway for the efficacy of ACE-031 could also be directed for trials in older individuals with sarcopenia. Although the life-long lack of myostatin (Mstn^{-/-}) appears to reduce the sarcopenic phenotype in mice [39], these mice still have some degree of age-associated muscle wasting [40]. Myostatin appears to be a key contributing factor to sarcopenia but is likely not the sole factor influencing the maintenance of muscle mass. In fact, a reduced sensitivity to anabolic signaling proteins such as growth hormone and IGF-1 is associated with increases in myostatin and SOCS3 protein which act to inhibit myogenesis [21]. Therefore, the interactions between inflammatory cytokines and growth factors, in addition to alterations in the satellite cell niche such as increased fibrosis [41] and decreased notch-delta signaling [42] suggest treatment strategies for preventing sarcopenia need to address multiple signaling pathways. At present it appears that exercise is still the most effective single countermeasure for human aging, partially reducing the aging phenotype [43,44]. Perhaps combining exercise with a pharmacological intervention such as myostatin blockade will be sufficient to rapidly restore muscle mass (faster or to a greater degree than resistance exercise alone) and

prevent the progression of sarcopenia that, at present, appears to be an inevitable consequence of aging [40].

5.5 NON-CANONICAL PATHWAYS FOR MYOSTATIN SIGNALING:

Although the majority of studies illustrate myostatin exerts is action on skeletal muscle and satellite cells via ActRIIb/Smad3 signaling [45–47], noncanonical pathways may also be activated by myostatin. Myostatin can activate Erk 1/2 through ActRIIB via Ras, which prevented the differentiation of C_2C_{12} myoblasts [48]. Furthermore, myostatin can induce the activation of p38 MAPK in a Smad-independent manner, mediated by TAK1-MKK6 in proliferating myoblasts, which acts to inhibit cell proliferation [49]. The TAK1-MKK4 pathway can also be activated by myostatin independently of Smad signaling, causing the activation of c-Jun n-terminal kinase (JNK), thus inhibiting the proliferation and differentiation of myoblasts in culture [50].

In chapter 4, myostatin occasionally appeared as punctate granules colocalized with myonuclei in muscle cross-sections. We confirmed the presence of myostatin with nuclear and cytoplasmic fractionation of whole muscle protein homogenates. It appears that myostatin is mainly in the cytosol of skeletal muscle, and appears as concentrated granules in some myonuclei. We also confirmed the nuclear localization of myostatin in primary human myoblast cultures. We noted myostatin in the cytosol and also smaller punctate granule clusters in the nuclei of many but not all primary cells. This is not the first time nuclear myostatin has been observed, the nuclear localization of myostatin was

demonstrated in C_2C_{12} cultures in response to dexamethasone treatment [51]. The authors of that study used both immunocytochemistry and nuclear and cytosolic fractionation to confirm this finding [51].



Figure 3: Known and Hypothesized Cellular Functions of Myostatin:

Figure 3: Known myostatin (MSTN) signaling and the proposed nuclear role of MSTN. Red lines denote inhibitory actions, green lines denote activation or stimulation. Question marks indicate a potential role of nuclear localized myostatin within the satellite cell, inhibiting the transcriptional activity of Myf5 and/or Pax7, inhibiting proliferation or maintaining quiescence.

Furthermore, both cytoplasmic and nuclear myostatin protein levels were elevated in the diaphragm of patients with chronic obstructive pulmonary disease (COPD), possibly contributing to the observed atrophy of the diaphragm [52] Immunohistochemical analysis of regenerating rat muscle demonstrates a diffuse fiber staining in normal muscle and concentrated myostatin co-localized with mononuclear cells in regenerating muscle [53]. The nuclear localization was further confirmed using nuclear and cytoplasmic fractionation of whole muscle, with myostatin detected in both fractions but, but to a greater degree in the nuclear fraction [53]. Although nuclear myostatin has been observed before, the reason for this is unknown. It may be myostatin is acting as a transcriptional repressor, inducing some satellite cells to maintain or return to quiescence by blocking the action of the MRFs or Pax7, however this is speculative and requires further investigation. The actions of known myostatin signaling pathways (canonical and non-canonical) in addition to the hypothesized nuclear function of myostatin are depicted in Figure 3.

5.6 INTEGRATIVE SIGNALING: THE IN VIVO DILEMMA:

One major limiting factor plaguing human physiologists is the issue of isolating single systems for critical analysis. Using a model of exercise to elicit muscle damage in a human, for the study of a single factor such as IL-6, and its contribution to myogenesis is inherently confounded with the redundancy and cross-talk between multiple signaling pathways making it almost impossible to definitively confirm the exact mechanisms that drive the response. The main signaling pathways related to this thesis are the IGF-1 and IGF-1 receptor (IGF-1R) mediated pathway(s), the IL-6/GP130/IL-6 receptor- α (IL-6R α) mediated pathway and the myostatin (MSTN)/ActRIIb mediated pathway. Although the

canonical pathways appear to be isolated from each other with IGF-1 activating PI3 kinase [14], IL-6 activating STAT3 [54] and MSTN activating Smad3 [47], closer examination demonstrates some redundancy and cross-talk between these pathways [55]. IGF-1/IGF-1R binding can phosphorylate STAT3 leading to STAT3 dimerization and nuclear translocation of the pSTAT3 homodimer. pSTAT3 DNA binding will induce the transcription of cyclin D1, cMyc, SOCS1 and SOCS3. Importantly, SOCS3 has been shown to directly inhibit IGF-1R mediated STAT3 phosphorylation and IRS-1 phosphorylation in a negative feedback manner [56]. IL-6/GP130 also induces STAT3 phosphorylation, which causes the upregulation of SOCS1 and SOCS3 [54]. Therefore, either IGF-1 or IL-6 mediated STAT3 phosphorylation will increase SOCS3 protein that will, in turn, inhibit GP130/ IL-6R α and IGF-1R action. GP130/ IL-6R α and IGF-1R activation of STAT3 leads to cell-cycle progression via the upregulation of cyclin D1 and cMyc, while IGF-1R activation of PI3 kinase/Akt induces the upregulation of MyoD and p21, a Cdk inhibitor which inhibits cell-cycle progression, promoting differentiation [56]. MSTN also induces p21 via Smad3 however MSTN also inhibits MyoD thus MSTN appears to promote the guiescence of muscle stem cells and inhibition of differentiation. The possible interaction of these pathways in response to injury may confound the discovery of causative mechanisms behind the actions of specific receptor binding. Figure 4 demonstrates the canonical signaling pathways involved in this thesis and their possible interactions as gathered from the literature.



Figure 4: The Potential Cross-talk Between IL-6, IGF-1 and MSTN in vivo:

Figure 4: Myostatin (MSTN) is secreted by the myofiber and satellite cell, acting on this activin receptor IIb (ActRIIb) which activates Smad3, leading to the induction of p21 and suppression of the cell-cycle. MSTN-Smad3 signaling also prevents the induction of MyoD thus promotes satellite cell quiescence. IGF-1 is produced by the liver, myofiber and satellite cell and acts on the IGF-1 receptor (IGF-1R), initiating PI3 kinase/Akt signaling. Akt induces p21 and MyoD thus favouring satellite cell differentiation. IGF-1-IGF-1R also causes the phosphorylation of STAT3, leading to the transcription of cyclin D1 and cMyc which initiate the cell-cycle, thus increasing the proliferation of satellite cells. IL-6 from the invading inflammatory cells (T-cells, macrophages, neutrophils), myofiber and satellite cell bind the GP130/IL-6R alpha subunits, phosphorylating JAK2 which phosphorylates STAT3, which translocates to the nucleus and activates the transcription of cell-cycle genes. Both IGF-1 and IL-6 induce SOCS3 via STAT3 signaling, which acts in a negative feedback manner to block the phosphorylation of JAK2, STAT3, Akt, and PI3 kinase. In addition, the actions of MSTN-Smad3 signaling inhibit IGF-1 mediated MyoD induction.

Aging also increases the complexity of these signaling pathways. For example, aging is associated with an increase in SOCS3, a decreased ability to phosphorylate Akt and an increase in myostatin [21]. This may decrease the sensitivity of both IL-6 and IGF-1 to initiate signaling and therefore reduce the myogenic response by inhibiting MyoD through increased myostatin and decreased IGF-1 signaling. Due to potential interactions and the redundancy in molecular signaling pathways, providing definitive mechanistic evidence in the human model is essentially impossible. Therefore, probing key factors such as one specific mediator from each step in the signaling cascade (as conducted in chapter 3 and 4) is an effective way to confirm that pathways isolated in animal and cell-culture models in fact apply to humans *in vivo*. In future, the use of flowcytometry and more advanced multiple labeling fluorescent microscopy techniques will provide new insights and greater resolution to the signaling events regulating satellite cell function *in vivo*.

5.7 CONCULSION:

The major findings of this thesis underline the complexity of the in vivo regulation of muscle stem cells as evidenced by the potential cross-talk between these seemingly isolated systems. Further classification of these factors and their interactions is absolutely necessary to: 1) identify the biological significance of the IGF-1 splice variants and how they regulate muscle stem cell proliferation and differentiation; 2) identify whether the increased systemic IL-6 associated with advanced age or other chronic low-grade inflammatory states impairs the

ability of the muscle stem cells to respond to myotrauma; 3) investigate the role of potential therapies such as myostatin blockade at mitigating the muscle stem cell impairment imposed by age-related myostatin signaling. In conclusion, the investigations discussed in this thesis have advanced our basic understanding of the regulation of human satellite cells and provide new avenues for additional

experiments involving the regulation of human muscle stem cells.

