SATELLITE CELL REGULATION IN RESPONSE TO ACUTE DAMAGING LENGTHENING CONTRACTION

SATELLITE CELL REGULATION IN RESPONSE TO ACUTE DAMAGING LENGTHENING CONTRACTION: INTERLEUKIN-6 MEDIATED STAT3 INDUCED PROLIFERATION

By

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TITLE: Satellite cell regulation in response to acute damaging lengthening contraction: Interleukin-6 mediated STAT3 induced proliferation

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Abstract

Background: Although the satellite cell (SC) is a key regulator of muscle adaptation following exercise, the regulation of human muscle SC function in response to damaging exercise remains largely unexplored. STAT3 signalling, mediated via interleukin-6 (IL-6), has recently come to the forefront as a potential regulator of SC proliferation. The early response of the SC population in human muscle to muscle-lengthening contractions (MLC) as mediated by STAT3 has not been studied.

Methodology/Principal Findings: Twelve male subjects (21 ± 2 y; 83 ± 12 kg) performed 300 maximal MLC of the quadriceps femoris at a velocity of $180°\cdot s^{-1}$ over a 55° range of motion with muscle samples (*vastus lateralis*) and blood samples (*antecubital* vein) taken prior to exercise (PRE), 1 hour (T1), 3 hours (T3) and 24 hours (T24) post-exercise. Cytoplasmic and nuclear fractions of muscle biopsies were purified and analyzed for total and phosphorylated STAT3 (p-STAT3) by western blot. p-STAT3 was detected in cytoplasmic fractions across the time course, peaking at T24 ($p<0.01$ vs. PRE). Nuclear total and p-STAT3 were not detected at appreciable levels. However, immunohistochemical analysis revealed a progressive increase in the proportion of SCs expressing p-STAT3 with ~60% of all SCs positive for p-STAT3 at T24 ($p<0.001$ vs. PRE). Additionally, *cMYC*, a STAT3 downstream gene, was significantly up-regulated in SCs at T24 versus PRE ($p<0.05$). Whole muscle mRNA analysis revealed induction of the STAT3 target genes *IL-6*, *SOCS3*, *cMYC* (peaking at T3, $p<0.05$), *IL-6Rα* and *GP130*

(peaking at T24, $p<0.05$). In addition, *MYF5* mRNA was up-regulated at T24 ($p<0.05$) with no appreciable change in *MRF4* mRNA.

Conclusions/Significant Findings: We have demonstrated that IL-6 mediated induction of STAT3 signaling occurred exclusively in the nuclei of SCs in response to MLC. An increase in the number of $cMYC^+$ SCs indicated that human SCs were induced to proliferate under the control of STAT3 signaling.

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LIST OF ABREVIATIONS

BSA	Bovine serum albumin
CK	Creatine kinase
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
G1	Gap 1 phase
GAS	γ-interferon activation sequence
HGF	Hepatocyte growth factor
HGFA	Hepatocyte growth factor activator
IGF-1	Insulin-like growth factor I
IL-4	Interleukin-4
IL-6	Interleukin-6
JAK2	Janus kinase 2
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
MGF	Mechano-growth factor
MLC	Muscle-lengthening contraction
MMP	Matrix metalloproteinase
MRFs	Myogenic regulatory factors
MRF4	Myogenic regulatory factor 4
MYF5	Myogenic factor 5
MyoD	Myogenic determination factor

NCAM	Neural cell adhesion molecule
NO	Nitric oxide
PAX3	Paired box transcription factor three
PAX7	Paired box transcription factor seven
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PIAS	Protein Inhibitors of Activated STATs
SOCS1	Suppressor of cytokine signalling 1
SOCS3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3

1: The Satellite Cell: Discovery, Identification and Regulation

a. Satellite Cells: An Overview

The skeletal muscle stem cell or satellite cell (SC) was identified almost fifty years ago [1]. Since then, this unique population of cells, which resides between the sarcolemma and basal lamina, have been shown to be indispensible for skeletal muscle repair [2], hypertrophy [3, 4], and hyperplasia [5, 6] in human and animal models. A better understanding of satellite cells (SCs) would allow us a greater understanding of muscular development, myofiber adaptation, and maintenance of normal functioning muscle [7].

b. Discovery and Identification of Satellite Cells

Prior to the discovery of SCs in 1961, it was thought that skeletal muscle was exclusively postmitotic with no capacity for repair or regeneration [1]. It is now understood that skeletal muscle is capable of repair following even the most traumatic of injuries via the SC. A key to the study of SCs and understanding their function is the ability to easily identify them through the different phases of the myogenic program as they are activated, proliferate and eventually differentiate, donating their nuclei to existing myofibers (figure I).

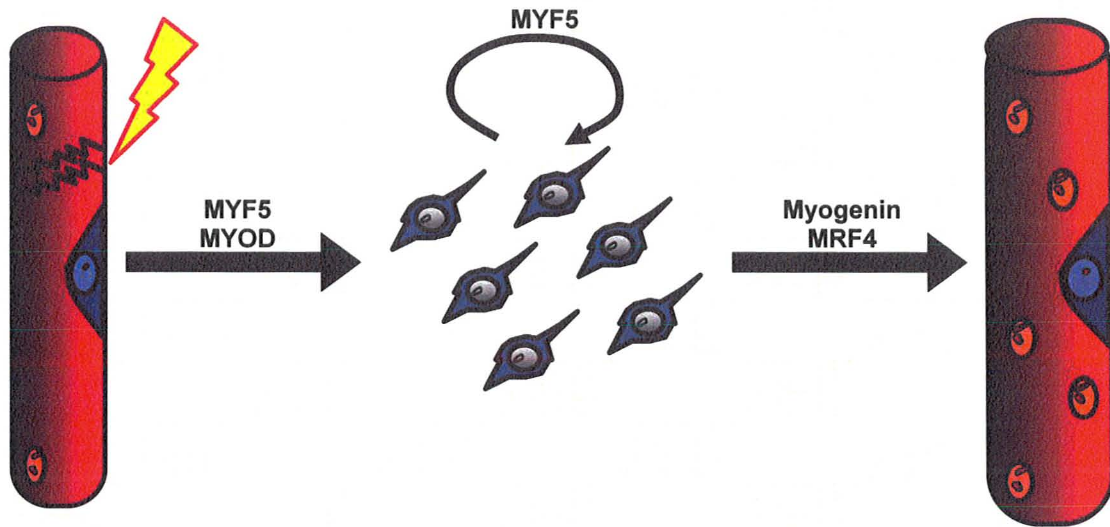

Figure I: The myogenic program. Muscle damage leads to the activation of SCs. Through the induction of MYF5 and MYOD the SCs proliferate. Differentiation is induced through up-regulation of Myogenin and MRF4 resulting in the repair of existing myofibers or the formation of new ones. Adapted from Parise et al. [8].

SCs are located between the basal lamina and the sarcolemma of individual muscle fibers [1, 9]. Additionally, electron microscopy has revealed that SCs possess a high nuclear-to-cytoplasmic ratio, relatively few organelles and a small nucleus that is abundant in heterochromatin when compared to non SC myonuclei. These characteristics are all congruent with the mitotically quiescent state that SCs are found in under basal conditions [7, 10]. Upon activation, these characteristics shift to include a reduction in nuclear heterochromatin, an increase in the cytoplasmic-to-nuclear ratio and number of intracellular organelles, as well as a morphological change characterised by swelling of the cell with processes extending from one or both of the poles [7, 10].

In addition to the aforementioned morphological characteristics, SCs can be identified via cell surface, cytoskeletal, and nuclear transcription markers. These approaches simplify the process of identifying SCs by enabling the use of light microscopy or flow cytometry for analysis. A number of different molecular markers have been used to identify SCs. The most commonly used markers include M-cadherin [11-13], c-MET [14, 13] and neural cell adhesion molecule (NCAM) [14, 15]. Nuclear transcription factors are also used such as myogenic factor 5 (MYF5) and myogenic determination factor (MYOD), which identify activated and proliferating SCs [16, 13, 17]. Paired box transcription factor 7 (PAX7) is also a common marker used to identify quiescent SCs in addition to those that have been activated and are undergoing proliferation [18]. The efficacy of defining SCs based on these markers continues to evolve with regard to human work. McKay et al. [14] using both immunohistochemistry and flow cytometry has shown that PAX7 is the most consistent marker of SCs when compared to c-MET and NCAM.

In addition to some of the morphological and biochemical signatures of SCs, their anatomical position is of great importance. As mentioned, SCs lie between the basal lamina and sarcolemma of muscle fibers. There are, however, additional positional characteristics that they tend to exhibit. For example, a greater number of SCs appear to congregate at motor neuron junctions and around capillaries. In humans 68% of satellite cells are found localized within 5μm from either a capillary or a vascular endothelial cell [19]. Interestingly there appears to be no correlation between fiber type and SC

distribution or mean cross-sectional area of a muscle fiber and SC distribution in healthy human muscle [20].

c. Developmental Regulation of Satellite Cells

Developmental studies of SC biology have revealed that PAX7 is a critical transcription factor in the commitment of high level progenitors to the SC lineage and the maintenance of the SC population. PAX7 is a member of the larger PAX family (1-9) of transcription factors all of which have similar characteristics. These include several conserved elements such as two DNA-binding domains, a paired domain, and a homeodomain [21, 22]. The PAX family encodes genes that are responsible for controlling the processes necessary for development [23, 24].

While both PAX3 and PAX7 appear to induce the expression of MYOD during embryogenesis, postnatally the job appears to fall solely to PAX7 [25]. PAX7 and not PAX3 is expressed in adult human primary myoblasts [23], and it has been shown that PAX7 is necessary for the commitment of muscle presatellite cells into fully functional SCs [26]. Seminal work done by Seale et al. revealed through *in situ* hybridization that *PAX7* mRNA was expressed in SCs residing in adult skeletal muscle [18]. Work by Braun and colleagues demonstrated that Pax7 was essential for renewal and maintenance of the SC population [27].

d. The Myogenic Program

Repair of damaged muscle tissue appears to mimic the later stages of embryonic myogenesis [28]. The repair process, from the point of satellite cell quiescence to terminal differentiation is referred to as the "myogenic program" [8]. Most of the work delineating the process of the myogenic program has come from animals specifically examining embryogenesis; however, it is thought that in the human, SC differentiation is a process that shares great similarities with myogenesis during embryonic development. There are four main transcription factors that orchestrate the myogenic program. Collectively they are called the myogenic regulatory factors (MRF). These transcription factors drive the SC from quiescence through to terminal differentiation.

MYF5 is the first of the MRFS to be up-regulated. It was shown that *MYF5* is an important target gene for PAX7 [29]. The expression of MYF5 is the defining characteristic of a SC that is fully committed to the myogenic lineage and has the capacity to proliferate [16, 30-32]. Recently it has been found that there are two sub-types of satellite cells; those that have never expressed MYF5 and those that have [33]. It is proposed that those that have never expressed MYF5 are SCs responsible for maintaining a basal SC number and only produce daughter cells that enter the myogenic program in situations where extreme damage occurs. Those that have expressed MYF5 are responsible for the normal myonuclear turnover that occurs as a result of everyday damage of skeletal muscle [34].

The other proliferative MRF is MYOD. MYOD is expressed during the late phases of proliferation and is required for the cell to begin differentiation [35, 36]. Once a SC expresses MYOD it is termed a myoblast. As differentiation begins, both MYOD and MYF5 are turned off [37]. It is of interest to note that newborn mice that are deficient in both MYF5 and MYOD lack both myoblasts and myofibers punctuating their importance to muscle development [38].

Following a decline in MYOD expression, myogenin production is induced during the early stages of differentiation. With the expression of myogenin, the myoblast is termed a myocyte and is ready to either fuse with existing myofibers or form new myotubes by fusing with other myocytes. Myogenin is essential for the terminal differentiation of myoblasts [13, 39, 40]. Finally myogenic regulatory factor 4 (MRF4) is up-regulated up to several days after the initial cues for the myocyte to differentiate allowing for terminal differentiation to take place and marking the end of the myogenic program [41, 42].

e. Contraction-induced Damage: Skeletal Muscle and the Satellite Cell Response

A lengthening contraction is characterized by an active muscle attempting to contract in a concentric manner but is forced, either voluntarily or due to the load exceeding the muscle's force generating capacity, instead to lengthen. During lengthening a muscle can produce more force than a concentrically contracting muscle. Furthermore, during lengthening contractions there are fewer active motor units and subsequently fewer active fibers [43, 44]. Because of the increase in force production

during an eccentric action, combined with the decrease in the number of active fibers, those that are active generate a much higher active force per active muscle cross-sectional area, which can lead to a greater stimulus for damage.

Lengthening contractions have been shown to lead to a greater degree of muscle damage than either concentric or isometric actions [45]. A number of studies have shown that this damage can take many different forms. These include primary or secondary sarcolemmal disruption, swelling or disruption of the T tubules, damage to myofibrillar contractile components, cytoskeletal damage including Z disk streaming and other extracellular changes to the myofiber matrix [46]. It is interesting to note that although both type I and type II fibers are affected it appears that type II fibers are more susceptible to damage [47]. It has been well documented that following lengthening contractions, serum creatine kinase (CK) levels are increased from 24-48 hours after exercise and may remain elevated for up to a week. Since CK is a muscle specific enzyme, its appearance in the blood can be interpreted as a disruption of the muscle fiber membrane [46] indicating that muscle damage has occurred.

The general adaptive response to the damage caused by lengthening contraction starts with inflammation. Exercise induced damage promotes the infiltration of leucocytes and monocytes from the circulation. These inflammatory cells respond to a number of different chemotactic signals including the release of interleukin-6 (IL-6) [48]. This infiltration of inflammatory cells leads to proteolytic digestion and the subsequent degradation of damaged structures [49, 50]. This transient degradation of the muscle,

however, enables tissue remodelling to take place with the potential for hypertrophy. The cells that are largely responsible for tissue remodelling are the SCs [46].