5.8 Reference List

- Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P (2008) Interleukin-6 is an essential regulator of satellite cellmediated skeletal muscle hypertrophy. Cell Metab 7: 33-44.
- Papasani MR, Wang G, Cheguru P, Julien DC, Hofwegen DV, Chapalamadugu KC, Brooking A, Read S (2009) Interactions of muscle regulatory factors and isoforms of insulin growth factor-1 in the muscle regenerative process. J Physiol 587: 2427-2428.
- Kadi F, Johansson F, Johansson R, Sjostrom M, Henriksson J (2004) Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle. Histochem Cell Biol 121: 329-334.
- Philippou A, Papageorgiou E, Bogdanis G, Halapas A, Sourla A, Maridaki M, Pissimissis N, Koutsilieris M (2009) Expression of IGF-1 isoforms after exercise-induced muscle damage in humans: characterization of the MGF E peptide actions in vitro. In Vivo 23: 567-575.
- Philippou A, Stavropoulou A, Sourla A, Pissimissis N, Halapas A, Maridaki M, Koutsilieris M (2008) Characterization of a rabbit antihuman mechano growth factor (MGF) polyclonal antibody against the last 24 amino acids of the E domain. In Vivo 22: 27-35.
- Yang SY, Goldspink G (2002) Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett 522: 156-160.

- 7. Philippou A, Halapas A, Maridaki M, Koutsilieris M (2007) Type I insulinlike growth factor receptor signaling in skeletal muscle regeneration and hypertrophy. J Musculoskelet Neuronal Interact 7: 208-218.
- Miyake M, Hayashi S, Sato T, Taketa Y, Watanabe K, Hayashi S, Tanaka S, Ohwada S, Aso H, Yamaguchi T (2007) Myostatin and MyoD family expression in skeletal muscle of IGF-1 knockout mice. Cell Biol Int 31: 1274-1279.
- 9. Matheny RW, Jr., Nindl BC, Adamo ML (2010) Minireview: Mechanogrowth factor: a putative product of IGF-I gene expression involved in tissue repair and regeneration. Endocrinology 151: 865-875.
- Rotwein P, Pollock KM, Didier DK, Krivi GG (1986) Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. J Biol Chem 261: 4828-4832.
- Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE (2005) Molecular interactions of the IGF system. Cytokine Growth Factor Rev 16: 421-439.
- Bell GI, Stempien MM, Fong NM, Rall LB (1986) Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acids Res 14: 7873-7882.
- Chew SL, Lavender P, Clark AJ, Ross RJ (1995) An alternatively spliced human insulin-like growth factor-I transcript with hepatic tissue expression that diverts away from the mitogenic IBE1 peptide. Endocrinology 136: 1939-1944.
- Mourkioti F, Rosenthal N (2005) IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
- Foulstone EJ, Savage PB, Crown AL, Holly JM, Stewart CE (2003) Role of insulin-like growth factor binding protein-3 (IGFBP-3) in the differentiation of primary human adult skeletal myoblasts. J Cell Physiol 195: 70-79.
- Hameed M, Orrell RW, Cobbold M, Goldspink G, Harridge SD (2003) Expression of IGF-I splice variants in young and old human skeletal muscle after high resistance exercise. J Physiol 547: 247-254.
- Hameed M, Toft AD, Pedersen BK, Harridge SD, Goldspink G (2007) Effects of eccentric cycling exercise on IGF-I splice variant expression in the muscles of young and elderly people. Scand J Med Sci Sports
- Toth KG, McKay BR, De LM, Little JP, Tarnopolsky MA, Parise G (2011) IL-6 induced STAT3 signalling is associated with the proliferation of human muscle satellite cells following acute muscle damage. PLoS One 6: e17392.
- 19. Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, Nevitt M, Harris TB (2002) Relationship of interleukin-6 and

tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. J Gerontol A Biol Sci Med Sci 57: M326-M332.

- 20. Pereira LS, Narciso FM, Oliveira DM, Coelho FM, Souza DG, Dias RC (2009) Correlation between manual muscle strength and interleukin-6 (IL-6) plasma levels in elderly community-dwelling women. Arch Gerontol Geriatr 48: 313-316.
- 21. Leger B, Derave W, De BK, Hespel P, Russell AP (2008) Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res 11: 163-175B.
- 22. Haddad F, Zaldivar F, Cooper DM, Adams GR (2005) IL-6-induced skeletal muscle atrophy. J Appl Physiol 98: 911-917.
- Payette H, Roubenoff R, Jacques PF, Dinarello CA, Wilson PW, Abad LW, Harris T (2003) Insulin-like growth factor-1 and interleukin 6 predict sarcopenia in very old community-living men and women: the Framingham Heart Study. J Am Geriatr Soc 51: 1237-1243.
- 24. Roubenoff R (2003) Catabolism of aging: is it an inflammatory process? Curr Opin Clin Nutr Metab Care 6: 295-299.
- 25. Steensberg A, van HG, Osada T, Sacchetti M, Saltin B, Klarlund PB (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. J Physiol 529 Pt 1: 237-242.
- Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK (2002) IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle. Am J Physiol Endocrinol Metab 283: E1272-E1278.
- 27. Hiscock N, Chan MH, Bisucci T, Darby IA, Febbraio MA (2004) Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. FASEB J 18: 992-994.
- Langberg H, Olesen JL, Gemmer C, Kjaer M (2002) Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans. J Physiol 542: 985-990.
- 29. Rosendal L, Sogaard K, Kjaer M, Sjogaard G, Langberg H, Kristiansen J (2005) Increase in interstitial interleukin-6 of human skeletal muscle with repetitive low-force exercise. J Appl Physiol 98: 477-481.
- Kelly M, Gauthier MS, Saha AK, Ruderman NB (2009) Activation of AMPactivated protein kinase by interleukin-6 in rat skeletal muscle: association with changes in cAMP, energy state, and endogenous fuel mobilization. Diabetes 58: 1953-1960.
- 31. Ruderman NB, Keller C, Richard AM, Saha AK, Luo Z, Xiang X, Giralt M, Ritov VB, Menshikova EV, Kelley DE, Hidalgo J, Pedersen BK,

Kelly M (2006) Interleukin-6 regulation of AMP-activated protein kinase. Potential role in the systemic response to exercise and prevention of the metabolic syndrome. Diabetes 55 Suppl 2: S48-S54.

- 32. McKay BR, De LM, Johnston AP, O'Reilly CE, Phillips SM, Tarnopolsky MA, Parise G (2009) Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. PLoS One 4: e6027.
- Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374: 1-20.
- Steensberg A, Dalsgaard MK, Secher NH, Pedersen BK (2006) Cerebrospinal fluid IL-6, HSP72, and TNF-alpha in exercising humans. Brain Behav Immun 20: 585-589.
- 35. Rose-John S, Mitsuyama K, Matsumoto S, Thaiss WM, Scheller J (2009) Interleukin-6 trans-signaling and colonic cancer associated with inflammatory bowel disease. Curr Pharm Des 15: 2095-2103.
- Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M (2009) IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 15: 103-113.
- 37. Bollrath J, Phesse TJ, von B, V, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR (2009) gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 15: 91-102.
- 38. McFarlane C, Gu ZH, Wong ZW, Lau HY, Lokireddy S, Ge X, Mouly V, Butler-Browne G, Gluckman PD, Sharma M, Kambadur R (2011) Human myostatin negatively regulates human myoblast growth and differentiation. Am J Physiol Cell Physiol
- 39. Siriett V, Platt L, Salerno MS, Ling N, Kambadur R, Sharma M (2006) Prolonged absence of myostatin reduces sarcopenia. J Cell Physiol 209: 866-873.
- 40. Morissette MR, Stricker JC, Rosenberg MA, Buranasombati C, Levitan EB, Mittleman MA, Rosenzweig A (2009) Effects of myostatin deletion in aging mice. Aging Cell 8: 573-583.
- 41. Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317: 807-810.
- 42. Conboy IM, Conboy MJ, Smythe GM, Rando TA (2003) Notch-mediated restoration of regenerative potential to aged muscle. Science 302: 1575-1577.

- 43. Melov S, Tarnopolsky MA, Beckman K, Felkey K, Hubbard A (2007) Resistance exercise reverses aging in human skeletal muscle. PLoS One 2: e465.
- 44. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, McConnell JP, Nair KS (2008) Endurance exercise as a countermeasure for aging. Diabetes 57: 2933-2942.
- 45. Ge X, McFarlane C, Vajjala A, Lokireddy S, Ng ZH, Tan CK, Tan NS, Wahli W, Sharma M, Kambadur R (2011) Smad3 signaling is required for satellite cell function and myogenic differentiation of myoblasts. Cell Res
- 46. McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (2003) Myostatin negatively regulates satellite cell activation and selfrenewal. J Cell Biol 162: 1135-1147.
- 47. Huang Z, Chen X, Chen D (2011) Myostatin: A novel insight into its role in metabolism, signal pathways, and expression regulation. Cell Signal 23: 1441-1446.
- 48. Yang W, Chen Y, Zhang Y, Wang X, Yang N, Zhu D (2006) Extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase pathway is involved in myostatin-regulated differentiation repression. Cancer Res 66: 1320-1326.
- 49. Philip B, Lu Z, Gao Y (2005) Regulation of GDF-8 signaling by the p38 MAPK. Cell Signal 17: 365-375.
- 50. Huang Z, Chen D, Zhang K, Yu B, Chen X, Meng J (2007) Regulation of myostatin signaling by c-Jun N-terminal kinase in C2C12 cells. Cell Signal 19: 2286-2295.
- Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K, Gonzalez-Cadavid NF (2002) Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in C2C12 skeletal muscle cells. J Cell Physiol 190: 170-179.
- 52. Testelmans D, Crul T, Maes K, Agten A, Crombach M, Decramer M, Gayan-Ramirez G (2010) Atrophy and hypertrophy signalling in the diaphragm of patients with COPD. Eur Respir J 35: 549-556.
- 53. Mendler L, Zador E, Ver HM, Dux L, Wuytack F (2000) Myostatin levels in regenerating rat muscles and in myogenic cell cultures. J Muscle Res Cell Motil 21: 551-563.
- 54. Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signaling pathway. J Cell Sci 117: 1281-1283.
- Al-Shanti N, Saini A, Faulkner SH, Stewart CE (2008) Beneficial synergistic interactions of TNF-alpha and IL-6 in C2 skeletal myoblasts--potential cross-talk with IGF system. Growth Factors 26: 61-73.
- 56. Himpe E, Kooijman R (2009) Insulin-like growth factor-I receptor signal transduction and the Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT) pathway. Biofactors 35: 76-81.

Appendix I: Manuscript 4

The Journal of Physiology. 2010 Sept. 1; 588 (17):3307-20

Satellite Cell Number and Cell-cycle Kinetics in Response to Acute Myotrauma in Humans: Immunohistochemistry versus Flow Cytometry

Bryon R. McKay¹, Kyle G. Toth¹, Mark A. Tarnopolsky² and Gianni Parise^{1, 3}

Departments of Kinesiology¹, Pediatrics² and Medical Physics & Applied Radiation Sciences³, McMaster University, Hamilton Ontario, L8S 4L8

Running Title: Analysis of muscle satellite cell enumeration and function

Keywords: Pax7, NCAM, C-Met, Muscle Damage, Propidium Iodide

Correspondence: Gianni Parise, PhD Departments of Kinesiology, and Medical Physics & Applied Radiation Sciences McMaster University Hamilton, Ontario, Canada L8S 4L8

Telephone:	(905) 525-9140 x27353
Fax:	(905) 523-6011
E-mail:	pariseg@mcmaster.ca

Abstract:

In humans, muscle satellite cell (SC) enumeration is an important measurement used to determine the myogenic response to various stimuli. To date, the standard practice for enumeration is immunohistochemistry (IHC) using antibodies against common SC markers (Pax7, N-CAM). Flow cytometry (FC) analysis may provide a more rapid and quantitative determination of changes in the SC pool with potential for additional analysis not easily achievable with standard IHC. In this study, FC analysis revealed Pax7⁺ cells/mg isolated from \sim 50 mg of fresh tissue increased 36% 24h after exercise-induced muscle injury (300 unilateral maximal eccentric contractions). IHC analysis of Pax7 and NCAM appeared to sufficiently and similarly represent the expansion of SC after injury (28-36% increase). IHC and FC data illustrated that Pax7 was the most widely expressed SC marker in muscle cross-sections and represented the majority of positive cells, while NCAM was expressed to a lesser degree. Moreover, FC and IHC demonstrated a similar % change 24h after injury (36% increase, Pax7; 28% increase, NCAM). FC analysis of isolated SCs revealed that the number of $Pax7^+$ cells/mg in G₂/M phase of the cell-cycle increased 202% 24h after injury. Cells/mg in G_0/G_1 and cells in S-phase increased 32% and 59% respectively. Here we illustrate the use of FC as a method for enumerating SC number on a per milligram tissue basis, providing a more easily understandable relation to muscle mass (vs. % of myonuclei or per myofiber). Although IHC is a powerful tool for SC analysis, FC is a fast, reliable and effective method for SC

quantification as well as a more informative method for cell-cycle kinetics of the SC population in humans.

Introduction:

Post-natal skeletal muscle is a terminally differentiated tissue, yet it retains a remarkable regenerative capacity. Although skeletal muscle fibers are postmitotic and unable to divide, a class of stem cells residing on the periphery of the fiber maintains the capacity for tissue remodeling and muscle regeneration. These stem cells, referred to as satellite cells (SC) based on their anatomical location between the basal lamina and sarcolemma, are directly responsible for post-natal muscle development, homeostasis and regeneration (Seale & Rudnicki, 2000;Charge & Rudnicki, 2004;Mauro, 1961;Zammit et al., 2006). Following muscle damage (or a hypertrophic stimuli), SC activate and undergo rounds of proliferation before differentiating and fusing with existing myofibers (Holterman & Rudnicki, 2005;Le Grand& Rudnicki, 2007). Progression of SC through the myogenic program is controlled by the coordinated up- and downregulation of the myogenic regulatory factors (MRF). The upregulation of Myf5 marks the earliest phase of myogenic commitment followed by the concomitant expression of MyoD which together mark the majority of newly activated SC (myoblasts) (Dhawan & Rando, 2005; Charge & Rudnicki, 2004). Following proliferation, these cells express markers of myoblast differentiation (MRF4 and Myogenin) and ultimately fuse, contributing their nuclei to existing myofibers or

giving rise to nascent myotubes, aiding in the repair process (Dhawan & Rando, 2005;Charge & Rudnicki, 2004).

Studies examining human muscle SC generally utilize immunohistochemistry (IHC) of muscle cross-sections to enumerate the SC response to a given stimuli such as acute myotrauma or resistance exercise training (McKay et al., 2009;O'Reilly et al., 2008;Dreyer et al., 2006;Crameri et al., 2004;Kadi et al., 2004). Although SC enumeration is an important measure, it provides limited data with regards to the regulatory events associated with SC function. In the majority of studies investigating human muscle SC, neural cell adhesion molecule (NCAM;CD56) was used to mark SC for enumeration (O'Reilly et al., 2008;Dreyer et al., 2006;Crameri et al., 2004;Mackey et al., 2007c;Kadi et al., 2004;Kadi & Thornell, 2000;Mackey et al., 2007a;Kadi et al., 1999;Eriksson et al., 2005;Mackey et al., 2007b;Mackey et al., 2009). More recently, the paired box transcription factor Pax7, which can activate transcription and control the expression of the MRF in guiescent and activated SC (Zammit, 2008), has been used to identify SC in human muscle cross-sections (McKay et al., 2009; Mikkelsen et al., 2009). Typical human SC analysis is more often than not, limited to a satellite cell marker (N-CAM or Pax7) co-stained with Ki67 or PCNA (proliferating cell nuclear antigen), which provides important, yet limited cell-cycle information (McKay et al., 2009;Mackey et al., 2009). Understanding the kinetics of SC entering and progressing through the cell-cycle during the

response to damage is imperative to understanding deficiencies in muscle repair and growth in conditions such as aging or cachexia.

Flow cytometry is routinely used to analyze cells based on multiple-antigen labeling *in vitro* and used extensively in stem cell research to purify obscure cell populations from a larger cellular milieu. We propose FC analysis on human tissue as an alternate means to objectively enumerate SC number on a per milligram of tissue basis, while allowing for the determination of SC cell-cycle kinetics (*in vivo*) with resolution virtually unobtainable by IHC techniques alone. Furthermore, we aim to validate the use of Pax7 as an accurate marker for human SC analysis through direct comparison with NCAM using both FC and IHC methods.

Materials and Methods:

Subjects:

Twelve healthy males (age 21 ± 2 y, height 185 ± 5 cm, weight 82 ± 11 kg) were recruited from the McMaster University community. Subjects underwent a routine screening, completed a health questionnaire, and were not involved in a lowerbody resistance exercise training program for at least six months prior to participating in the study. Subjects were told to refrain from exercising throughout the time-course of the study. All subjects were informed of the procedures and potential risks associated with the study and gave their written informed consent to participate. This study was approved by the Hamilton Health Sciences Research Ethics Board and conforms to the *Declaration of Helsinki* concerning the use of human subjects as research participants.

Muscle Damage Protocol:

To induce muscle damage we employed a protocol involving maximal isokinetic unilateral muscle-lengthening contractions of the *quadriceps femoris* performed on a Biodex dynamometer (Biodex-System 3, Biodex Medical Systems, Inc., USA) at 3.14 rad.s⁻¹ as previously described (McKay *et al.*, 2009). Briefly, for each subject, the dominant leg was selected to complete the protocol. Subjects were required to perform 30 sets of 10 maximal muscle lengthening contractions with one minute of rest between sets for a total of 300 lengthening contractions.

It has been well documented that this protocol induces some level of skeletal muscle damage with some cellular disruption evidenced by z-band streaming, discontinuous desmin staining, an increase in plasma creatine kinase, an infiltration of leukocytes, macrophage and neutrophils, and a significant myogenic regulatory factor (MRF) and muscle satellite cell response (Beaton *et al.*, 2002b;O'Reilly *et al.*, 2008;McKay *et al.*, 2008;Mahoney *et al.*, 2008;McKay *et al.*, 2009;Lauritzen *et al.*, 2009;Paulsen *et al.*, 2010). Although overt damage is sometimes observed from this type of exercise, it may not induce a significant level of necrosis as evidenced by a lack of regenerating fibers expressing embryonic myosin heavy chain (indicative of a newly formed or regenerated fiber) 8 days following 200 maximal eccentric contractions of the guadriceps (Mikkelsen

et al., 2009). Furthermore, it was shown that electrical stimulation of human quadriceps will induce far more necrosis and thus represents a model of tissue degeneration / regeneration compared with normal physiological damage observed with eccentrically-biased exercise (Crameri *et al.*, 2007).

Muscle Biopsies:

Two percutaneous needle biopsies were obtained from the mid-portion of the *vastus lateralis* under local anesthetic (1% lidocaine) using manual suction (Hennessey *et al.*, 1997;Bergstrom, 1975;Bourgeois & Tarnopolsky, 2004). One muscle biopsy was obtained from the non-working leg prior to the intervention. Baseline measures were generated from the pre-intervention biopsy (PRE). The other biopsy was obtained from the working leg 24 h post-intervention. Approximately 25 mg of each biopsy sample was mounted in Optimum Cutting Temperature (OCT) compound and frozen in isopentane cooled in liquid nitrogen. Approximately 50 mg (50.8 \pm 3.4 mg) of fresh muscle tissue was placed in ice-cold Ham's (F-10) culture media (Gibco, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) for immediate flow cytometry preparation.