A number of studies in humans have reported a SC response following an acute bout of eccentrically biased exercise [51-54]. Although the direct mechanisms leading to the increase in SC number following muscle damage remains unclear, there is little doubt that the SC response is closely tied to myotrauma. It has been shown that ultrastructural muscle damage can result in SC activation and that individuals who exhibit the greatest ultrastructural damage also appear to show the greatest number of activated SCs [55]. This provides very compelling evidence that the SC response is tightly linked to the necessary repair and adaptation of muscle to exercise.

f. Molecular Regulation of Human Muscle Satellite cells

Animal models have been extensively used to delineate the regulation of SCs, however, relatively little is known about the molecular regulation of SCs in humans. The regulation of SCs is very complex and depends on a number of factors including the perturbation itself, type/amount of damage, age, gender and individual genetic factors. Furthermore, the continuum from activation to full differentiation requires different molecular regulators working in concert with each other to achieve the desired goal of muscle repair and potential hypertrophy. While a number of molecules have recently been identified as having the potential to regulate SC function (figure II) very few have been studied and shown to affect human satellite cells *in vivo*. Those that have include

hepatocyte growth factor (HGF), insulin-like growth factor 1(IGF-1), interleukin-4 (IL-4) and interleukin-6 (IL-6).

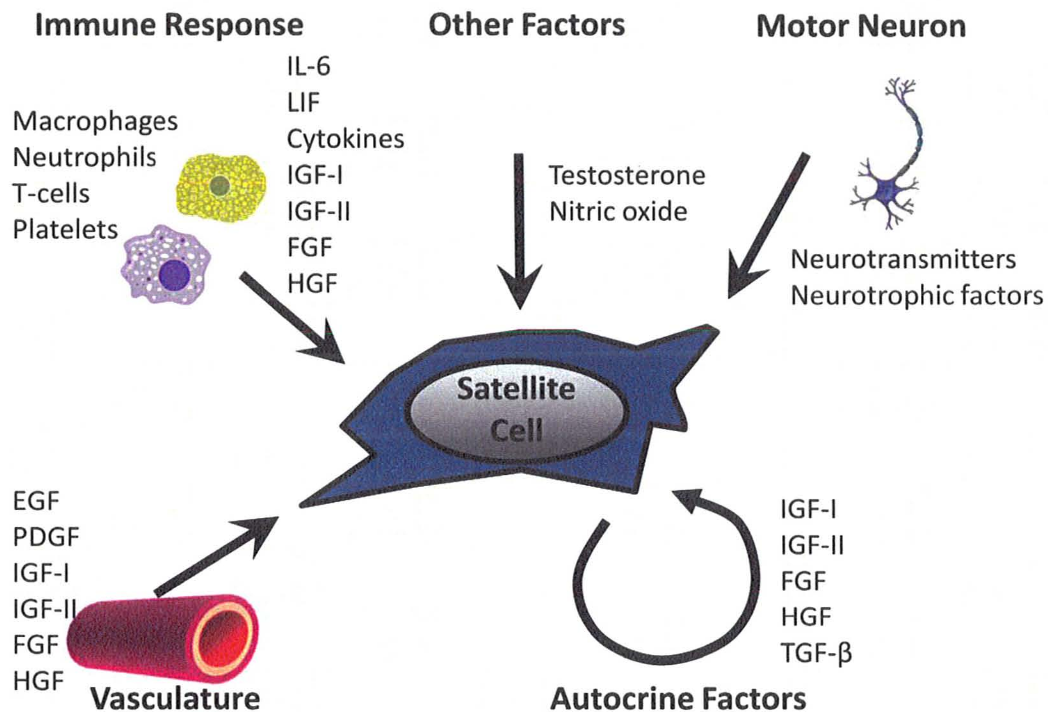

Figure II: Factors regulating SC function. A number of different factors have been shown to regulate SC function but few have been studied *in vivo* in humans. These factors have been shown to be released from a number of different sources making the SC niche an important area of study. Adapted from Hawke and Garry [7].

i. Hepatocyte Growth Factor (HGF)

HGF appears to be important to the activation of SCs. In response to micro-damage or myofiber stretch, nitric oxide can be released, which in turn leads to the release of HGF from the muscle extracellular matrix [56, 57]. It has been shown through

immunoneutralization experiments in rat SCs that HGF is the primary factor capable of inducing quiescent SCs to become activated and enter the cell cycle [58]. Work in humans has demonstrated that SCs express the HGF receptor, c-met [14]. A study by O'Reilly and colleagues reported an increase in serum HGF 4 hours following muscle damaging exercise. While no significant change was observed in whole muscle HGF protein concentration, HGF activator (HGFA) protein showed a significant up-regulation 24 hours after muscle damage with HGFA inhibitor-1 and -2 not peaking until 72 hours post muscle damage [53]. These data showed that muscle does produce HGF and that its early expression coincided with an increase in NCAM$^+$ cells. Once HGF had led to the activation of SCs, it appears that HGFA-1 and -2 then turn off the signalling cascade allowing for differentiation to occur.

ii. Insulin-like Growth Factor 1 (IGF-1)

There are three different splice variants of IGF-1 in the human; IGF-1Ea, Eb and Ec (otherwise known as mechano-growth factor (MGF)). Each of these variants appears to have differing effects on human SC function. Both *IGF-1Ea* and *IGF1eB* were shown to peak 72 hours post muscle damage and remained elevated at 120 hours post [52]. *MGF* was up-regulated early peaking at 24 hours post and was then rapidly down-regulated. The regulation of these splice variants also correlated with *MRF* mRNA expression [52]. It was shown that *IGF-1Ea* and *-Eb* correlated significantly with *MRF4*, potentially linking them with the process of differentiation. Conversely, *MGF* was significantly correlated with *MYF5* and therefore may play a role in proliferation. A pan-

IGF-1 antibody was used for immunohistochemical co-localization of IGF-1 to PAX7 which showed there were IGF-1^{+}/PAX7^{+} cells at T24 and T72, potentially showing the MGF splice variant co-localized early and the IGF-1Ea or -Eb variant co-localized later following the muscle damaging protocol [52].

iii. Interleukins

Interleukins and their effect on SCs is an area of great interest. Again following 300 eccentric contractions, interleukin-4 (IL-4) protein was shown to be co-localized with PAX7^{+} cells 4, 24, and 72 hours post damage. Furthermore, *IL-4* and *IL-4 receptor* mRNA species showed a significant 4.4 fold increase in expression. In addition, suppressor of cytokine signalling 1 (*SOCS1*), a downstream target gene of the IL-4 signalling cascade and a negative regulator of the IL-4 mediated signalling, was up-regulated 2.9 fold 4 hours post damage [59]. These data provide evidence that IL-4 may potentially regulate SC function in response to acute myotrauma possibly to recruit other myoblasts and promote their fusion [60].

IL-6 is the interleukin with the most compelling evidence as a regulator of SCs in humans. McKay and colleagues [51] showed that following 300 lengthening contractions serum IL-6, *IL-6* mRNA and *IL-6 receptor* mRNA were up-regulated 4 hours post muscle damage. This was followed by a significant increase in IL-6^{+}/PAX7^{+} cells at 24 hours post muscle damage. Furthermore, p-STAT3 was shown to co-localize with PAX7. In addition, *CYCLIN- D1* and *SOCS3* mRNA species were up-regulated, both downstream genes regulated by IL-6 induced STAT3 signalling. These data suggest that the IL-

6/STAT3 signalling cascade may be responsible for the proliferation of SCs in response to acute myotrauma, however STAT3 downstream intermediate gene expression was only measured in whole muscle and it remains unknown whether all aspects of the STAT3 pathway are localized to SCs.

2: Interleukin-6 and the Satellite Cell

a. The Discovery of Interleukin-6

IL-6 was first discovered in 1980 and was named interferon-β2 [61]. It was subsequently identified and named on eleven different occasions due to its pleiotropic effects on a variety of different cell types including B cells, T cells, hepatocytes, macrophages, and hybridoma cells. In 1989 it was found that these variously named cytokines were identical and the name IL-6 became the standard [62]. Traditionally IL-6 was seen as an inflammatory cytokine due to its role in a number of inflammatory states such as seen in those afflicted with asthma, chronic low-grade inflammatory conditions like Type 2 diabetes or with severe infection [63].

b. Interleukin-6 Production in Human Muscle

Not until relatively recently was it understood that IL-6 was also released from skeletal muscle into the circulation in response to exercise [64]. With this discovery, IL-6 became known as a "myokine" [65, 66]. The manner in which IL-6 was released from muscle into circulation, however, was still not understood. It was believed that the

release was a consequence of an immune response whereby exercise led to cytoskeletal damage and resident immune cells in skeletal muscle were induced to release IL-6. The IL-6 then acted as a chemotactic stimulus for the migration of additional immune cells and additional release of IL-6 [67, 49]. The other school of thought was that it was the muscle fibers themselves that were releasing IL-6 [65]. It is of interest to note the difference between the general systemic cytokine response between sepsis and exercise conditions. Under septic conditions IL-6 release was preceded by tumour necrosis factor α release, which was not the case during exercise where it was observed that IL-6 was the first cytokine to be up-regulated. In both conditions, however, the increase in systemic IL-6 concentration was much greater, which provides at least temporal evidence that it plays an important role in the general cytokine response [66].

The true cellular source of IL-6 release was first revealed by Penkowa et al. in 2003 [68]. In response to cycling at progressively increasing workloads, muscle cross-sections were immunostained for IL-6. From PRE to 21 hours post exercise, IL-6 was present in muscle fibers [68] and was later found to be released predominantly by type I fibers [69]. These two studies provide at least qualitative evidence supporting the concept of skeletal muscle production and release of IL-6. Definitive evidence of IL-6 release from individual myonuclei was provided through an *in situ* hybridization experiment demonstrating *IL-6* mRNA elevated in skeletal muscle fibers [70]. In contrast to previous work, both IL-6 mRNA and protein appeared to co-localize to a greater degree to type II fibers [70].

While IL-6 has been shown to be released from human muscle fibers in response to exercise, there appears to be an interplay between inflammatory cells and muscle cells in using the released IL-6. Inflammatory cells allow for proteolytic digestion of muscle structures in response to damage. These inflammatory cells follow the chemotactic stimulus of IL-6 released from damaged muscle fibers. Then, chemotactic agents released from the inflammatory cells, signal newly activated SCs to migrate to the area of damage leading to its repair [49]. Furthermore, because the inflammatory cells phagocytose the damaged muscle structures breaking down collagenous and fibrous tissue, the SCs are able to migrate to the sites of injury with greater efficiency expediting the repair process [49]. While it has not been definitively proven that human SCs produce IL-6 *in vivo*, it has been shown *in vitro* suggesting that IL-6 may signal in an autocrine manner [50]. It is believed that a similar mechanism exists *in vivo*.

c. An Emerging Role for Interleukin-6 in Human Satellite Cell Function

The work on IL-6 mediated signalling in human muscle is relatively limited. Keller and colleagues [71] first reported that the *IL-6 receptor* was expressed in contracting human skeletal muscle. More recently, the IL-6 response in humans following a damaging bout of muscle lengthening contractions was characterized. It was reported that SC number was significantly increased 24 hours post damage and that IL-6 positive SCs were significantly increased at that time as well. In addition, IL-6 mRNA and protein both peaked 4 hours following damage. Finally, p-STAT3 was shown to co-localize with SCs 4 hours post damage. The increase in SC number, which followed an

up-regulation of *IL-6* mRNA and protein, suggested a major regulatory role for IL-6 in the proliferation of SCs [51]. It has also been previously reported that MYF5 and MYOD mRNA, following the same muscle damaging protocol, peaked at 4 and 24 hours respectively [53]. Collectively, this suggests that IL-6 signalling through a JAK2/STAT3 mediated pathway may play a role in inducing the proliferation of SCs while its absence may be necessary for differentiation to occur in humans following damaging muscle lengthening contractions.

d. Regulation of the JAK/STAT Pathway

The JAK/STAT signalling pathway is a highly conserved cascade that has been shown to transduce a wide variety of signalling events from the *Drosophila* to the human [72, 73]. JAKs function via binding on the intracellular domain of transmembrane receptors that have JAK binding domains. This leads to tyrosine residue phosphorylation on the receptor complexes as well as of STAT molecules [74]. JAKs allow for STATs to be phosphorylated by cytokine receptors as they are not able to phosphorylate kinases themselves [75].

STAT proteins are found sequestered in the cytoplasm where they remain dormant until activated by receptor stimulation. STAT3 specifically, is activated by phosphorylation of the tyrosine residue 705 located near the C-terminus [74] . Once phosphorylated, STATs can then form dimers via reciprocal phosphotyrosine and Src homology 2 domain interactions. These dimers then enter the nucleus via interaction with the nuclear import proteins importin α and importin β [76]. Once in the nucleus STAT dimers are able to bind to DNA. Most STAT dimers have a particular affinity for

an 8-10 base pair sequence of 5'-TT(N_{4-6})AA-3' which has been termed a GAS element after its initial discovery as a γ-interferon activation sequence.

e. Interleukin-6 Mediated JAK2/STAT3 Signalling

The JAK/STAT signalling family members induced by IL-6 in skeletal muscle are JAK2 and STAT3 [77, 51, 78]. The IL-6/JAK2/STAT3 pathway (figure III) serves to up-regulate a number of different genes including *IL-6* itself, *GP130*, *IL-6Rα*, *SOCS3*, *cMYC* and *CYCLIN-D1*.