Fluorescence Activated Cell Sorting (FACS) and Flow Cytometry:

Determination of Myogenic Satellite Cell Number and Cell Cycle Kinetics using Pax7 and propidium iodide (PI): PRE and 24 h muscle biopsy tissue was prepared for FACS analysis using standard methods and criteria (Alexander *et*

al., 2009; Parise et al., 2008; Megeney et al., 1996; Conboy et al., 2010). Briefly, after weighing, muscle samples were mulched using sterile surgical scissors in 3.5 cm tissue culture plates in a sterile tissue culture hood. After tissue was adequately mulched, 1 mL of a collagenase/dispase solution (10mg/mL Collagenase B, Roche Diagnostics, Germany; 2.4U/mL Dispase, Gibco USA; with 5µL/mL of 0.5M CaCl₂.) was added to the plate and placed in a tissue culture incubator for 10 minutes (5% CO₂, 37°C). Another 0.5 mL of collagenase/dispase was added to the plate and the mixture was manually triturated through a 5 mL and 1 mL plastic serological pipette to further disrupt the tissue. The mixture was then incubated for 5-10 minutes. Following the incubation, 5 mL of ice-cold Ham's (F-10 with 20% FBS and 1% penicillin/streptomycin) culture media was added and the mixture was triturated several times and filtered through a 50 µm mesh filter (BD biosciences, BD Canada) into a 50 mL conical bottom tube. The mixture was then centrifuged at 800 g for 5 min to obtain a pellet containing mononuclear cells. The pellet was re-suspended in 1 mL of 1x phosphate-buffered saline (10mM PBS, pH 7.4) and a 10 µL sample was taken for analysis of total mononuclear cell concentration using a hemacytometer (Hausser Scientific, USA). The cell suspension was added drop-wise to ice-cold 70% ethanol under gentle agitation for fixation. Total time of tissue removal from the subject to fixation was no longer than one hour. as great care was taken to reach fixation as guickly as possible to maintain the in vivo cell-cycle characteristics. After fixation, cells were pelleted and re-

suspended in a blocking solution (2% BSA, 5% FBS, 0.2% Triton-X 100, 0.1% sodium azide) and incubated on ice for 10 min. Cells were then pelleted and resuspended in 500 µL of anti-human Pax7 primary antibody (cell supernatant from hybridoma cells obtained from DSHB, USA; $50 \pm 2 \mu g/mL mAb$). After incubation on ice for 45 min, cells were pelleted and washed before being re-suspended in 2 mL of the appropriate secondary antibody (Alexafluor 488, Invitrogen, USA, 1:500 for 45 min). After incubation in the secondary antibody cells were then pelleted and washed several times before being re-suspended in 300 µL of a propidium iodide (PI) solution (0.1mg/mL PI (Calbiochem, USA), 0.2mg/mL RNase A (Roche Diagnostics, Germany) and 0.1% Triton-X 100 (Sigma-Aldrich, Canada) in 1x PBS). Flow cytometry was conducted using a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, USA). Optical alignment and fluidics of the cytometer were verified daily using Flow-Check fluorospheres (Beckman-Coulter, USA) and optimum instrument settings were verified daily using a Flow-Set kit (Beckman-Coulter, USA) by a trained technician. Gating strategies were optimized through multiple experiments which included various unstained, secondary only, and P.I. only samples (Example see Fig. S2). Furthermore, gates were set to include only cells with DNA (P.I. positive), thus gating out cellular debris commonly observed in isolating cells from solid tissues. The population of interest encompassed cells with a wide spread of side-scatter (SS) and forward scatter (FS), which we expected based on the fact that guiescent SCs have a very low cytoplasmic and organelle volume, while activated and

cycling SCs have varying degrees of organelle and cytoplasmic volume (Charge & Rudnicki, 2004). Furthermore, cells beginning to differentiate upregulate different cellular machinery, altering SS and FS compared to quiescent SCs making discrete SS and FS gating (as with done with blood cells) more difficult. Repeated running of controls periodically throughout the study helped ensure the consistency of our gating strategies. Specific fluorochrome details are as follows: P.I. excitation = 520 nm, emission = 610 nm; Pax7 (Alexa 488) excitation = 494 nm, emission = 519 nm; NCAM (PE-Cy5.5) excitation = 488 nm, emission = 690 nm.

Determination of Pax7 and NCAM Cell Number: For co-staining with Pax7 and NCAM, tissue from a group of 4 subjects $(22.5 \pm 1.0 \text{ y})$ was isolated as stated above. Preparation was the same as above except during the primary antibody incubation, cells were incubated with a cocktail of Pax7 (cell supernatant from hybridoma cells obtained from DSHB, USA; ~50 µg/mL mAb) and neural cell adhesion molecule (NCAM, CD56, 35-0569 eBioscicence CD56-PE Cy5.5 conjugated mouse anti-human mAb, titrated to 2µg/1 million cells). Appropriate positive, single stained and unstained control samples were re-run to establish the correct gates for N-CAM and Pax7.

FACS of Pax7 Positive Cells: For sorting Pax7⁺ cells, tissue from 4 subjects (22.5 \pm 1.0 y) was isolated as above, however cells were not re-suspended in PI, rather cells were re-suspended in 1x PBS and sorted on Pax7 only. Cells were

sorted onto silane (aminoalkysilane) coated slides (Sigma-Aldrich, Canada) and into 1.5 mL culture tubes and cytospun (StatSpin Cytofuge 2, IRIS International, USA) onto silane-coated slides for immunocytochemistry of desmin content (see below). Sorting was conducted using a Beckman-Coulter Epics Altra Cell Sorter (Beckman-Coulter, USA). Gating strategies, optimization and controls were conducted as above.

Verification of Myogenic Lineage using Desmin: Mononuclear cells isolated from muscle biopsies and FACS-sorted Pax7⁺ cells were then stained with an antibody against desmin, a muscle specific marker (Bar et al., 2004). Briefly, slides were washed with 1xPBS prior to incubation in the primary antibody. Slides were incubated in the primary antibody (anti-human desmin mAb, 1:500, Abcam, USA) overnight at 4°C. Following three 5 min washes, slides were incubated in the appropriate secondary antibody (Alexafluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA) for 2 h. Mononuclear cells were then cytostained with Pax7 as described below. Slides were then washed with PBS and DAPI (Sigma-Aldrich, Canada) for nuclear staining. Staining was verified using the appropriate positive and negative controls to ensure specificity of staining. Stained slides were viewed with the Nikon Eclipse 90*i* Microscope (Nikon Instruments, Inc., USA) and images were captured and analyzed using the Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., USA). The mononuclear cell preparation served as an additional confirmation that the sorted Pax7 cells were, indeed, of the myogenic lineage.

Muscle Tissue Titration Experiments: To determine the optimal amount of tissue needed to accurately reproduce $Pax7^+$ cell number and $Pax7^+$ cells/mg tissue, we conducted a series of separate titration experiments using the above methods. Briefly, we divided muscle biopsies obtained PRE and 24h after damage from a separate subject group (n = 5) into 5 different tissue weight classes (approximately 15, 25, 50, 75 and 100 mg). Each sample was compared to the other weights for reproducibility of the number of cells/mg tissue value at each time point. The PRE samples were compared against tissue obtained 24 h after injury to determine the reproducibility of the expansion of the number of Pax7⁺ cells/mg tissue for each subject. We determined that 40-50 mg of fresh tissue was sufficient for satellite cell analysis using FACS since there was little variance observed in Pax7⁺ cell/mg with tissue weights ranging between 40 and 110 mg.

Immunofluorescence:

7 μm muscle cross-sections were stained with antibodies against Pax7 (neat; cell supernatant from cells obtained from the DSHB, USA); neural cell adhesion molecule (NCAM/CD56/ Leu-19) (1:100, 559043, BD, Canada); C-Met (2μg/mL; 71-8000 Rabbit anti-C-Met, Zymed Laboratories, USA); and Laminin (1:1000, L8271, Sigma-Aldrich, Canada). Secondary antibodies used were: Pax7 (AlexaFluor 488 or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA or when using two mouse primary antibodies an immunoglobulin biotinylated secondary antibody, 1:200, Dako Canada, Inc., Canada; followed by a streptavidin-FITC fluorochrome, 1:100, Biosource. USA); NCAM (CD56)

(AlexaFluor 488 or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., USA); C-Met (Texas Red, 1:500, Invitrogen, Molecular Probes Inc., USA) and Laminin (AlexaFluor 488, 1:500, Invitrogen, Molecular Probes Inc., USA). Histochemical methods were adapted from previously published methods from our lab (McKay et al., 2009;O'Reilly et al., 2008;McKay et al., 2008). Briefly, for co-immunofluorescent staining (Pax7 and NCAM, Pax7 and Laminin, Pax7 and C-Met, NCAM and Laminin, or C-Met and Laminin), sections were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Canada) for 10 min followed by several washes in PBS. Sections were then covered for 60 min in a blocking solution containing, 2% BSA, 5% FBS, 0.2% Triton-X 100, 0.1% sodium azide. Following blocking, sections were incubated in the primary antibody at 4°C overnight. After several washes, sections were then incubated in the appropriate secondary antibodies. Sections were then re-fixed in 4% PFA (Sigma-Aldrich, Canada) to prevent migration of the secondary antibodies and re-blocked in 10% GS in 0.01% Triton-X 100 (Sigma, USA). The sections were then incubated in the second primary antibody, followed by incubation in the appropriate secondary antibody. Sections were then washed with PBS and 4',6-diamidino-2phenylindole (DAPI, 1:20000) (Sigma-Aldrich, Canada) for nuclear staining. Staining was verified using the appropriate positive and negative controls to ensure specificity of staining. Stained slides were viewed with the Nikon Eclipse 90*i* Microscope (Nikon Instruments, Inc., USA) and images were captured and

analyzed using the Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., USA).