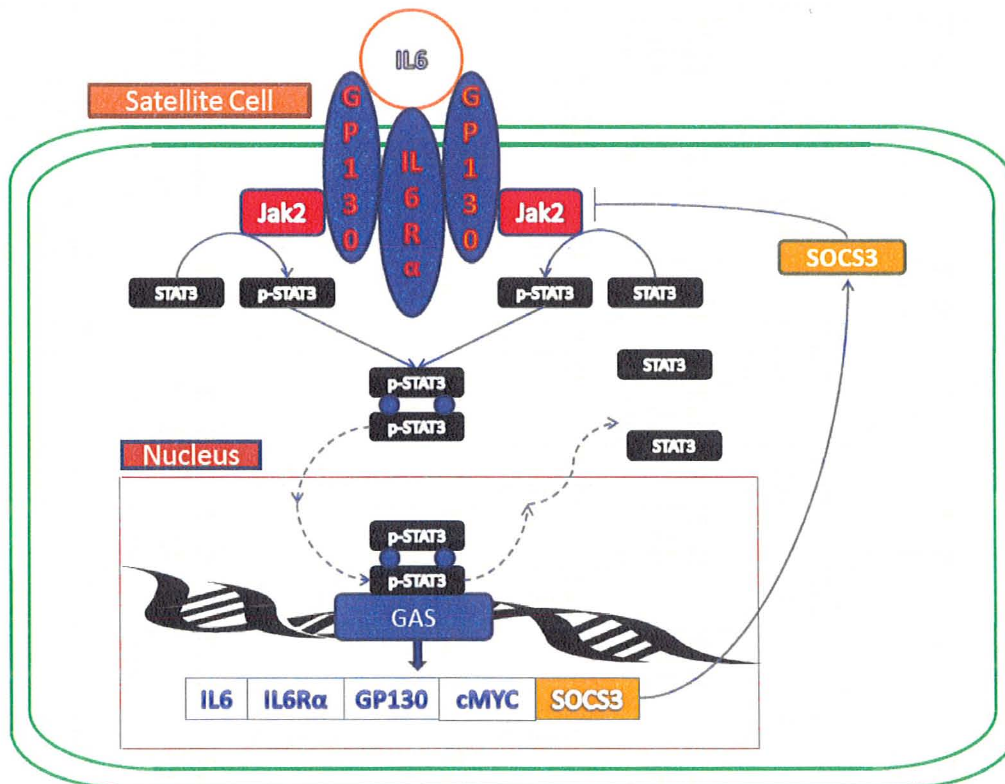

Figure III: The IL-6/JAK2/STAT3 signalling cascade. IL-6 binds to its receptor which is composed of the two subtypes IL-6Ra and GP130. This leads to the phosphorylation and homodimerisation of STAT3. STAT3 then translocates to the nucleus, binds to the GAS element and leads to the up-regulation of a number of downstream genes including *IL-6* itself, the receptor subtypes, *SOCS3* and *cMYC*.

The proliferative capacity of the IL-6 mediated JAK2/STAT3 signalling pathway means that there must also be a very definite means of controlling that capacity to avoid the onset of pathological events such as tumour growth. For example, *cMYC* is a proliferative gene that has been linked to many different forms of cancer when it's usual regulatory influences no longer inhibit it from uncontrolled proliferation [79, 80]. This risk is mitigated by a number of different and sometimes redundant means, controlled by the JAK2/STAT3 pathway.

Production of both IL-6 and its receptor complexes serve as the initial regulator of the pathway [81, 75]. JAK2 has its own negative regulator called suppressor of cytokine signalling 3 (SOCS3). SOCS3 acts via a classic feedback inhibition whereby its production is up-regulated in response to JAK2/STAT3 circuitry. As a result of this up-regulation, SOCS3 aids in the suppression of JAK2 signalling via binding phosphotyrosines on the cytokine receptor complexes, binding directly to JAK2 and through recruitment of ubiquitin-transferases [74]. These three processes serve to either reduce the ability of JAK2 to be phosphorylated, phosphorylate downstream signalling cascade members, or lead to proteosomal degradation of the JAK2 protein [74, 66]. Furthermore, STAT function is limited by protein tyrosine phosphatases and by protein inhibitors of activated STATs (PIAS). These methods function via dephosphorylating STAT dimers [82, 83] and by inhibiting STAT dimers from binding to a promoter region respectively [84]. Finally, SOCS3 can also bind phosphotyrosines on the cytokine receptor complex rendering it impossible for STAT3 to be recruited and phosphorylated [74]

f. STAT3 Signalling as a Regulator of Satellite Cell Proliferation

STAT3 has recently been identified as a potential regulator of SC function in animal models. It was found, via co-stain with MYOD that p-STAT3 was present in myoblasts during proliferation and was absent once the process of differentiation began [78]. In addition, it was determined that intact and uninjured mouse muscle homogenate had no detectable p-Stat3 but after muscle damage, its presence was detected [77]. Furthermore, using IL-6 knockout mice it was found that there was almost full inhibition of STAT3 signalling after a chronic overload stimulus as the number of p-STAT3^{+} SCs remained at baseline levels [77]. This indicated an impaired proliferative capacity due to a lack of IL-6 mediated STAT3 signalling.

Following this work, *in vivo* data in humans has now shown similar results whereby STAT3 signalling occurred in activated myonuclei in response to acute resistance exercise [85]. They expanded upon this discovery by examining potential differences in young and elderly populations to identify dysregulation of the pathway as a potential mechanism explaining delayed recovery and ultimately the sarcopenic phenotype observed with aging [86]. Increased p-STAT3 signalling was observed in the elderly human population, specifically with an increase seen in SOCS3 production. This indicates the potential for differential regulation of muscle between younger and older men in response to resistance exercise. This work by Trenerry and colleagues [85, 86] however, did not make an attempt at SC quantification. McKay and colleagues followed these observations and showed that human SCs expressed p-STAT3 in response to a damaging bout of exercise and that a number of the downstream genes regulated by

STAT3 were also up-regulated [51]. This provided the first evidence that p-STAT signalling was occurring in the human SCs potentially leading to their proliferation.

g. Rational for Research

While our understanding of the human SC continues to grow, many aspects of SC regulation remain unknown. IL-6 has been shown to be imperative for SC proliferation and subsequent hypertrophy in mice. Furthermore, recent work has shown a role for IL-6 in the human SC. IL-6 has come a long way from being thought of solely as playing a role in the inflammatory response. We now know that as muscle is damaged, IL-6 is released from the muscle fibers. This leads to the infiltration of a number of inflammatory cells to help phagocytose damaged structures and initiate the repair process [49]. The released IL-6 can also be utilized by the SCs to either increase their own proliferative capacity and/or to produce additional IL-6 to further the proliferative response. Having these different yet sequential processes sharing common signalling molecules allows for greater control of the end result which is muscle repair and adaptation. Because of its diverse effects and potential to play a significant role in many processes of muscle repair and adaptation IL-6 and its downstream intermediates such as STAT3 are excellent candidates to study in greater detail.

While focused research is needed to understand how the SC is regulated, a broader understanding of the potential impacts and the potential therapeutic benefit that IL-6 related research can have is imperative. Muscular dystrophy is debilitating and affects millions of people the world over [87]. In addition, as the average age of the population increases, sarcopenia is becoming an even greater threat not only to the

elderly who are experiencing it but also to the healthcare system as it bends under the burden of age-related pathology. The understanding of SC regulation will aid in the development of therapeutic strategies for diseases associated with muscle wasting and aging.

h. Statement of Research Questions and Hypothesis

It has been shown in animal models that IL-6 is necessary for hypertrophy and that this hypertrophy is caused by the proliferative capacity of SCs. What is less clear are the exact mechanisms through which IL-6 regulates SCs in the human. The goal of this study was to expand upon previous work done by McKay and colleagues [51] to better understand the downstream signalling events initiated by IL-6 leading to the observed increase in the SC population. Through the analysis of the IL-6/JAK2/STAT3 signalling cascade, we aimed to better understand the effects this pathway has on SC proliferation. By collecting muscle biopsies after an exercise protocol that has been shown to produce muscle damage, we examined the mRNA signature of this pathway to see the timeline of its up- and down-regulation. We also looked at functional proteins that are produced as a consequence of this pathway being active via both immunohistochemical and western blot techniques. Furthermore, through both serum and plasma collection we will be able to understand the systemic response of IL-6 to the muscle damaging protocol. Most importantly we will be able to quantify the SC response as a result of the muscle damage and the IL-6/JAK2/STAT3 signalling cascade specifically in the SC compartment.

We propose the following hypotheses:

1. IL-6^{+}/PAX7^{+} cells will be increased from PRE to 24 hours post.
2. The nuclear portion of whole muscle homogenate will show no change in p-STAT3 in response to muscle damage. The immunohistochemical stains, however, will show p-STAT3^{+}/PAX7^{+} cells with very few, if any, other cells expressing p-STAT3.
3. p-STAT3+/PAX7^{+} cells will show a similar increase to that seen with IL-6^{+}/PAX7^{+}.
4. The mRNA species of the *IL-6/JAK2/STAT3* pathway along with some of their downstream genes will be up-regulated in response to muscle damage. The pathway members will show a sequential up-regulation in line with the pathway being activated starting with *IL-6* itself up early at 1 or 3 hours post and followed by the downstream genes including *cMYC* up-regulated 24 hours post.
5. The myogenic regulatory factor member *MYF5* will be up-regulated by 24 hours post while *MRF4* will remain at baseline expression over the time-course.

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The following manuscript has been submitted to PLoS ONE for review

IL-6 Induced STAT3 Signalling Promotes Proliferation of Human Muscle Satellite Cells Following Acute Muscle Damage

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Abstract

Background: Although the satellite cell (SC) is a key regulator of muscle growth during development and muscle adaptation following exercise, the regulation of human muscle SC function remains largely unexplored. STAT3 signalling mediated via interleukin-6 (IL-6) has recently come to the forefront as a potential regulator of SC proliferation. The early response of the SC population in human muscle to muscle-lengthening contractions (MLC) as mediated by STAT3 has not been studied.

Methodology/Principal Findings: Twelve male subjects (21 ± 2 y; 83 ± 12 kg) preformed 300 maximal MLC of the quadriceps femoris at a velocity of $180° \cdot s^{-1}$ over a 55° range of motion with muscle samples (*vastus lateralis*) and blood samples (*antecubital* vein) taken prior to exercise (PRE), 1 hour (T1), 3 hours (T3) and 24 hours (T24) post-exercise. Cytoplasmic and nuclear fractions of muscle biopsies were purified and analyzed for total and phosphorylated STAT3 (p-STAT3) by western blot. p-STAT3 was detected in cytoplasmic fractions across the time course peaking at T24 ($p<0.01$ vs. PRE). Nuclear total and p-STAT3 were not detected at appreciable levels. However, immunohistochemical analysis revealed a progressive increase in the proportion of SCs expressing p-STAT3 with ~60% of all SCs positive for p-STAT3 at T24 ($p<0.001$ vs. PRE). Additionally, *cMYC*, a STAT3 downstream gene, was up-regulated in SCs at T24 versus PRE ($p<0.05$). Whole muscle mRNA analysis revealed induction of the STAT3 target genes *IL-6, SOCS3*, *cMYC* (peaking at T3, $p<0.05$), *IL-6Rα* and *GP130* (peaking at

T24, $p<0.05$). In addition, *MYF5* mRNA was up-regulated at T24 ($p<0.05$) with no appreciable change in *MRF4* mRNA.

Conclusions/Significant Findings: We demonstrate that IL-6 mediated induction of STAT3 signaling occurred exclusively in the nuclei of SCs in response to MLC. An increase in the number of cMYC^{+} SCs indicated that human SCs were induced to proliferate under the control of STAT3 signaling.

Introduction

Muscle satellite cells (SCs) are a population of cells that reside between the sarcolemma and basal lamina of myofibres [1] and have been shown to play an integral role in skeletal muscle repair [2], hypertrophy [3, 4], and hyperplasia [5, 6]. Although SCs are key regulators of muscle growth during development and muscle adaptation following exercise [7-15], the cellular regulation of human muscle SC function remains largely unexplored. Undoubtedly, the orchestration of events that govern SC function following damage involves a complex milieu of factors originating from the SC in addition to niche factors extrinsic to the SC [16]. Identified regulators of human SCs include insulin like growth factor-1 [17], hepatocyte growth factor [18], transforming growth factor beta [19] and Notch/Delta [7]. Recently, interleukin-6 (IL-6) has been implicated as playing a role in the regulation of human SCs in response to damaging lengthening contractions [20].

Traditionally, IL-6 was considered an inflammatory cytokine [21], however, recent work has shown that IL-6 is produced by muscle [22], released into circulation

[23] and can act on the muscle cells themselves. As such, IL-6 is now also referred to as a "myokine" [24, 25]. Importantly, IL-6 knockout mice demonstrated a greatly reduced hypertrophic response and less SC-mediated myonuclear accretion compared to wild-type mice following compensatory hypertrophy [26]. 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) signalling [26]. We have recently reported an increase in IL-6 protein localized in SCs 24 hours following a damaging bout of muscle-lengthening contractions (MLC) in humans coinciding with an increase in *CYCLIN-D1* expression and SC number [20]. These data indicate that IL-6, acting via the janus kinase 2 (JAK2)/STAT3 signalling pathway, may be involved in SC proliferation/activation.

STAT3 is a downstream target of IL-6 [27, 28], and in response to IL-6 binding, STAT3 is phosphorylated via JAK2. This leads to the subsequent homodimerization and translocation of p-STAT3 to the nucleus [29]. Once in the nucleus, p-STAT3 binds to the γ-interferon activation sequence (GAS) element where it then promotes the transcription of downstream genes [30]. These genes have been shown to be responsible for a number of cellular functions including proliferation, migration, as well as anti-apoptotic functions [26]. *cMYC* is a downstream target gene in the STAT3 signalling cascade. It has been shown to regulate cell-cycle kinetics through the up-regulation of a number of Cyclin proteins which are involved in the cell growth phase G1 [31-33]. Furthermore, STAT3 also regulates a number of its upstream signalling cascade members including IL-6, GP130, IL-6Rα and suppressor of cytokine signalling 3 (SOCS3). The STAT3 pathway

is regulated in a negative feedback loop through interactions with JAK2 [34]. SOCS3 can bind phosphotyrosines on JAK2 and physically block STAT3 from binding to JAK2. Additionally, SOCS3 can recruit ubiquitin-transferases leading to the ubiquitination and degradation of JAK2 [29].

Based on previous work by McKay and colleagues [20] we aimed to elucidate the time course of SC localized STAT3 signalling. We hypothesized that the STAT3 family members would be up-regulated early following the MLC protocol along with a similar increase, as seen previously, in the IL-6^{+}/PAX7^{+} cell population. Furthermore, using a time course directed at capturing STAT3 phosphorylation, we hypothesized that we would observe an increase in p-STAT3 specifically in the SC population coinciding with an increase in SC number. In addition, we hypothesized that cMYC, a downstream product of STAT3 signalling, would be detected with mRNA up-regulated in whole muscle and protein co-localized to SCs following an increase in STAT3 signalling in the SC population.