Blood Measures:

A resting blood sample was obtained from the antecubital vein immediately prior to the intervention and at 24 h post. Approximately 20 mL of blood was collected and separated into one serum and one plasma vacutainer tube (BD, Canada) at each time-point. Samples were separated into 50 µL aliquots and stored at -80 °C for analysis at a later date. Serum samples were thawed on ice and analyzed for creatine kinase (CK) protein concentration using a commercially available kit according to manufactures' instructions (Pointe Scientific Inc., USA).

Statistical Analysis:

Statistical analysis was performed using SigmaStat 3.1.0 analysis software (Systat, SPSS Inc., USA). Serum creatine kinase concentrations, immunohistochemistry and flow cytometry were analyzed using a 1-way repeated-measures analysis of variance (ANOVA). Statistical significance was accepted at P < 0.05. Significant interactions and main effects were analyzed using the Tukey's HSD post hoc test. Data are presented as mean ± SD.

<u>Results:</u>

Tissue Titration and Myogenic Lineage Determination:

To ensure specificity of the anti-body labeling, we preformed a series of confirmatory experiments. Pax7⁺ cells were sorted and subsequently cytostained with the muscle-specific intermediate filament protein desmin. Of the 1615 Pax7⁺ cells sorted, 100% of the Pax7⁺ cells were positive for desmin (cells that were Pax7⁻ were negative for desmin). Figure S1a shows the localization of desmin (red) to the cytoplasm of Pax7⁺ sorted cells. The results of the FACS analysis were further confirmed using whole mononuclear preps isolated from muscle biopsies and cytostained for both Pax7 or NCAM and desmin. 100% of the cells which stained positive for Pax7 or NCAM were also positive for desmin, confirming that Pax7 (and NCAM) were specific for mononuclear cells restricted to the myogenic lineage (Fig. S1b, c). Furthermore, of the mononuclear cells, Pax7 negative cells did not show any desmin staining (fig. S1d). Following confirmation of the specificity of the antibody, we conducted a series of titration experiments to determine the optimal amount of tissue needed to accurately reproduce the SC enumeration. The number of Pax7⁺ cells/mg muscle tissue was consistent from approximately 110 mg down to 50 mg with no significant loss in the reproducibility of cell number per mg of tissue. At tissue weights below 40-50 mg there was an abrupt loss in cell number in both resting and 24h postdamage samples (Fig. S1e). Thus it was determined that a minimum of ~50 mg of muscle was needed for reproducibility and that an average of 50.8 ± 11.7 mg used in the present study.

Enumeration of Pax7 Positive Cells using Flow Cytometry following Myotrauma:

The number of Pax7⁺ cells /mg of fresh muscle tissue were analyzed before (PRE) and 24h after a bout of damaging muscle contractions via flow cytometry. The extent of muscle damage was assessed using a creatine kinase (CK) assay. Muscle CK in the blood increased 212% 24h after damaging exercise (167.1 ± 112.2 IU/L; PRE to 522.3 ± 252.2 IU/L; 24h). The number of total mononuclear cells tended to increase 24h post-damage (Fig. 1A: 27 155.7 ± 8 221.1 cells·mg⁻¹; PRE to 34 824.2 \pm 11 495.1 cells·mg⁻¹ 24h; p = 0.08). The number of Pax7⁺ cells·mg⁻¹ increased 36% 24h post-damage (Fig. 1B: 17 407.0 \pm 5 953.1 Pax7⁺ cells·mg⁻¹; PRE to 23 664.6 \pm 8 547.2 Pax7⁺ cells·mg⁻¹; 24h, p = 0.04), with a visible shift in the mean fluorescence of the $FITC^+$ (Pax7⁺) cell population (Fig. 1C). Furthermore, there was a detectable change in both the forward scatter (FS) and side scatter (SS) of the FITC⁺ cell population, which indicates an increase in the size (FS) and granularity (SS or complexity i.e. increased organelle volume; Fig. 1D) which would be expected as the SCs exit quiescence and enter the cell-cycle.

Cell-Cycle Analysis of Isolated Pax7 Positive Cells following Myotrauma:

In the resting condition (PRE) cell-cycle analysis using propidium iodide (P.I.) staining of individual cells isolated from fresh tissue revealed that the majority of $Pax7^+$ cells were in the G_0/G_1 phase of the cell-cycle (84.6 ± 4.4 %
G_0/G_1) with very few cells actively dividing (i.e. in G_2/M ; 2.6 ± 1.7 %; Fig. 2A). There was a significant increase in Pax7⁺ cells entering the cell-cycle at 24h, evidenced by an increase in the proportion of cells in S-phase and G_2/M -phase of the P.I. DNA histogram (Fig. 3B). The P.I. data translated into a 32% increase in Pax7⁺ cells·mg⁻¹ in G_0/G_1 , 59% increase in Pax7⁺ cells·mg⁻¹ in S-phase and 202% increase in Pax7⁺ cells·mg⁻¹ in G_2/M 24h after myotrauma (P < 0.001 for each phase; Fig. 2C-F).

Immunohistochemical Analysis of Pax7, NCAM, and C-Met following Myotrauma:

To validate the expansion of the SC pool quantified using flow cytometry, we utilized common immunohistochemical analysis of several known SC markers. Muscle cross-sections were stained with Pax7, NCAM, or C-Met alone (with laminin to determine proper anatomical location of the SCs) and sections were also co-stained with Pax7 and NCAM or Pax7 and C-Met to determine the co-expression of these markers on SCs (Fig. 3A-C). There was no change in the average number of myonuclei per myofiber following contraction-induced myotrauma (Fig. 3D). Each marker (Pax7, NCAM, C-Met) illustrated a significant increase in the number of positive cells per100 myofibers (MF) (Fig. 3E). However, there were significantly fewer C-Met positive SCs in the PRE condition compared to either Pax7 or NCAM (Fig. 3E, F). Furthermore, the number of SCs increased when expressed either as SCs per 100 MF (Fig. 3E) or as a percentage of total myonuclei (MN; Fig 3F) regardless of marker used except for

the increase in NCAM⁺ SCs as % MN, which did not reach statistical significance (p = 0.06, Fig. 3B). In agreement with Lindstrom and Thornell (Lindstrom & Thornell, 2009), expressing SCs per MF (or per 100 MF) appears to be a more relevant expression of SC number, which is more relatable to the FACS data which is expressed per mg of tissue. However, to allow for comparisons with other studies we have provided SC enumeration as both % MN and per 100 MF.

The co-expression of Pax7 and NCAM or Pax7 and C-Met are depicted in figure 4A and B respectively. The number of Pax7⁺/NCAM⁺ cells was higher at PRE compared to Pax7⁺/C-Met⁺ cells (p = 0.003; Fig. 4C). However, the number of Pax7⁺/C-Met⁺ cells significantly increased 24h after muscle damage (p = 0.016), while the number of Pax7⁺/NCAM⁺ cells showed a strong trend to increase (p = 0.07). In both cases there were several SCs that only stained positive for one of the two SC markers suggesting some heterogeneity in the SC pool (e.g. Fig. S3). There were significantly more Pax7⁺ only cells compared to either NCAM⁺ or C-Met⁺ only cells (p = 0.001; as % MN, Fig. 4D). Following myotrauma there was no detectable increase in any of the single-marker positive cell populations, however the number of Pax7⁺ only cells was no longer significantly higher than NCAM⁺ or C-Met⁺ only cell populations (p = 0.065, Fig. 4D).

The commonly used marker N-CAM co-localized to approximately 95% of Pax7⁺ cells using immunohistochemical techniques (data not shown). To verify that both markers were in fact binding to the same cells, we isolated mononuclear

cells from fresh muscle and stained them for both NCAM and Pax7 and analyzed them via flow cytometry. Figure 4E is a histogram illustrating the percent colocalization of Pax7 and NCAM using flow cytometry. This experiment verified the immunohistochemical findings where approximately 95% of the Pax7⁺ cells also stained positive for NCAM (section E2, Fig. 4E). In addition, we also observed more Pax7⁺ only cells (section E4, Fig 4E), compared with NCAM⁺ only (section E1, Fig. 4E) further verifying our finding that Pax7 appears to be expressed in a larger proportion of the SC pool then NCAM alone during the time-course of our experimental protocol.

Comparison of Flow Cytometry and Immunohistochemistry for Muscle Satellite Cell Enumeration:

Both flow cytometry on fresh muscle samples and IHC on frozen muscle cross-sections illustrated that expression of Pax7 and NCAM similarly increased 24h after acute myotrauma (Fig. 1B and 3E). The comparison of the expansion of both Pax7 and NCAM positive cells expressed as a percent change from PRE reveled that both techniques (FACS or IHC) report similar increases in SC number. For Pax7, flow cytometry (or FACS) reported a 36% increase in Pax7⁺ cells·mg⁻¹ (p = 0.04), and IHC reported a 36% increase in Pax7⁺ cells per 100 myofibers (p < 0.05, Fig. 4F). In addition, NCAM followed a similar trend increasing approximately 27% using flow cytometry (p < 0.05) and increasing 28% using immunohistochemistry (NCAM⁺ cells per 100 MF, p < 0.05; Fig. 4F).

Discussion:

We have demonstrated a significant shift of human muscle SC from G₀/G₁ to Sand G₂/M-phase of the cell-cycle *in vivo* in as little as 24h following contractioninduced muscle injury. Furthermore, we report that FC or FACS analysis of freshly isolated human SC is an objective and reliable method for SC enumeration. Further, we demonstrate that analysis by FC allows for the accurate determination of cell-cycle kinetics of SC at a resolution which is virtually unobtainable by IHC. Finally, we demonstrate that analysis of either Pax7 or NCAM result in similar increases in the SC pool following acute myotrauma; however Pax7 appears to be more widely expressed as compared to NCAM or C-Met.