Results:

To confirm that the MLC protocol caused muscle damage we examined serum creatine kinase (CK) levels over the time course. Serum CK peaked 24 hours post MLC, increasing over 200% from PRE levels ($p<0.05$ vs. PRE) (figure 1a). Serum IL-6 also peaked 24 hours post-MLC ($p>0.05$ vs. PRE), as well, increasing over 100% from PRE (figure 1b). These measures were correlated indicating a relationship between CK release and serum IL-6 (R^2=0.3055; $p<0.001$) (supplemental figure 1)

In response to acute muscle damage we observed a 26.6% increase in $PAX7^+$ cells 24 hours following the MLC protocol with $PAX7^+$ cells per 100 myofibers increasing from ~15.5 at PRE to ~19.6 ($p<0.05$) 24 hours post exercise. When expressed as a percentage of total myonuclei we observed a 60.3% increase in satellite cell number (~3% at PRE to ~5% 24 hours post; $p>0.05$) (figure 1d). Satellite cells were quantified using a PAX7/Laminin co-stain (figure 1e) to ensure that all $PAX7^+$ cells were in the SC niche. Furthermore, myogenic regulatory factor 5 (*MYF5*), known for its role in SC proliferation was significantly up-regulated 1.8 fold 24 hours following the MLC ($p<0.05$) (figure 1c), while no significant change was observed for myogenic regulatory factor 4 (*MRF4)* (data not shown), known for its role in differentiation.

The number of SCs expressing IL-6 was quantified (figure 2a-d). While there were some co-positive SCs PRE, there was a significant increase in $IL\text{-}6^+/PAX7^+$ cells at 3 and 24 hours post MLC vs. PRE ($p<0.05$) with the peak observed at 3 hours post where 75% of $PAX7^+$ cells were co-positive for IL-6 (figure 2e).

Previous attempts to quantify the STAT3 response did not show a significant change at the whole muscle level, thus the nuclear and cytoplasmic fraction of whole muscle was examined for a more location-specific analysis. Purity was assessed using lactate dehydrogenase (LDH) as a cytoplasmic marker and p84 as a nuclear marker (supplemental figure 2). Cytoplasmic p-STAT3 was significantly up-regulated 24 hours vs. PRE ($p<0.05$) when measured against t-STAT3 which remained unchanged over the time course (figure 3a). When the nuclear fractions were analysed; however, no

detectable p-STAT3 and only traces of t-STAT3 were observed (figure 3b). JAK2 was also analyzed in the cytoplasmic fraction (figure 3c). The ratio of p-JAK2 to t-JAK2 was not significantly different at any time point (figure 3d).

Although changes in p-STAT3 were not detectable in the enriched nuclear fractions via western blot, immunofluorescent analysis of p-STAT3 (figure 4a-b) illustrated an increase in p-STAT3$^+$/PAX7$^+$ cells as a percentage of total PAX7$^+$ cells ranging from 20% PRE to 40% at 1 hour, 50% at 3 hours, and 60% at 24 hours (all significantly different vs. PRE $p<0.05$) (figure 4c). Diffuse p-STAT3 staining was observed throughout the muscle fibres and appeared to intensify over the time course (figure 4a-b), which agreed with the increased cytoplasmic p-STAT3 at 24 hours post observed using western blot analysis. Importantly, the diffuse p-STAT3 observed in the fibre almost never co-localized with non-satellite cell myonuclei. To further verify that p-STAT3 signalling was indeed occurring, downstream target genes of STAT3 were analyzed. *IL-6* mRNA peaked at 3 hours, up approximately 150 fold from PRE ($p<0.05$) and remained elevated, up ~80 fold from PRE ($p<0.05$) at 24 hours (figure 5a). Both *IL-6Rα* (figure 5b) and *GP130* (figure 5c) mRNA showed significant increases peaking at 24 hours up 7 fold from PRE ($p<0.05$) and 4.5 fold from PRE ($p<0.05$) respectively. *SOCS3* also increased, peaking at 3 hours, up 13 fold ($p<0.05$) and remaining elevated at 24 hours up 8 fold ($p<0.05$) (figure 5d) from PRE. Furthermore, the expression of *SOCS3* was positively correlated ($R^{2}=0.5984$, $p<0.001$) with the expression of *IL-6* across time (supplemental figure 3). The cell-cycle related gene *cMYC* was robustly up-regulated from PRE over the entire time course peaking at 4 hours up 15 fold ($p<0.05$) (figure 6a).

cMYC mRNA was positively correlated with *IL-6* mRNA ($R^2 = 0.2876$, $p<0.001$) (supplemental figure 4) and *SOCS3* mRNA ($R^2 = 0.5406$, $p<0.001$) (supplemental figure 5) illustrating a positive relationship in the temporal expression of these STAT3 target genes.

To further verify the mRNA data illustrating an increased expression of *cMYC*, immunohistochemical analysis of cMYC was coupled with PAX7 in serial sections (figure 6c-d) and the number of $cMYC^+/PAX7^+$ cells was quantified PRE and 24 hours following MLC. The percentage of $cMYC^+/PAX7^+$ cells was significantly increased at 24 hours (~40%) versus PRE (~9%) (figure 6b). This provides additional evidence that the STAT3 signalling cascade was active as a consequence of the bout of MLC in SCs leading to proliferation of the SC population.

Discussion

Satellite cell (SC) proliferation is a complex process regulated by a number of factors, such as IL-6, IGF-1, HGF and TNFα [17, 35, 26, 18, 36], among others. It is a process vital for continued functional capacity and overall tissue survival of skeletal muscle. Skeletal muscle fibres are post-mitotic thus the SC is the exclusive source of new nuclei for the maintenance of healthy skeletal muscle. Here we illustrate the significance of STAT3 signalling in promoting SC proliferation following acute muscle damage in humans. We have demonstrated that following muscle damage, p-STAT3 in SCs increases early (within one hour), inducing downstream target genes (i.e. *GP130* and *SOCS3*), which further regulate the increase in STAT3 production and response (as

induced via IL-6), leading to increased *cMYC* expression, which drives cell proliferation. The increase in p-STAT3$^+$/PAX7$^+$ cells over the time course as measured via immunohistochemistry and lack of p-STAT3 in the nuclear fraction strongly supports the premise that p-STAT3 signalling occurs almost exclusively in SCs in human muscle. Further evidence is provided by the expansion of the cMYC$^+$/PAX7$^+$ cell population from PRE to 24 hours expressed as a percentage of total PAX7$^+$ cells. Collectively, the time-course utilized in this study was successful in capturing a rapid up-regulation in p-STAT3 signalling in SCs, which was associated with an increase in SC number following exercise induced muscle injury.

In agreement with a previous study [20], up-regulation of IL-6 mRNA, increased IL-6 in the blood, and more IL-6 co-localized to the SC was observed in response to MLC. The ~1.5 fold increase in serum IL-6 supports a number of studies reporting that IL-6 is released from the muscle into circulation in response to MLC [20, 25, 37]. *IL-6* mRNA was significantly up-regulated 3 hours and 24 hours post MLC, up ~150 fold and ~100 fold respectively. Finally, IL-6$^+$/PAX7$^+$ cells increased from PRE, where there were relatively few co-positive cells, to a peak at 3 hours where approximately 75% of all SCs were IL-6$^+$. IL-6$^+$/PAX7$^+$ cells remained significantly up-regulated from PRE at 24 hours, with approximately 60% of PAX7$^+$ cells expressing IL-6. It is known that SCs express the IL-6Rα [38, 20] allowing for IL-6 signalling to occur. Thus, an increase in SC mediated IL-6 synthesis suggests that IL-6 signalling occurs in an autocrine/paracrine fashion. As a consequence, we suggest that increased IL-6 signalling leads to the

induction of p-STAT3 signalling in the SC as evidenced by the increases in the p-STAT3$^+$/PAX7$^+$ population and the up-regulation of p-STAT3 regulated genes.

Previously p-STAT3 was found to be localized in the SC compartment; however, quantification of p-STAT3 over a time-course was not conducted in that study [20]. To verify that p-STAT3 could be regulating the SC response, we quantified p-STAT3 immunofluorescence. The proportion of p-STAT3$^+$ SCs was significantly elevated at T1 with ~40% of SCs co-positive and peaking at T24 where ~60% of SCs were co-positive, similar to that of IL-6$^+$ SCs. Importantly, this early increase in p-STAT3$^+$ SCs verifies the rapid signalling of this system and confirms that the timing of these events were indeed occurring early as proposed by McKay et al. [20]. This provides temporal evidence that following a bout of damaging exercise, STAT3 is phosphorylated leading to downstream signalling events resulting in proliferation of human SCs. The significant increase in PAX7$^+$ cells, the up-regulation of *MYF5* mRNA (a major regulator of SC proliferation) coupled with no significant change in *MRF4* mRNA (a major regulator of SC differentiation) and an increase in the percentage of cMYC$^+$ SCs 24 hours after the MLC provide further evidence that human SC proliferation involved p-STAT3 signalling. In addition, because almost no other nuclei other than SCs were positive for p-STAT3 and the nuclear fraction western blots detected virtually no p-STAT3, it would appear that the IL-6 induced STAT3 signalling cascade occurred almost exclusively in SCs. This supports the observational report from McKay and colleagues who found that p-STAT3 was co-localized to SCs 4 hours after subjects had preformed 300 MLC [20] and work done by Kami & Senba who co-localized p-STAT3 to c-MET$^+$ cells in rats

following freeze crush injury [39]. Trenerry and colleagues also showed a transient response in myonuclei expressing p-STAT3 2 hours following leg extension exercise [28], however no SC-specific analysis was conducted in that study.

There was a progressive increase in p-STAT3 staining in the muscle fibers over the duration of the experiment, which is in line with the stepwise increase in p-STAT3 observed in the cytoplasmic fraction. This may be explained by additional cellular functions of p-STAT3 which has been implicated in microtubule remodelling, matrix metalloproteinase production [40, 41] and focal adhesion protein production [42, 41], leading to increased migratory potential of different cell types such as inflammatory and SCs. In addition, p-STAT3 has also been shown to play a role in regulating the general hypertrophic response through its interactions with the leptin receptor in skeletal muscle [43] and angiotensin II in cardiac muscle [44]. Possibly, in response to muscle fiber damage, STAT3 is phosphorylated in damaged myofibers acting as a localized chemotactic stimulus for the SC and inflammatory cell populations to migrate toward. This allows for expeditious repair via phagocytosis of necrotic or damaged tissues [45], breakdown of connective tissues through matrix metalloproteinase activity [46] and efficient migration of SCs to the areas of damage [47].

There was no significant increase in p-JAK2 protein at any of the time points. As an upstream regulator of STAT3 phosphorylation we would expect a temporal profile for JAK2 phosphorylation similar to that of STAT3. Since cytoplasmic p-STAT3 peaked at

24 hours following MLC it is possible that we missed the increase in p-JAK2 as it may have occurred sometime between 3 and 24 hours.

In addition to the increase in serum IL-6 and *IL-6* mRNA, we also observed an up-regulation of several STAT3-related downstream genes. *SOCS3* expression, downstream of STAT3 signalling serves as a negative regulator of JAK/STAT signalling. *SOCS3* functions via feedback inhibition. Production of SOCS3 is up-regulated in response to JAK/STAT signalling whereby it then acts to suppress JAK/STAT signalling by inhibiting the activity of JAK2 kinase [34, 29]. Importantly, the temporal expression of *SOCS3* was closely related to the temporal expression of *IL-6* with a peak at 3 hours and remaining elevated at 24 hours post MLC. A gene known to promote proliferation, *cMYC,* and that is expressed downstream of STAT3 [32], was also temporally associated with the same pattern of expression as *IL-6* and *SOCS3* following MLC, as demonstrated through significant correlations with each of these genes (supplemental figures 4 & 5). Two other necessary members of this signalling cascade are the receptor sub-complexes *IL-6Rα* and *GP130* which both showed increases in their mRNA expression over the time course with a peak at 24 hours. This is important as both of these mRNA species are up-regulated in response to STAT3 signalling and serve to autoregulate the pathway.

Providing yet another level of support for STAT3 mediated SC proliferation was the increase in the number of $cMYC^{+}/PAX7^{+}$ cells observed at T24 versus PRE. Previous work by McKay and colleagues [20] did not localize the downstream signalling events of the STAT3 pathway to the SC, looking only at different mRNA species in

whole muscle. Importantly, the present work definitively shows that the change observed in *cMYC* mRNA translates into significant protein expression specifically in the SCs during a time when these cells were proliferating. This implicates STAT3 as a primary inducer of SC proliferation in human muscle following MLC. The $cMYC^{+}/PAX7^{+}$ population was only analysed PRE and 24 hours after MLC as this was the time when the highest levels of proliferation were occurring as evidenced by the significant increase in $PAX7^{+}$ cells. cMYC is a critical regulator of the cell cycle, responsible for the transition from G1 to S phase [48]. It has also been shown to be up-regulated in response to IL-6 signalling [49] and in a STAT3 induced GP130 mediated manner [32]. One of its main downstream functions is to regulate the production of CYCLIN-D1, a known regulator of the cell cycle, which we have previously reported to increase in response MLC. The increase in SCs expressing cMYC serves as direct evidence that p-STAT3 signalling is occurring in SCs leading to their proliferation.

The JAK2/STAT3 mediated signalling cascade as induced by IL-6, has been shown here to be an important regulator for the proliferation of SCs early following a muscle damaging protocol. Collectively, we have shown the potential for SCs to up-regulate IL-6 mRNA and protein following damage which likely acts in an autocrine/paracrine fashion to promote proliferation through STAT3 signalling. This IL-6 induced STAT3 signalling is evidenced by the up-regulation of the downstream genes regulated by p-STAT3. Furthermore, with p-STAT3 being observed almost exclusively in SCs, not in other myonuclei and only to a large degree at least 1 hour after muscle damage, it appears that it acts in a very specific manner through IL-6 leading to the

proliferation of SCs and subsequent repair of muscle damage. Thus, as evidenced by the induction of cMYC protein it appears that STAT3 is a key signalling molecule in human SCs in response to muscle damage and contributes to the robust proliferation of SCs in the acute period following damage.

Methods and Materials:

Ethics Statement:

All subjects were informed of the procedures and the potential risks associated with the study and gave written informed consent. This study was approved by the Hamilton Health Sciences Research Ethics Board (08-413) and conformed to the Declaration of Helsinki regarding the use of human subjects as research participants.

Subjects:

Twelve healthy males (age 21.2±1.6 yrs, height 178.2±5.5cm and weight 82.6±11.5kg) were recruited from the McMaster University community. Subjects were sedentary having done no lower body resistance exercise for at least the past 6 months and were non-smokers. Subjects were told to refrain from doing any moderate or strenuous exercise for two days prior to and during the study.

Muscle Damage Protocol:

The muscle damage protocol was preformed as described previously [50]. Briefly, subjects completed 300 unilateral isokinetic eccentric contractions of the *quadriceps femoris* using a Biodex dynamometer (Biodex-System 3, Biodex Medical

Systems, Inc., Shirley USA) at $180°\cdot s^{-1}$ over a 55° range of motion [20, 17, 18]. The experimental leg was chosen as the dominant leg. During each set investigators provided verbal encouragement so as to elicit maximal effort from the subjects. The entire duration of the protocol was approximately 30 min of which the muscle was under tension for about 10 min.

The protocol employed in the present study has previously been shown to cause physiological muscle damage as evidenced by increased serum creatine kinase (CK) [50-52], z-band streaming [53, 54], desmin disruption, a significant infiltration of the inflammatory related macrophages and neutrophils, as well as a significant myogenic response leading to an increase in satellite cell number based on both Pax7 and NCAM staining [20, 17, 18].

Muscle Biopsies:

Subjects reported to the lab at either 7, 7:30, or 8am having completed an overnight fast. Upon arrival to the lab subjects rested, had their PRE blood draw taken from an *antecubital vein* and then underwent the muscle damaging protocol. Once the subjects had completed the protocol they had one hour to rest. One hour post muscle damage the subjects had a second blood draw taken as well as two muscle biopsies; one from the exercise leg (T1) and one from the control leg (PRE). Three hours after the protocol the subjects had a third blood draw taken along with another biopsy from their exercise leg (T3). Subjects were then given a meal replacement beverage (Ensure, Abbott Laboratories, Abbott Park, Illinois, U.S.A.) The subjects were instructed to

refrain from taking any anti-inflammatory drugs or doing any vigorous exercise. The subjects arrived the next morning at 8, 8:30, or 9am in a fasted state for the final (T24) blood draw and biopsy from the exercised leg.

Muscle biopsies were obtained using the percutaneous needle [55, 56] method using manual suction from the vastus lateralis under local anaesthetic (1% lidocaine) as described previously [57]. In total, four muscle biopsies were obtained from each subject, three in the exercise leg and one in the control leg (non-exercise leg). The muscle tissue was dissected free of adipose and connective tissue. The tissue was immediately divided into four sections for RNA and protein (western blotting and immunohistochemical) analysis, three of which were flash-frozen in liquid nitrogen and stored at -80°C for later analysis and the final piece embedded in Optimal Cutting Temperature (OCT) compound embedding medium and frozen in liquid nitrogen cooled isopentane.

Blood Measures:

Blood samples were obtained from the antecubital vein immediately prior to the intervention and then again concurrently with the muscle biopsies one, three, and twenty-four hours after the muscle damage protocol. Approximately 8mL of blood was taken from each subject at each time point. 4mL was drawn into a heparinised tube while the other 4mL was drawn into a non-heparinized tube to obtain plasma and serum samples respectively. Samples were allowed to sit on ice (plasma) or at room temperature

(serum) for 15 minutes, centrifuged at 4000 RPM for 15 minutes, aliquoted into 600 μL aliquots and frozen down at -80°C for later analysis.

Serum analysis was conducted for both creatine kinase activity and IL-6 protein. Creatine kinase activity was analysed using a commercially available kit (Pointe Scientific, Inc., Canton USA) with modifications to the protocol to allow for running the samples at 25°C. This included adding additional sample using a ratio obtained from the International Federation of Clinical Chemistry (IFCC) for conversion between 37°C and 25°C. Additionally, sample absorbance was measured every twenty seconds for twenty minutes to obtain the slope of the change in absorbance/minute. This value was used to calculate the concentration of creatine kinase in international units (U/L) which is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute.

Serum IL-6 was analysed using a commercially available high sensitivity Quantikine Enzyme-Linked ImmunoSorbent Assay (ELISA) kit according to the manufacturer's instructions (R&D systems Inc., Minneapolis USA). Samples were run in duplicate with all subjects on the same plate and an intra-assay CV of 6.9%.

RNA Isolation:

RNA was isolated was conducted as previously described [20]. RNA isolation was conducted using Trizol Reagent (Invitrogen Corporation, Carlsbad USA) and RNA purification was done using the Qiagen RNeasy mini kit (Qiagen Sciences, Mississauga

Canada). The RNA was then quantified using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc., Wilmington USA).

Reverse Transcription (RT):

RNA was transcribed to cDNA using Applied Biosciences High Capacity cDNA reverse Transcription Kit (Applied Bioscience, Foster City USA). Individual samples in 20μL reactions were reverse transcribed into cDNA using an Eppendorf Mastercycle epigradient thermal cycler (Eppendorf, Mississauga Canada).

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

qRT-PCR was performed in 25μL reactions using SYBR Green/Rox master mix (SuperArray Bioscience Corp., Frederick USA). Primers were custom made using published sequences (Supplemental table 1). They were resuspened in 1X TE buffer (10mM Tris-HCL, 0.11mM EDTA) and frozen at -20°C until use. Using 0.2mL PCR tubes (Axygen Inc., Union City USA) 12.5μL of SYBR green, 2μL forward primer, 2μL reverse primer, 6.5μL or 7.5μL of H_2O depending on the cDNA template volume, 1μL or 2μL was added depending on the amount necessary for the particular gene of interest (25ng cDNA or 50ng cDNA). qRT-PCR was performed using a Stratagene Mx3000P real-time PCR system (Stratagene, Santa Clara USA) and Stratagene MXPro QPCR Software Version 3.00 (Stratagene, Santa Clara USA). Changes in gene expression over time were expressed as fold changes ± SEM from pre values using the delta delta CT method [58] with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a

housekeeping gene. *GAPDH* expression was not different from PRE at any of the post-intervention time-points.

Nuclear and Cytoplasmic Extraction:

Nuclear and cytoplasmic extraction was performed using a commercially available kit with minor modifications (NE-PER Nuclear and Cytoplasmic Extraction Reagents – Thermo Fischer Scientific Inc., Wilmington USA). Between 20mg and 60mg of tissue were transferred into a 2mL microcentrifuge tube while still frozen. 10X the volume in µL of the weight of the sample was added to the sample for CER I buffer (from kit with the addition of one protease and one phosphatase tablet dissolved in it). The muscle was then minced using mincing scissors in the 2mL microcentrifuge tube for about 15 seconds. The sample was then homogenized four times for five seconds using a rotary homogenizer (PRO250, PRO Scientific Inc., Oxford USA). The homogenate was then transferred to a 1.5mL microcentrifuge tube and vortexed for fifteen seconds. Following vortexing, 20µL of CER II buffer was added per sample (from kit with the addition of half a protease and half a phosphatase tablet dissolved). The sample was then vortexed for five seconds and put on ice for one minute. The tube was again vortexed for five seconds and then centrifuged for five minutes at 16 000g at 4°C. The supernatant (cytoplasmic extract) was immediately transferred to pre-chilled 1.5mL microcentrifuge tubes and frozen down at -80°C. The remaining pellet was then washed with 400µL of a PBS cocktail containing one protease and one phosphatase tablet dissolved, spun for five minutes at 16 000g at 4°C. The liquid was decanted with the wash repeated three more

times. On the last wash the pellet was resuspended prior to the final spin. After the final spin the pellet was suspended in 200μL of ice-cold NER buffer (from kit with the addition of 50μL of 10% SDS). The pellet was then broken up using Teflon pestles. The samples were then vortexed for 15 seconds and placed on ice for ten minutes. This was repeated three more times. The samples were then centrifuged at 16 000g for ten minutes at 4°C. The supernatant (nuclear extract) was then immediately transferred to a pre-chilled 1.5mL microcentrifuge tube and frozen down at -80°C. Bradford analysis was then conducted on the samples to obtain the concentration using a spectrophotometer (UltraSpec 300 pro, Biochrome Ltd., Cambridge UK).

Western Blotting:

Equal amounts (75 μg) of cytoplasmic or nuclear homogenate in 4x Laemlli buffer were boiled at 95°C for 5 minutes then loaded in the wells of at 7.5% gel. Nuclear samples were precipitated via the acetone precipitation [59] method to increase their concentrations so that 75 μg of nuclear protein could be loaded in each well. Briefly, samples were mixed with four volumes of ice cold acetone and incubated for 1 hour. Samples were spun at 13,000 g for 10 minutes and the resulting pellet was dried for 20 minutes. Dried pellets were reconstituted in 4x Laemlli buffer and ddH_2O. Phosphorylated and total JAK2 were analyzed in the cytoplasm only, while phosphorylated and total STAT3 were analyzed in both the nucleus and cytoplasm. Nuclear p-STAT3 was run with cytoplasmic p-STAT3 from the same subject. Gels were run at 125 V for approximately 1 hour, and then transferred to polyvinylidene fluoride

(PVDF; Millipore, Etobicoke, Canada) membranes at 70 V for 1 hour. Membranes were blocked with 5% non-fat powdered milk in PBS (10mM, pH 7.4) for 1 hour at 4°C, then incubated in primary, phospho-specific antibody (phospho-Stat3 Tyr705, 1:1000, and phospho-JAK2 Tyr1007/1008, 1:500, Cell Signaling Technology, Boston, USA) overnight in 5% bovine serum albumin for cytosolic fractions and 5% non-fat dry milk in PBS for nuclear STAT3 blots (BSA, Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C. After multiple washes, blots were incubated in goat anti-rabbit HRP (1:50,000; Abcam Inc., Cambridge USA) in 5% BSA for 90 minutes at room temperature. After multiple washes, proteins were detected with ECL (SuperSignal West Dura; Thermo Fisher Scientific, Rockford, USA) using FluorChem SP (Alpha Innotech Corporation, San Leandro, USA). After detection of phosphorylated proteins, blots were washed and stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, USA) for 20 minutes at room temperature. Membranes were washed and re-probed with total-specific antibodies (STAT3, 1:1000, and JAK2 D2E12 rabbit mAb, 1:500, Cell Signaling Techonlogy, Boston, USA) in the same manner as phospho-specific protein detection. Following total protein detection, ponceau staining confirmed equal loading. Protein bands corresponding to the predicted molecular weight of JAK2 (~125 kDa) and STAT3 (~86 kDa) were quantified using the AlphaEase FC Software, Version 5.0.2 (Alpha Innotech Corporation, San Leandro, USA) with background correction. As we were interested in determining the changes in the amount of activated protein, the ratio of phosphorylated to total protein was determined.

Immunohistochemistry:

7 μm muscle cross-sections were stained with antibodies against Pax7 (neat; cell supernatant from cells obtained from the DSHB, Iowa City USA); IL-6 (500 ng/mL, MAB 2061, R&D Systems, Minneapolis USA); p-STAT3 (1:100, Cell Signaling Technologies Inc., Danvers USA) and laminin (1:1000, L8271, Sigma-Aldrich, Oakville Canada). Secondary antibodies used were: Pax7 (AlexaFluor 488 or AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., Camarillo USA or when using two mouse primary antibodies an immunoglobulin biotinylated secondary antibody, 1:200, Dako Inc., Mississauga Canada; followed by a streptavidin-FITC fluorochrome, 1:100, Carlsbad Biosource. USA); and Laminin (AlexaFluor 594, 1:500, Invitrogen, Molecular Probes Inc., Camarillo USA). Histochemical methods were adapted from previously published methods from our lab [50, 17, 18, 20]. Briefly, for co-immunofluorescent staining (PAX7 and IL-6, PAX7 and Laminin), sections were fixed with 2% paraformaldehyde (PFA, Sigma-Aldrich, Oakville 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 2% PFA (Sigma-Aldrich, Oakville Canada) to prevent migration of the secondary antibodies and re-blocked in 10% GS in 0.01% Triton-X 100 (Sigma-Aldrich, Oakville Canada). 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, 1:20000) (Sigma-Aldrich, Oakville 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., Melville USA) and images were captured and analyzed using the Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., Melville USA). For cMYC immunodetection, serial sections were used to visualize both PAX7 (with Laminin) and cMYC. PAX7/Laminin staining was performed as described above, and cMYC was stained as follows: slides were dried and fixed in acetone for 10 minutes. Slides were then washed several times in 1x PBS followed by quenching of endogenous peroxidases for 30 minutes using 0.3% H_2O_2 solution. After washing, sections were then blocked in 10% goat serum in a 0.2% Triton-X 100 (Sigma-Aldrich, Oakville Canada) solution for 30 minutes. cMyc antibody (cell supernatant, DSHB, Iowa City USA) was used at 1:2 in a 1% BSA solution and was then incubated for 2 hours at room temperature. Slides were then washed several times in PBST. The secondary IGB goat anti-mouse (DAKO Inc., Mississauga Canada) was incubated at 1:200 for an hour followed by incubation in the Vectastain Elite ABC kit (Vector Laboratories, Burlington Canada) according to the manufacturer's instructions for 30 minutes. Following several washes in PBST, slides were developed using the DAB kit (Vector Laboratories, Burlington Canada) according to the manufacturer's instructions. Slides were counterstained using Mayer's hematoxylin (Sigma-Aldrich, Oakville Canada).