In order to verify that FC analysis is an accurate tool to quantify the SC response to muscle damage, we chose to use a muscle-lengthening contraction protocol which we have previously shown to induce a significant increase in SC (McKay *et al.*, 2009;O'Reilly *et al.*, 2008), MRF mRNA expression (McKay *et al.*, 2008) and an induction of some level of muscle damage (Beaton *et al.*, 2002b;Beaton *et al.*, 2002a;Mahoney *et al.*, 2008). Muscle damage from our protocol was verified with a 212% increase in CK observed in the blood 24h after the single-leg exercise bout. Myogenic lineage of Pax7⁺ cells was verified using the muscle intermediate filament protein desmin. Although it is unknown if all SCs *in vivo* express desmin, it has been shown that desmin expression precedes that of MyoD and may be regulated by Myf5 and thus is one of the earliest

markers of myogenic commitment (Bar et al., 2004). Previously it has been suggested that SCs expressing desmin may be a subpopulation of fusion competent cells which do not proliferate but rather are committed to differentiation, whereas desmin negative cells were able to undergo rounds of cell division (Rantanen et al., 1995). However, data from human SCs in culture indicated that both subpopulations (cells committed to differentiation and nonfusion competent myoblasts) expressed desmin (Baroffio et al., 1995). Furthermore, native SCs (freshly isolated from human muscle) constituted a homogeneous cell population which expressed desmin and desmin has been shown to be expressed along with Pax3 and Pax7 in proliferating myogenic precursors isolated from human muscle. (Baroffio et al., 1995;Conboy et al., 2010). Of the mononuclear cells isolated from human muscle in the present study all of the desmin⁺ cells were Pax7⁺. In addition, those cells sorted for Pax7 all stained positive for desmin in the cytosol. Thus it appears that in humans, $Pax7^{+}$ cells are committed to the myogenic lineage, but whether or not these cells can be further separated into subpopulations based on fusion competency remains unknown.

Cell-cycle analysis is particularly important when evaluating the SC response to a stimulus. However, to date, SC cell-cycle analysis has been limited to the description of SC that are either in the cell-cycle (i.e. active; Ki67 positive) or not active (Ki67 negative). Currently the use of Ki67 or proliferating cell nuclear antigen (PCNA) in muscle cross-sections is routinely used to

determine the percentage of SC actively proliferating (McKay et al., 2009;Mackey et al., 2009). However both Ki67 and PCNA provide limited data with regards to cell-cycle kinetics. Ki67 is apparent in the nucleus during all phases of the cellcycle, thus its expression does not discern cells in discrete phases of the cellcycle. PCNA is only expressed in the nuclei of cells in the DNA synthesis phase (S-phase) and in late G₁ of the cell-cycle (Bruno *et al.*, 1992) and therefore may underestimate the number of cycling cells in a muscle cross-section. FC analysis using propidium iodide (P.I.) allows for a more precise measurement of cell-cycle kinetics based on DNA content of each cell. Therefore the use of P.I. allows for the description of the proliferation characteristics of SC as they were in vivo allowing for the determination of the number of cells in each discrete phase. Our data shows that acute myotrauma is sufficient to induce an increase in the number of SC in all phases of the cell-cycle. There was a 32% increase in the number of SC in G_0/G_1 of the cell-cycle, which suggests that 24h was sufficient for a proportion of the SC to activate and complete one round of cell division. This finding is supported by the 36% increase in Pax7⁺ cells·mg⁻¹ tissue 24h after injury. Furthermore, there was a significant increase in the number of SC progressing through the cell-cycle towards mitosis (59% increase in SCs in Sphase, 202% increase in SCs in G_2/M). These data support previous reports showing a detectable expansion of the SC pool at 24h which progresses until at least 72h after induced-myotrauma (McKay et al., 2009;O'Reilly et al., 2008). However, here we clearly illustrate that the active cells are not synchronized (but

vary in cell-cycle phase) and thus differences in activation and proliferation between different groups or populations (i.e. elderly vs. young) may be readily detectable using this method. In addition to SC number, determination of cellcycle kinetics would be invaluable to discern if there is a delay in the SC response in human elderly muscle (or clinical populations, etc.).

Through a series of experiments we determined that the average tissue required for accurate reproducibility of the FC analysis was ≥50 mg. Although more tissue is needed for FC as compared to immunohistochemistry, this technique allows for multiple antigen labeling of SC, which allows for not only SC enumeration but also cell-cycle analysis and potentially the determination of SCspecific expression of many key regulators (such as IGF-1, IL-6 etc.) in a single analysis. The time and subjectivity involved in this analysis is significantly less than with IHC (i.e. ~4h for FC vs. several weeks for IHC), resulting in virtually instantaneous results. FC analysis of SC expansion was verified by IHC and through the use of multiple markers (i.e. Pax7, NCAM and C-Met). FC analysis of both NCAM and Pax7 reported identical percent changes in the SC pool compared with IHC techniques. However, Pax7 was consistently expressed on ~5% more cells than NCAM, which is in contrast to the staining pattern observed by others (Mackey et al., 2009;Mikkelsen et al., 2009;Lindstrom & Thornell, 2009) who reported a slightly higher level of NCAM staining compared to Pax7 in human muscle cross-sections. We verified the IHC finding using flow cytometry, where 95% of Pax7⁺ cells expressed NCAM with ~5% of cells that were

Pax7⁺/NCAM⁻. This finding supports previous work where muscle SC, identified in cross-section by co-labeling with NCAM and Pax7 showed that 94-96% of SC were positive for both NCAM and Pax7 (Lindstrom & Thornell, 2009; Verdijk et al., 2007). However, our data suggests that $Pax7^+$ cells are present to a greater degree than NCAM⁺ cells. This finding contrasts the work by Lindstrom and Thornell (2009), who show, in cross-section, that NCAM⁺ cells represent a greater proportion of cells than Pax7⁺ cells (5% of SC total was NCAM⁺/Pax7⁻). Our data show the opposite, with NCAM⁷/Pax7⁺ cells representing approximately 5% of the total SC pool. This finding was consistent between FC and IHC, and was repeated with separate sections to ensure accuracy of our findings. It is unclear at this time why this discrepancy exists since the same source for antibodies were used in both studies (Lindstrom & Thornell, 2009). The stringent staining protocol used by Lindstrom and Thornell (2009) showed a higher NCAM expression in 18 of the 19 subjects. Thus reconciling the differences between these studies may not be solely due to enumeration methods. Our previous studies employing the same damage protocol, utilized either NCAM (O'Reilly et al., 2008) or Pax7 (McKay et al., 2009) as the IHC marker for SC. As in the present study, Pax7 illustrated a larger response from the same relative damage compared to NCAM (McKay et al., 2009;O'Reilly et al., 2008).

Some of the discrepancy between Pax7 and NCAM staining may be partially attributed to the fact that NCAM was recently shown to mark SC committed to differentiation (Capkovic *et al.*, 2008). Capkovic et al. (2008), were

able to separate homogeneous primary myoblast cultures into proliferating and differentiating fractions based on NCAM expression. Furthermore, in vitro studies indicated that NCAM is mainly involved in early myoblast differentiation of cell-cell fusion leading to enhanced myotube formation (Suzuki et al., 2003). In addition, NCAM is not a SC specific marker; labeling intramuscular nerves, motor unit end terminals, Schwann cells and regenerating myofibers (Cashman et al., 1987;Mechtersheimer et al., 1992;Illa et al., 1992;Lindstrom & Thornell, 2009). These studies suggest that NCAM may not be the most appropriate marker for SC enumeration alone, especially during the early stages of muscle repair. Thus, the fact that NCAM is expressed to a higher degree in SC committing to terminal differentiation may explain why there were more NCAM⁺/Pax7⁻ cells observed by others (Mikkelsen et al., 2009;Lindstrom & Thornell, 2009). As the majority of SC progress towards differentiation they may begin to lose Pax7 and increase NCAM to allow for differentiation to occur (Kuang et al., 2008;Capkovic et al., 2008). Depending on the sampling time-point, the SC pool may differentially express NCAM and Pax7. For example, a muscle sample at 4-8d post exercise may express NCAM to a greater degree as cells progress towards differentiation, whereas the sampling time-point of 24 h may only capture the onset of the proliferative phase of the myogenic program. It has been previously demonstrated that Pax7 is a known modulator of MyoD and Myf5 expression (McKinnell et al., 2008;Zammit et al., 2006;Relaix et al., 2006), and therefore may represent the SC population to a greater degree at earlier time-points. Further

research is warranted to determine the role of NCAM in the SC response to physiological damage.

The use of any single satellite cell marker alone for enumeration may inherently underestimate total SC number. Importantly, for consistency in reporting SC number via IHC, the use of laminin to identify the SC niche is critical especially when using NCAM as a SC marker (Lindstrom & Thornell, 2009). Pax7 has been shown to be expressed on the majority of human SC (Mikkelsen et al., 2009;Eriksson et al., 2005;Verdijk et al., 2007;Reimann et al., 2004), representing approximately 95% of the SC pool. Thus to examine this heterogeneity further, the hepatocyte growth factor (HGF) receptor C-Met was also examined in the current study as another potential marker for human SC enumeration. The number of C-Met⁺ cells or C-Met⁺/Pax7⁺ cells were significantly lower PRE vs. NCAM⁺ or NCAM⁺/Pax7⁺ or Pax7⁺ cells, suggesting that the expression of C-Met is either very low in quiescent human SC or that there is significant heterogeneity in the guiescent SC pool. C-met⁺ cells increased 80% 24hr after exercise, which likely represented $Pax7^+$ cells that upregulated C-Met expression rather than an 80% increase in total SC. This suggests that C-met is highly inducible and not likely an effective immunohistochemical marker of SC number since it does not appear to be basally expressed (or expressed at detectable levels) on all human SC. Additionally, our data suggests that the expansion of the SC pool cannot be accounted for by a cell population expressing only a single SC marker. When

examining cells expressing only Pax7, C-met, or NCAM (Fig. 4D) no increase in the SC pool could be detected. This suggests that the expansion of the SC pool was accounted for by cells expressing multiple markers, which may be interpreted as an expansion of cells with greater relative complexity (i.e. greater myogenic commitment). For example, the percentage of myonuclei represented by cells only expressing Pax7 was approximately 1.4% at baseline and this did not change 24h following damage. However, since the total number of Pax7⁺ cells increased 36% following damage, then the increase could be accounted for by cells that expressed multiple markers and were likely progressing through the cell cycle. It may be that the single positive cells represent quiescent or more primitive progenitor cells (i.e. C-Met⁺ or Pax7⁺ only cells) or represent cells near terminal differentiation [i.e. NCAM⁺ only cells, (Capkovic *et al.*, 2008)]. Further work is needed to investigate C-Met expression and the role of HGF/C-Met signaling in response to damage. Previous work from our lab illustrated that components of the HGF signaling pathway were upregulated 4-24h after damaging exercise, suggesting that HGF is playing a role in the SC response to injury in humans (O'Reilly *et al.*, 2008), thus the increase in HGF signaling may have lead to the increase in C-Met observed at 24h.

For IHC, it appears that multiple labeling (Pax7 and NCAM or Pax7 and C-Met) provides the best description of the SC pool and the response to muscle damage. However, the use of multiple markers can become very difficult in IHC especially with multiple nuclear antigens. Thus, the use of FC to quantify the

expression of SC specific markers in addition to other measures such as cellcycle analysis through the application of multiple antibodies and the availability of positive and negative sorts for enhanced analysis seems preferable.

In conclusion, the use of FC and FACS has proven to be an objective, reproducible and precise tool for the investigation of SC cell-cycle kinetics. We report the expansion of SC in discrete phases of the cell-cycle from cells freshly isolated from human muscle and immediately fixed to preserve the *in vivo* characteristics of these cells. The cell-cycle analysis illustrated not only the initial expansion in SC number (32% increase in cells in G₀/G₁) but also that 202% more cells were in G₂/M of the cell-cycle progressing toward cell division. Furthermore, FC analysis illustrated the same percent expansion of the SC pool as IHC, with SC number reported as the number of SC per milligram of tissue. Our findings with both IHC and FC support previous work illustrating that NCAM and Pax7 mark approximately 95% of the SC pool and that both are sensitive enough to detect subtle changes in SC number as early as 24h after muscle damage or exercise stimuli (Mikkelsen *et al.*, 2009;Lindstrom & Thornell, 2009;Verdijk *et al.*, 2007).

Acknowledgements:

Funding for this study was provided by CIHR (90915) and NSERC (327073-06) through grants awarded to Dr. G. Parise. B.R. McKay currently holds an Alexander Graham Bell Doctoral CGS through NSERC.

The Pax7 hybridoma cells developed by Dr. A. Kawakami were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242 USA.

We would like to acknowledge Nicole MacFarlane and Dr. Doug Boreham for their technical assistance with the flow cytometry and FACS analysis

Reference List:

Alexander CM, Puchalski J, Klos KS, Badders N, Ailles L, Kim CF, Dirks P, & Smalley MJ (2009). Separating stem cells by flow cytometry: reducing variability for solid tissues. *Cell Stem Cell* **5**, 579-583.

Bar H, Strelkov SV, Sjoberg G, Aebi U, & Herrmann H (2004). The biology of desmin filaments: how do mutations affect their structure, assembly, and organisation? *J Struct Biol* **148**, 137-152.

Baroffio A, Bochaton-Piallat ML, Gabbiani G, & Bader CR (1995). Heterogeneity in the progeny of single human muscle satellite cells. *Differentiation* **59**, 259-268.

Beaton LJ, Allan DA, Tarnopolsky MA, Tiidus PM, & Phillips SM (2002a). Contraction-induced muscle damage is unaffected by vitamin E supplementation. *Med Sci Sports Exerc* **34**, 798-805.

Beaton LJ, Tarnopolsky MA, & Phillips SM (2002b). Contraction-induced muscle damage in humans following calcium channel blocker administration. *J Physiol* **544**, 849-859.

Bergstrom J (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35**, 609-616.

Bourgeois JM & Tarnopolsky MA (2004). Pathology of skeletal muscle in mitochondrial disorders. *Mitochondrion* **4**, 441-452.

Bruno S, Gorczyca W, & Darzynkiewicz Z (1992). Effect of ionic strength in immunocytochemical detection of the proliferation associated nuclear antigens p120, PCNA, and the protein reacting with Ki-67 antibody. *Cytometry* **13**, 496-501.

Capkovic KL, Stevenson S, Johnson MC, Thelen JJ, & Cornelison DD (2008). Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation. *Exp Cell Res* **314**, 1553-1565.

Cashman NR, Covault J, Wollman RL, & Sanes JR (1987). Neural cell adhesion molecule in normal, denervated, and myopathic human muscle. *Ann Neurol* **21**, 481-489.

Charge SB & Rudnicki MA (2004). Cellular and molecular regulation of muscle regeneration. *Physiol Rev* **84**, 209-238.

Conboy MJ, Cerletti M, Wagers AJ, & Conboy IM (2010). Immuno-analysis and FACS sorting of adult muscle fiber-associated stem/precursor cells. *Methods Mol Biol* **621**, 165-173.

Crameri RM, Aagaard P, Qvortrup K, Langberg H, Olesen J, & Kjaer M (2007). Myofibre damage in human skeletal muscle: effects of electrical stimulation versus voluntary contraction. *J Physiol* **583**, 365-380.

Crameri RM, Langberg H, Magnusson P, Jensen CH, Schroder HD, Olesen JL, Suetta C, Teisner B, & Kjaer M (2004). Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. *J Physiol* **558**, 333-340.

Dhawan J & Rando TA (2005). Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment. *Trends Cell Biol* **15**, 666-673.

Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, & Wiswell RA (2006). Satellite cell numbers in young and older men 24 hours after eccentric exercise. *Muscle Nerve* **33**, 242-253.

Eriksson A, Kadi F, Malm C, & Thornell LE (2005). Skeletal muscle morphology in power-lifters with and without anabolic steroids. *Histochem Cell Biol* **124**, 167-175.

Hennessey JV, Chromiak JA, Della VS, Guertin J, & MacLean DB (1997). Increase in percutaneous muscle biopsy yield with a suction-enhancement technique. *J Appl Physiol* **82**, 1739-1742.

Holterman CE & Rudnicki MA (2005). Molecular regulation of satellite cell function. *Semin Cell Dev Biol* **16**, 575-584.

Illa I, Leon-Monzon M, & Dalakas MC (1992). Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. *Ann Neurol* **31**, 46-52.

Kadi F, Eriksson A, Holmner S, Butler-Browne GS, & Thornell LE (1999). Cellular adaptation of the trapezius muscle in strength-trained athletes. *Histochem Cell Biol* **111**, 189-195.

Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, & Andersen JL (2004). The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *J Physiol* **558**, 1005-1012.

Kadi F & Thornell LE (2000). Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. *Histochem Cell Biol* **113**, 99-103.

Kuang S, Gillespie MA, & Rudnicki MA (2008). Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell* **2**, 22-31.

Lauritzen F, Paulsen G, Raastad T, Bergersen LH, & Owe SG (2009). Gross ultrastructural changes and necrotic fiber segments in elbow flexor muscles after maximal voluntary eccentric action in humans. *J Appl Physiol* **107**, 1923-1934.

Le Grand F & Rudnicki MA (2007). Skeletal muscle satellite cells and adult myogenesis. *Curr Opin Cell Biol* **19**, 628-633.

Lindstrom M & Thornell LE (2009). New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. *Histochem Cell Biol* **132**, 141-157.

Mackey AL, Esmarck B, Kadi F, Koskinen SO, Kongsgaard M, Sylvestersen A, Hansen JJ, Larsen G, & Kjaer M (2007a). Enhanced satellite cell proliferation with resistance training in elderly men and women. *Scand J Med Sci Sports* **17**, 34-42.

Mackey AL, Kjaer M, Charifi N, Henriksson J, Bojsen-Moller J, Holm L, & Kadi F (2009). Assessment of satellite cell number and activity status in human skeletal muscle biopsies. *Muscle Nerve* **40**, 455-465.

Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH, Schroder HD, & Langberg H (2007b). The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol* **103**, 425-431.

Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH, Schroder HD, & Langberg H (2007c). The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol* **103**, 425-431.

Mahoney DJ, Safdar A, Parise G, Melov S, Fu M, MacNeil L, Kaczor J, Payne ET, & Tarnopolsky MA (2008). Gene expression profiling in human skeletal muscle during recovery from eccentric exercise. *Am J Physiol Regul Integr Comp Physiol* **294**, R1901-R1910.

Maura A (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* **9**, 493-495.

McKay BR, De Lisio M, Johnston AP, O'Reilly CE, Phillips SM, Tarnopolsky MA, & Parise G (2009). Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans. *PLoS One* **4**, e6027.

McKay BR, O'Reilly CE, Phillips SM, Tarnopolsky MA, & Parise G (2008). Coexpression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans. *J Physiol* **586**, 5549-5560.

McKinnell IW, Ishibashi J, Le GF, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ, & Rudnicki MA (2008). Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. *Nat Cell Biol* **10**, 77-84.

Mechtersheimer G, Staudter M, & Moller P (1992). Expression of the natural killer (NK) cell-associated antigen CD56(Leu-19), which is identical to the 140-kDa isoform of N-CAM, in neural and skeletal muscle cells and tumors derived therefrom. *Ann N Y Acad Sci* **650**, 311-316.

Megeney LA, Kablar B, Garrett K, Anderson JE, & Rudnicki MA (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev* **10**, 1173-1183.