Immunohistochemical Analysis:

Immunohistochemical quantification and enumeration was performed at all time points (PRE, T1, T3, T24) for $IL6^+/Pax7^+$ (n=12) and $pSTAT3^+/PAX7^+$ (n=9) and images were taken at 40X magnification. For $cMYC^+/PAX7^+$ stain 7 subjects were analysed (n=7) at PRE and T24 with images being taken at 20X magnification using full muscle cross-section stitched images. Two separate blinded reviewers quantified the co-localization of PAX7 and cMYC. Slides were viewed and images captured with the Nikon Eclipse 90*i* Microscope (Nikon Instruments, Inc., Melville USA) and Nikon NIS Elements 3.0 software (Nikon Instruments, Inc., Melville USA)

Immunohistochemical quantification:

Satellite cells were enumerated via double labelling with an anti-PAX7 antibody and DAPI. Only those cells that were co-positive were counted as satellite cells. Furthermore, only cells associated with individual myofibers, that were not in the interstitial space, were counted as positive. $IL6^+/PAX7^+$ cells were only counted as positive if they were triple immunolabeled with DAPI and antibodies against IL-6 and PAX7. $pSTAT3^+/PAX7^+$ cells were only counted as positive if they were triple immunolabeled with DAPI and antibodies against pSTAT3 and PAX7. $cMYC^+/PAX7^+$ cells were only counted as positive if they were triple immunolabeled with Mayers Hemotoxylin and antibodies again cMYC and PAX7 with Laminin defining the SC niche. Non-fiber associated nuclei (interstitial nuclei) were not included in the quantification. All data are represented as a percentage of $PAX7^+$ cells.

Statistical Analysis:

Statistical analysis and graphing were performed using Sigmstat 3.1.0 analysis software (Systat, SPSS Inc., San Jose USA) and Prism5 for Windows - version 5.01 (GraphPad Software Inc., La Jolla USA). mRNA, protein, IL-6 and creatine kinase plasma concentrations, IL-6^{+}/PAX7^{+} and pSTAT^{+}/PAX7^{+} enumeration were analysed using a 1-way repeated measures analysis of variance (ANOVA). cMYC^{+}/PAX7^{+} enumeration was analysed via a two-tailed T-test. Statistical significance was set at $P<0.05$. Tukey's HSD post hoc test was used to analyse main effects and significant interactions. Results are presented as mean ±SEM.

Acknowledgements:

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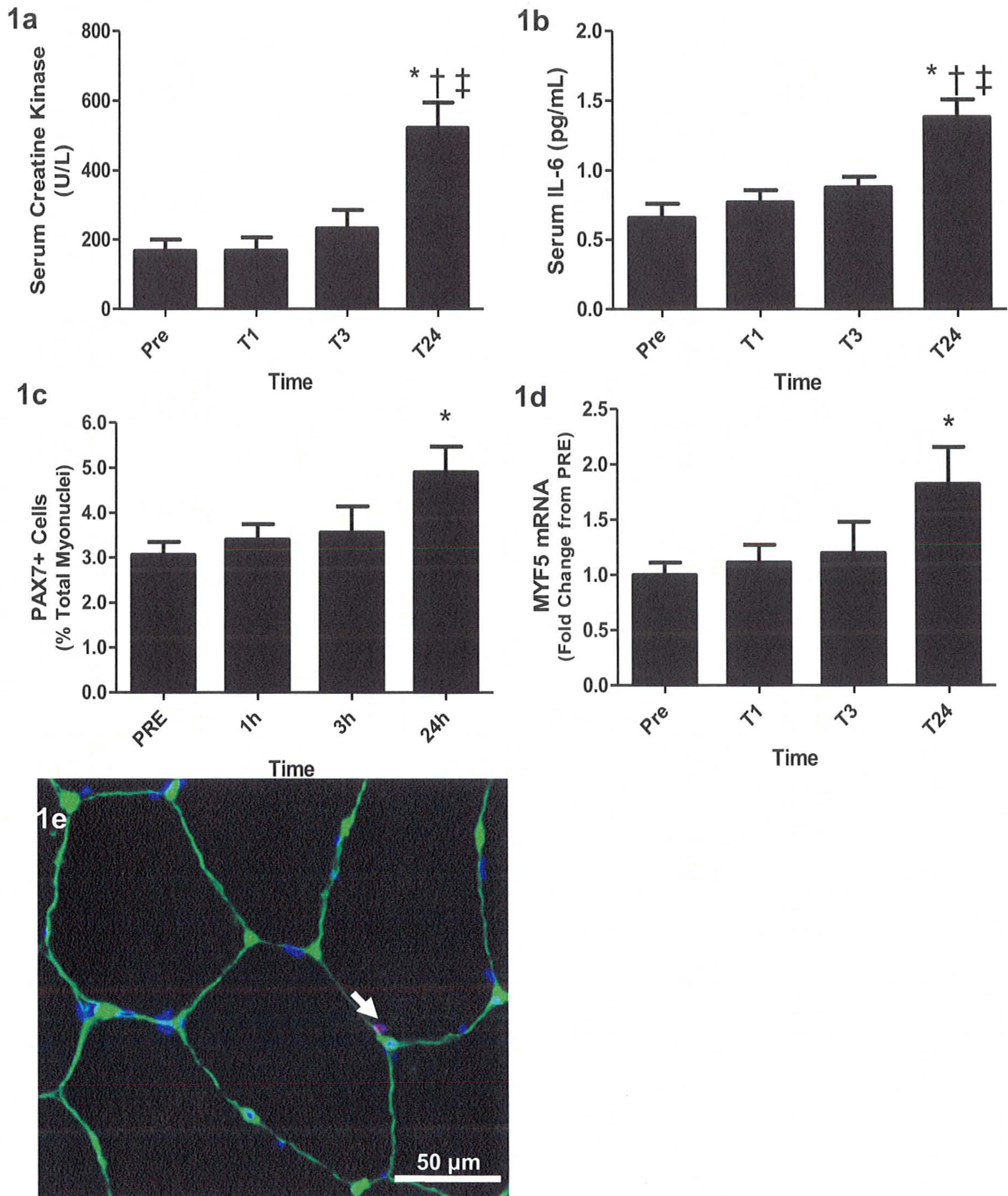
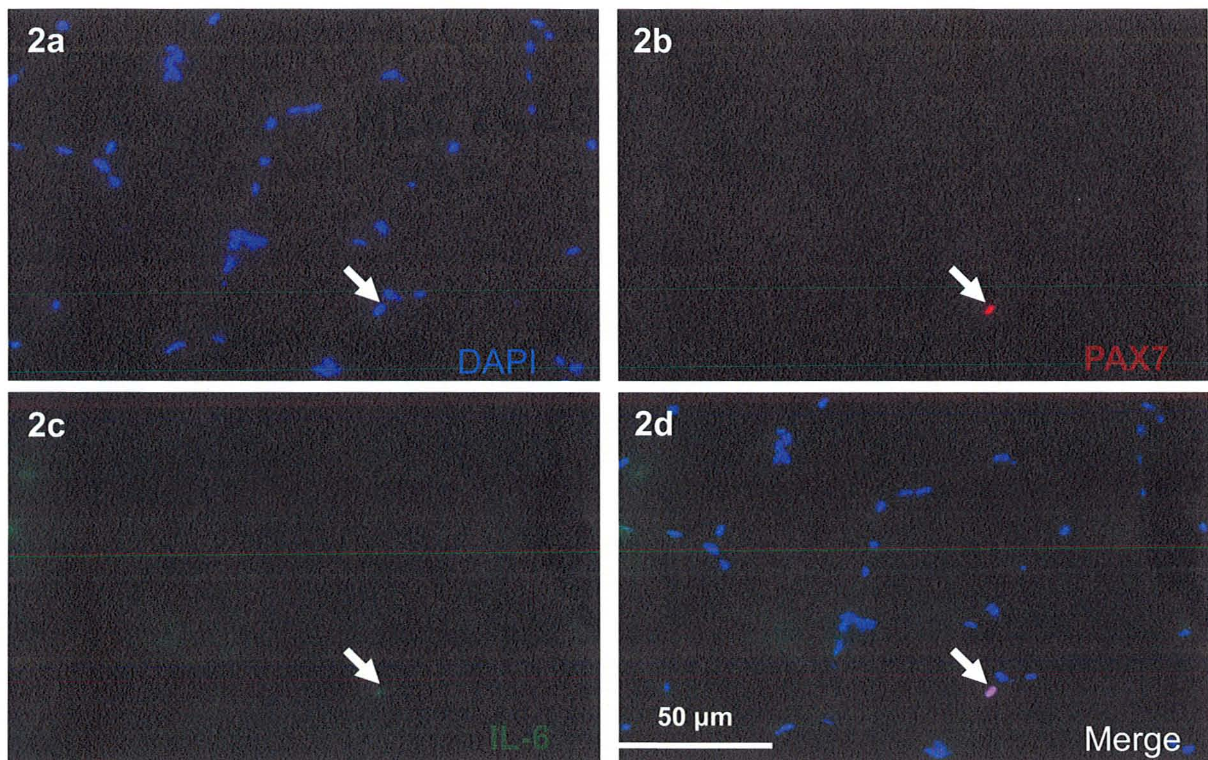


Figure 1. Serum measures and Pax7 positivity. (1a) Average serum CK response in U/L. **(1b)** Average serum IL-6 response in pg/mL; note the similar serum responses between IL-6 and CK. **(1c)** PAX7^{+} cells as a percentage of total myonuclei over the time-course. **(1d)** *MYF5* mRNA expression relative to *GAPDH*, expressed as fold change from PRE. **(1e)** Representative image of a PAX7/Laminin stain with PAX7 in red, Laminin in green and DAPI in blue. Values are reported as mean±S.E.M. *$p<0.05$ vs. PRE; † $p<0.05$ vs. T1, ‡ vs. T3.



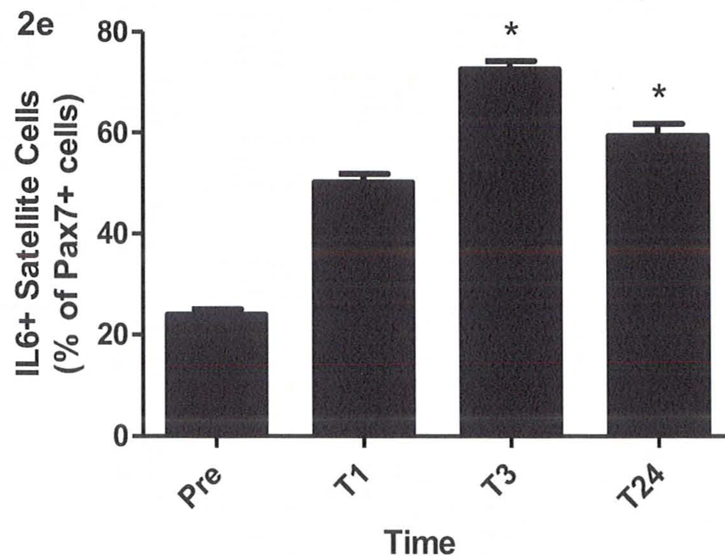


Figure 2. IL-6/PAX7 co-localization. (2a-d) Representative images of a $DAPI^{+}$, $PAX7^{+}$, $IL\text{-}6^{+}$ and merge. **(2e)** $IL\text{-}6^{+}$ cells as a percentage of $PAX7^{+}$ cells. Values are reported as mean±S.E.M. *$p<0.05$ vs. PRE.

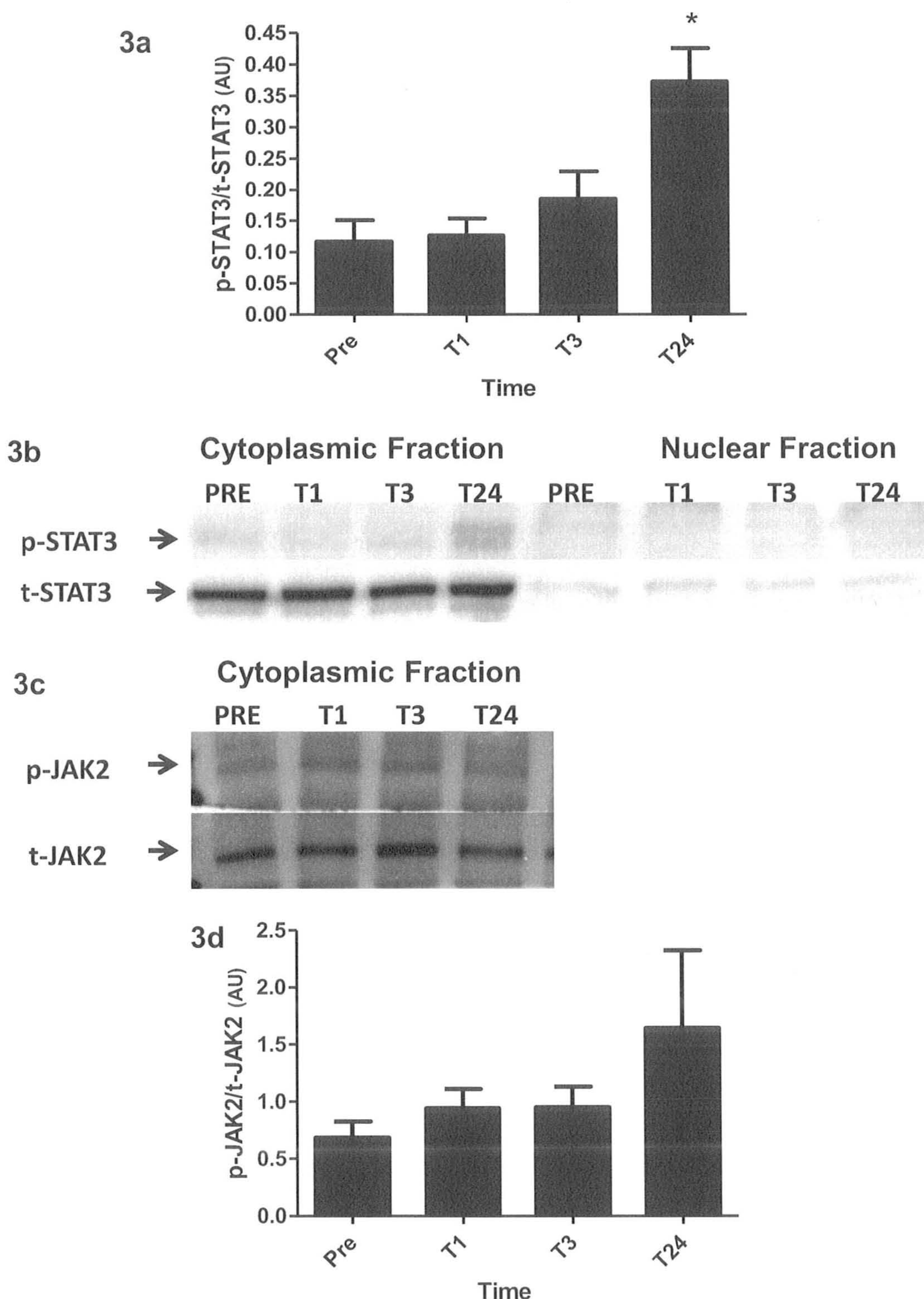


Figure 3. Nuclear and cytoplasmic expression of STAT3 and JAK2. (3a) Ratio of phosphorylated to total STAT3 protein in the cytoplasmic fraction. **(3b)** Representative images of p-STAT3 and t-STAT3 in both the cytoplasmic and the nuclear fraction. **(3c)** Representative images of p-JAK2 and t-JAK2 in the cytoplasmic fraction. **(3d)** Ratio of phosphorylated to total JAK2 in the cytoplasmic fraction. Values are reported as mean±S.E.M. *$p<0.05$ vs. PRE.

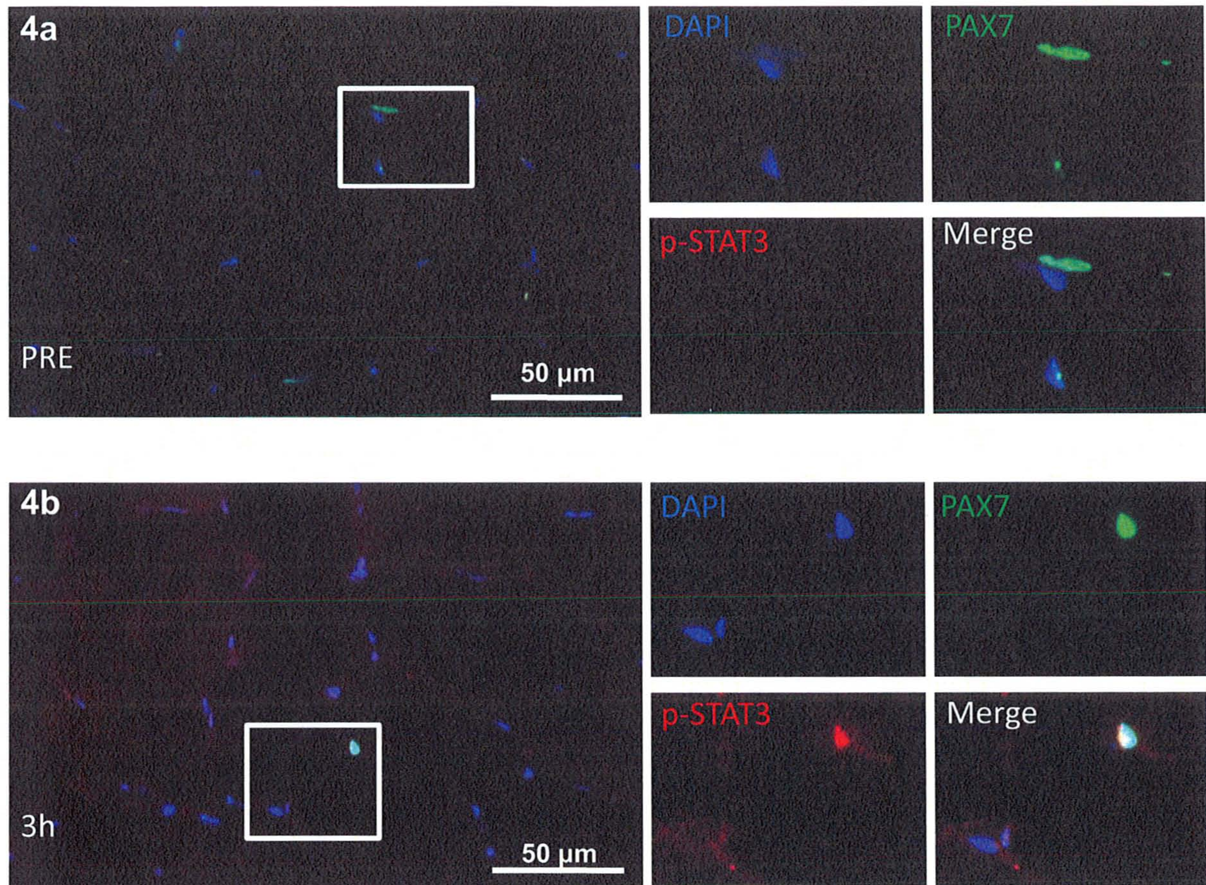


Figure 4. p-STAT3^{+}/PAX7^{+} cells quantified over the time course. (4a & b) Representative images of p-STAT3 not present at PRE but co-localizing with PAX7 at T3. **(4c)** Percentage of p-STAT3+ SC as quantified over the time course peaking at T24. Values are reported as mean±S.E.M. *$p<0.05$ vs. PRE.

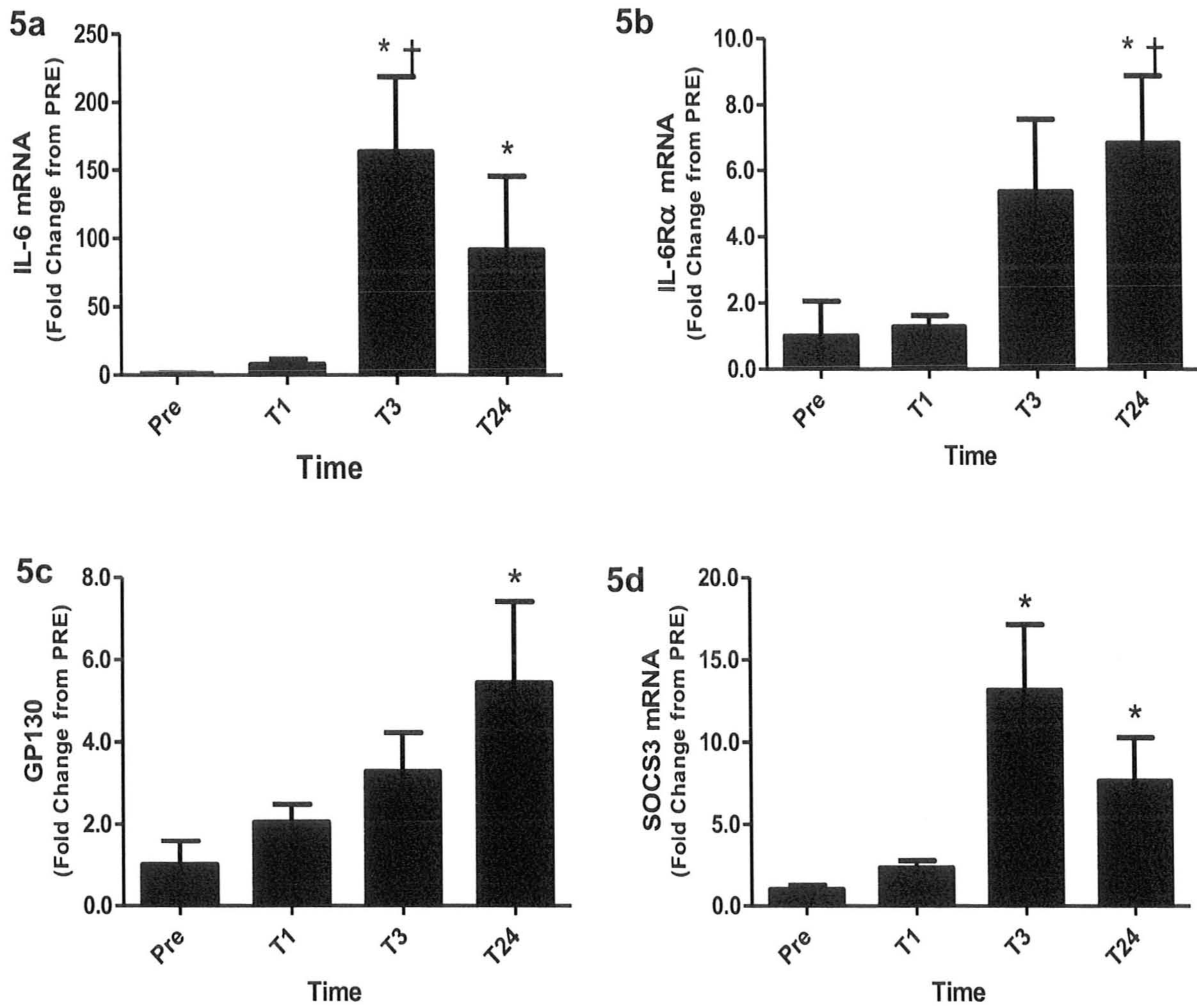


Figure 5. STAT3 downstream genes. (5a) *IL-6*, **(5b)** *IL-6Rα*, **(5c)** *GP130* and **(5d)** *SOCS3* mRNA expression relative to *GAPDH*, expressed as fold change from PRE. Values are reported as mean±S.E.M. *$p<0.05$ vs. PRE; † $p<0.05$ vs. T1, ‡ vs. T3.

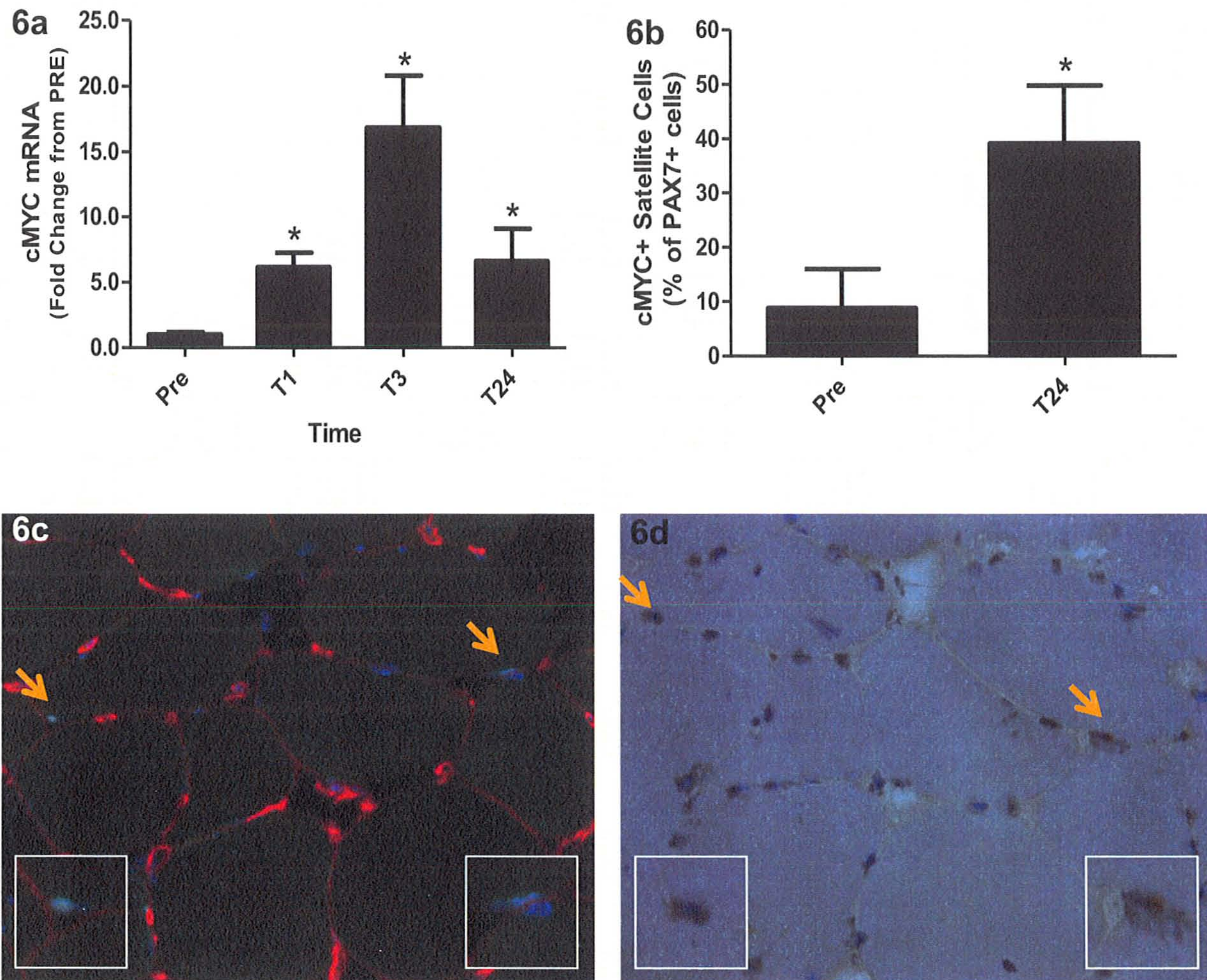
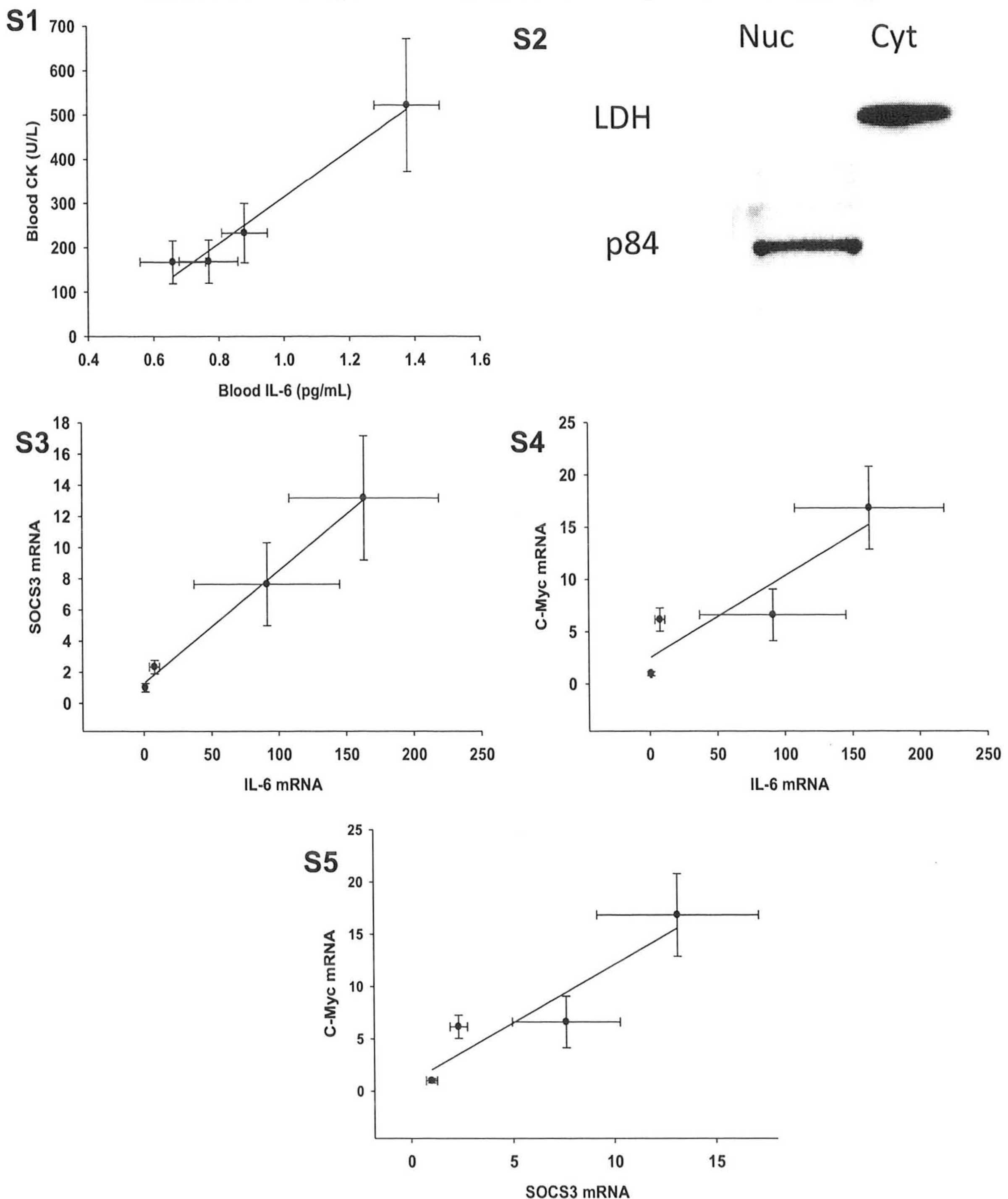