Mikkelsen UR, Langberg H, Helmark IC, Skovgaard D, Andersen LL, Kjaer M, & Mackey AL (2009). Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise. *J Appl Physiol* **107**, 1600-1611.

O'Reilly C, McKay B, Phillips S, Tarnopolsky M, & Parise G (2008). Hepatocyte growth factor (HGF) and the satellite cell response following muscle lengthening contractions in humans. *Muscle Nerve* **38**, 1434-1442.

Parise G, McKinnell IW, & Rudnicki MA (2008). Muscle satellite cell and atypical myogenic progenitor response following exercise. *Muscle Nerve* **37**, 611-619.

Paulsen G, Crameri R, Benestad HB, Fjeld JG, Morkrid L, Hallen J, & Raastad T (2010). Time course of leukocyte accumulation in human muscle after eccentric exercise. *Med Sci Sports Exerc* **42**, 75-85.

Rantanen J, Hurme T, Lukka R, Heino J, & Kalimo H (1995). Satellite cell proliferation and the expression of myogenin and desmin in regenerating skeletal muscle: evidence for two different populations of satellite cells. *Lab Invest* **72**, 341-347.

Reimann J, Brimah K, Schroder R, Wernig A, Beauchamp JR, & Partridge TA (2004). Pax7 distribution in human skeletal muscle biopsies and myogenic tissue cultures. *Cell Tissue Res* **315**, 233-242.

Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, & Buckingham M (2006). Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* **172**, 91-102.

Seale P & Rudnicki MA (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol* **218**, 115-124.

Suzuki M, Angata K, Nakayama J, & Fukuda M (2003). Polysialic acid and mucin type o-glycans on the neural cell adhesion molecule differentially regulate myoblast fusion. *J Biol Chem* **278**, 49459-49468.

Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, & van Loon LJ (2007). Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Endocrinol Metab* **292**, E151-E157.

Zammit PS (2008). All muscle satellite cells are equal, but are some more equal than others? *J Cell Sci* **121**, 2975-2982.

Zammit PS, Partridge TA, & Yablonka-Reuveni Z (2006). The skeletal muscle satellite cell: the stem cell that came in from the cold. *J Histochem Cytochem* **54**, 1177-1191.

Figure Legend

Figure 1:

Satellite cell enumeration via flow cytometry before (PRE) and 24h after muscle damage: 1a) Total mononuclear cells isolated from whole muscle biopsies. 1b) Total Pax7⁺ cells·mg⁻¹ isolated from whole muscle biopsies. Pax7⁺ cells·mg⁻¹ increased 36% (p = 0.04; mean ± SD, N = 12). 1c) Representative flow cytometry histograms of total FITC (Pax7) fluorescence versus cell number of a secondary only control (CON), a pre-exercise (PRE) and 24h post-exercise (24h) cell sample. 1d) Representative flow cytometry histograms of forward-

scatter (FS, y-axis) vs. side-scatter (SS, x-axis) of an ungated sample and Pax7⁺ cells (gated on Pax7 and propidium iodide PRE and 24h).

Figure 2:

Cell-cycle analysis via flow cytometry: 2a & b) Representative DNA histograms of SC (Pax7⁺ cells) isolated from muscle biopsies before (**a**, PRE) and 24h after (**b**, 24h) muscle damage. Cells in G_0/G_1 of the cell-cycle are depicted in blue, cells with increasing DNA content (S-phase) are depicted in purple and cells with 2 compliments of DNA (cells in G_2/M) are depicted in red. **2c)** Total number of Pax7⁺ cells·mg⁻¹ which are in G_0/G_1 of the cell-cycle before (PRE) and 24h after muscle damage. **2d)** Total number of Pax7⁺ cells·mg⁻¹ in S-phase of the cell-cycle before (PRE) and 24h after muscle damage (24h). **2e)** Total number of Pax7⁺ cells·mg⁻¹ in G_2/M of the cell-cycle before (PRE) and 24h after muscle damage (24h). **2f)** Total number of Pax7⁺ cells·mg⁻¹ in each phase of the cell-cycle as a proportion of the total number of SCs before (PRE) and 24h after muscle damage. * denotes a significant difference from PRE (p < 0.05). All data are mean ± SD, N = 12

Figure 3:

Immunohistochemical analysis of the satellite cell response to muscle damage before (PRE) and 24h after muscle damage: 3a) Representative image of Pax7/laminin co-immunofluorescent stain. Arrow denotes a Pax7⁺ nuclei (red) beneath the basal lamina (green). Scale bar = 50 μ m. **3b)** Representative image of a C-Met/laminin co-immunofluorescent stain. Arrow denotes a C-Met⁺ nuclei (green) beneath the basal lamina (red). Scale bar = 50 μ m. **3c)** Representative image of NCAM/laminin co-immunofluorescent stain. Arrow denotes a NCAM⁺ cell (red) beneath the basal lamina (green). Scale bar = 50 μ m. **3d)** Total number of myonuclei per muscle. **3e)** SC number (per 100 myofibers) by SC marker. * denotes a main effect of time (p < 0.05). **3f)** SC number (as a percentage of total myonuclei) by SC marker. * denotes a significant effect of time between for Pax7 and C-met (p < 0.05). † denotes a significant difference between Pax7 and C-met at PRE (p < 0.05). All data are mean ± SD, N = 12.

Figure 4:

Co-labeling of satellite cell markers before (PRE) and 24h after muscle damage: **4a)** Co-localization of C-Met and Pax7 with DAPI, scale bar = 50µm; higher magnification of box: **i)** C-Met (red), **ii)** nucleus (DAPI, blue), **iii)** Pax7

(green), and iv) merged image showing co-localization. 4b) Co-localization of NCAM and Pax7 with DAPI, scale bar = 50μ m; higher magnification of box: i) NCAM (red), ii) nucleus (DAPI, blue), iii) Pax7 (green), and iv) merged image showing co-localization. 4c) Co-positive SC number by marker as a percentage of total myonuclei. * denotes a significant difference between NCAM/Pax7 and C-Met/Pax7 at PRE (p<0.05). † denotes a significant effect of time for C-Met/Pax7 (p<0.05). 4d) Single positive cells (cells expressing only one SC marker) as a percentage of total myonuclei. * denotes a significant difference at PRE between Pax7 only vs. C-Met and NCAM only (p<0.05). 4e) Representative flow cytometry histogram of muscle derived mononuclear cells stained for NCAM and Pax7. Cells expressing both NCAM and Pax7 (E2) represent approximately 95% of all positive cells, while NCAM only cells (E1) and Pax7 only (E4) represent approximately 5% of all positive cells. Cells negative for both SC markers are shown in guadrant E3. 4f) Comparison of flow cytometry and IHC enumeration techniques: Comparison of the percent change in SC number 24h after muscle damage analyzed by flow cytometry and IHC for Pax7 (5a) and NCAM (5b). * denotes a significant effect of time (PRE vs. 24h: p<0.05), All data presented as mean \pm SD, N = 12.

Supplemental Figure Legend

Figure S1:

Verification of myogenic lineage: S1a) Human satellite cell (Pax7⁺; green) sorted onto a slide and co-stained with desmin (red). Nuclei stained with DAPI (blue). Scale bar = 10 μ m. S1b) Mononuclear cells isolated from human muscle biopsies stained for nuclei (DAPI; blue) Pax7 (red) and desmin (green) Scale bar = 50 μ m and for inset = 10 μ m. S1c) Mononuclear cells isolated from human muscle biopsies stained for NCAM (red) and desmin (green) Scale bar = 10 μ m. S1d) Low magnification of mononuclear cells isolated from human muscle biopsies stained for Pax7 (green) and desmin (red) as indicated by the arrows Scale bar = 50 μ m Note Pax7⁻/desmin⁻ nuclei (DAPI, blue). S1e) Muscle tissue weight titration experiment: Number of Pax7⁺ cells·mg⁻¹ isolated as a function of tissue weight before (closed circles) and 24h after exercise (open circles). Note the loss of cell number as tissue weight falls below 50 mg.

Figure S2:

Pax7 and NCAM co-staining controls: S2a &b) Cells stained positive for NCAM (PC5⁺) and Pax7 (FITC/488⁺) positive controls. **S2c)** Histogram showing

unstained control of mononuclear cells isolated from a fresh human muscle biopsy.

Figure S3:

Single positive satellite cell: S3) A muscle cross-section stained for Pax7 (green), C-Met (red) and DAPI (blue). Scale bar = 50µm; higher magnification of box: **i)** C-Met (red), **ii)** nucleus (DAPI, blue), **iii)** Pax7 (green), and **iv)** merged image showing lack of Pax7 co-localization.











257



Supplemental Figures:









Secondary Only Control





Appendix II: Preliminary Data





Main Effect for TIME and AGE:

*p<0.05 vs. OLD **p<0.05 vs. PRE ***p<0.001 vs. PRE and 3h



Appendix II: (**A**): Serum IL-6 following acute unilateral-leg leg exercise before (PRE), 3h, 24h, 48h post-exercise in young (~21y) compared to older men (~70y). Effect of age *p<0.05 vs. Young; effect of time **p<0.05 vs. PRE, ***p<0.05 vs. PRE, 3h and 24h; bar indicates effect of time for both groups. Mean±SEM. (**B**): IL-6 mRNA expression (a.u.) at baseline between young and older males calculated using 2^{-ΔCt} method using β2-microglobulin as the housekeeping gene. (**C**): SOCS3, (**D**): SOCS1 and (**E**): cMyc mRNA expression following acute unilateral-leg leg exercise before (PRE), 3h, 24h, 48h post-exercise in young (~21y) compared to older men (~70y). Effect of age *p<0.05 vs. Old; effect of time **p<0.05 vs. PRE, ***p<0.05 vs. PRE, and 3h; bar indicates effect of time for both groups. Calculated using 2^{-ΔCt} method using β2-microglobulin as the housekeeping gene, Mean±SEM.