Figure 6. cMYC$^+$/PAX7$^+$ quantification at Pre and T24. (5a) *cMYC* mRNA relative to *GAPDH*, expression represented as fold changes from PRE. **(5b)** cMYC$^+$ SC as a percentage of PAX7$^+$ cells between Pre and T24, n=7. **(5c)** Representative image of two cMYC$^+$ cells **(5d)** which are also PAX7$^+$. *$p<0.05$ vs. PRE.



Supplemental Figures. (S1) Pearson correlation between the serum concentrations of IL-6 (pg/mL) and CK (U/L); R^2=0.3055; p<0.001. The correlation is representative of the individual data points presented as mean values ± SD (error bars). **(S2)** Representative image of nuclear and cytoplasmic preparations with the cytoplasmic marker LDH present only in the cytoplasm and the nuclear marker p84 present only in the nuclear fraction. **(S3 – S5)** Pearson correlation between the mRNA regulation (fold change) of *IL-6* and *SOCS3*; R^2 = 0.5984, p<0.001, *IL-6* and *cMYC*; R^2 = 0.2876, p<0.001 and *SOCS3* and *cMYC*; R^2 = 0.5406, p<0.001. The correlations are representative of the individual data points presented as mean values ± SD (error bars).

Gene Name	Forward Sequence	Reverse Sequence	cDNA (ng)	Annealing Temperature (°C)
IL-6	GAAAGCAGCAAAGAGGCACT	AGCTCTGGCTTGTTCCTCA	50	62
IL-6Rα	GACAATGCCACTGTTCACTG	GCTAACTGGCAGGAGAACTT	50	60
GP130	AGAGTGGGACCAACTTCCTG	CCTTCCCACCTTCATCTGTG	25	60
SOCS3	GACCAGCGCCACTTCTTCA	CTGGATGCGCAGGTTCTTG	25	60
MYF5	ATGGACGTGATGGATGGCTG	GCGGCACAAACTCGTCCCCAA	25	55
cMYC	CGTCTCCACACATCAGCACAA	TCTTGGCAGCAGGATAGTCCTT	25	62
MRF4	CCCCTTCAGCTACAGACCCAA	CCCCCTGGAATGATCGGAAAC	25	55

Supplemental Table 1. mRNA species that were analysed with their forward and reverse sequences, cDNA (ng) concentration used and annealing temperature (°C).

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4: Expanded Discussion

a. Major Findings

The current work provides evidence describing an IL-6/JAK2/STAT3 pathway in human SCs following MLC-induced damage promoting the proliferation of satellite cells. We have shown the presence of this pathway using a multifaceted approach. In the serum fraction of whole blood, IL-6 protein increased 24 hours after MLC. In whole muscle, several mRNA species related to the IL-6/JAK2/STAT3 pathway were shown to be up-regulated with significant correlations between *IL-6* and *SOCS3*, *IL-6* and *cMYC*, and *cMYC* and *SOCS3*, suggesting controlled regulation of the pathway. Using a nuclear fraction of whole muscle homogenate, no detectable p-STAT3 and only traces of t-STAT3 were observed, while immunohistochemical data suggested p-STAT3 was almost exclusively co-localized with nuclei of satellite cells. This may suggest that the changes in the mRNA likely reflect changes specifically in the SCs. Support for SC specific signalling of the IL-6/JAK2/STAT3 pathway was further bolstered by immunohistochemical data demonstrating that all aspects of the pathway co-localized with SCs; IL-6/PAX7, p-STAT3/PAX7, and cMYC/PAX7. Finally, a significant increase in $PAX7^{+}$ cells was observed 24 hours after MLC showing the proliferative response as regulated by the IL-6/JAK2/STAT3 signalling cascade. Taken together we propose the following model of IL-6 regulation of SCs in human muscle (figure i).

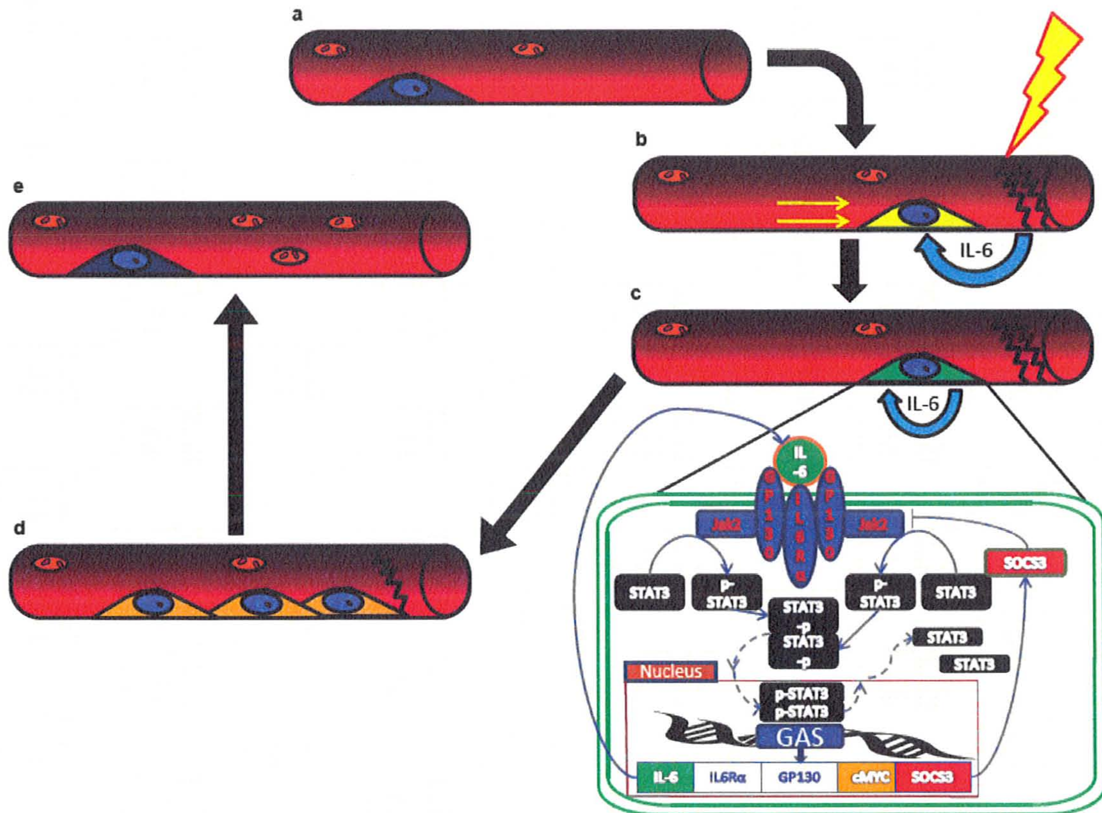

Figure i: A theoretical model of the series of events that occur as a result of muscle lengthening damage. **(a)** The quiescent SC (blue) lies between the basal lamina and the sarcolemma on a muscle fiber. **(b)** In response to a damaging stimulus, such as muscle lengthening exercise, IL-6 is released from the muscle fiber stimulating the activation and migration of the SCs (yellow) closest to the damage. **(c)** The SCs themselves then begin to produce IL-6 (green) used in an autocrine signalling cascade. IL-6 binding with its receptor on the SC leads to the phosphorylation of STAT3 and its transport into the nucleus. Once there it binds with the GAS element. This leads to the up-regulation of a number of the substrates involved in the pathway itself including cMYC. **(d)** cMYC then promotes the SC to proliferate (orange). **(e)** Some of these new SCs donate their nuclei to the myofiber leading to its repair while others return to quiescence (blue) maintaining the SC population.

b. Limitations

A limitation of the present study is the apparent discrepancy with that of McKay et al (2008). The first difference observed was in the mRNA responses of some of the IL-6/JAK2/STAT3 pathway members. The current study showed that *IL-6* mRNA was up-regulated ~150 fold 3 hours post MLC. Previously, however, we reported a ~4.5 fold increase 4 hours after the same protocol [1]. In addition, in the current study we reported a ~7 fold increase in *IL-6Rα* mRNA at 24 hours post MLC while the work of McKay and colleagues reported that *IL-6Rα* had already been down-regulated to baseline levels by this time. Finally, while 24 hours after the MLC protocol both of the studies showed the same up-regulation of *SOCS3*, the current study reported a peak in *SOCS3* expression at 3 hours post and not at 24 hours post.

The second area of discrepancy was in the p-STAT3 western blots. The previous work showed no change in p-STAT3 in whole muscle homogenate. The current study, however, showed an increase in p-STAT3 protein in the cytoplasmic fraction of whole muscle, peaking 24 hours after the MLC protocol. JAK2, however, was similar between the two studies.

There are a number of reasons why these discrepancies may have occurred. First, the subject characteristics of the two studies varied significantly. For the present study we recruited subjects that were sedentary having not engaged in resistance training in the past six months. In the study by McKay and colleagues, "recreationally" active individuals were recruited, which included several experienced cyclists. This change

alone could explain the differences in the mRNA species and p-STAT3 content as it might be expected that the individuals who were less exercise-naive would exhibit a blunted response to the MLC protocol [2].

Another potential cause for discrepancy could be the dietary controls implemented in the present study that were not used in the study by McKay and colleagues. Subjects in the current study reported to the lab in the fasted state, and were provided with a liquid meal replacement drink 3 hour post MLC. It has been shown that protein synthesis is stimulated when a bolus of 10g of protein is ingested [3]. This is the same protein content in the meal replacement drink provided to subjects. Furthermore, the carbohydrate content of the meal replacement could have stimulated the release of insulin. Insulin has been shown, *in vitro*, to stimulate protein synthesis in SCs [4]. The additive effects of both the protein and carbohydrate may explain the differences observed between the studies.

A final reason for the differences may simply be due to the fact that McKay and colleagues used tissue that was stored in the freezer for up to 3 years. In the present study, we used tissue that was stored in the freezer for weeks. The potential rate of breakdown of certain mRNA species and proteins over the course of 3 years may explain the differential results [5]. It may be that certain mRNA species and proteins degrade at varying rates helping to resolve the different magnitudes or lack of change seen in certain measures and not in others. Despite these minor differences between the studies it is

important to emphasize that while the magnitude of change may have been different, the directional changes of each of these genes was identical.

A strong argument was made for IL-6 mediated STAT3 signalling. Three levels of the signalling pathway were shown to be co-localized with SCs; IL-6 which starts the signalling cascade, p-STAT3 in the middle further propagating the signal and cMYC at the end leading to increased proliferation. There are still, however, questions that remain to be answered. One of the main challenges of demonstrating such a signalling cascade *in vivo* is the use of human subjects and the inherent limitations that are imposed on such research. It is impossible to control all the necessary variables to ensure only one cause and effect. Because of this, some criticism may be levelled toward this work. One criticism may be that the p-STAT3 signalling observed to be co-localized to the SC was in fact random. In other words p-STAT3 released from other cells or the fiber itself only appeared to co-localize with SCs when in fact the p-STAT3 was actually above or below the actual SC. Although possible, we don't believe that this is the case. First, if in fact the p-STAT3 co-localization with the SCs was random we would not expect to see any significant change in the proportion of p-STAT^{+}/PAX7^{+} cells at any point in the time-course. Second, the virtually identical staining pattern seen with both PAX7 and p-STAT3 at 100X magnification, suggest that it is not random staining that is observed but in fact targeted to the p-STAT3 in the SC (figure ii).

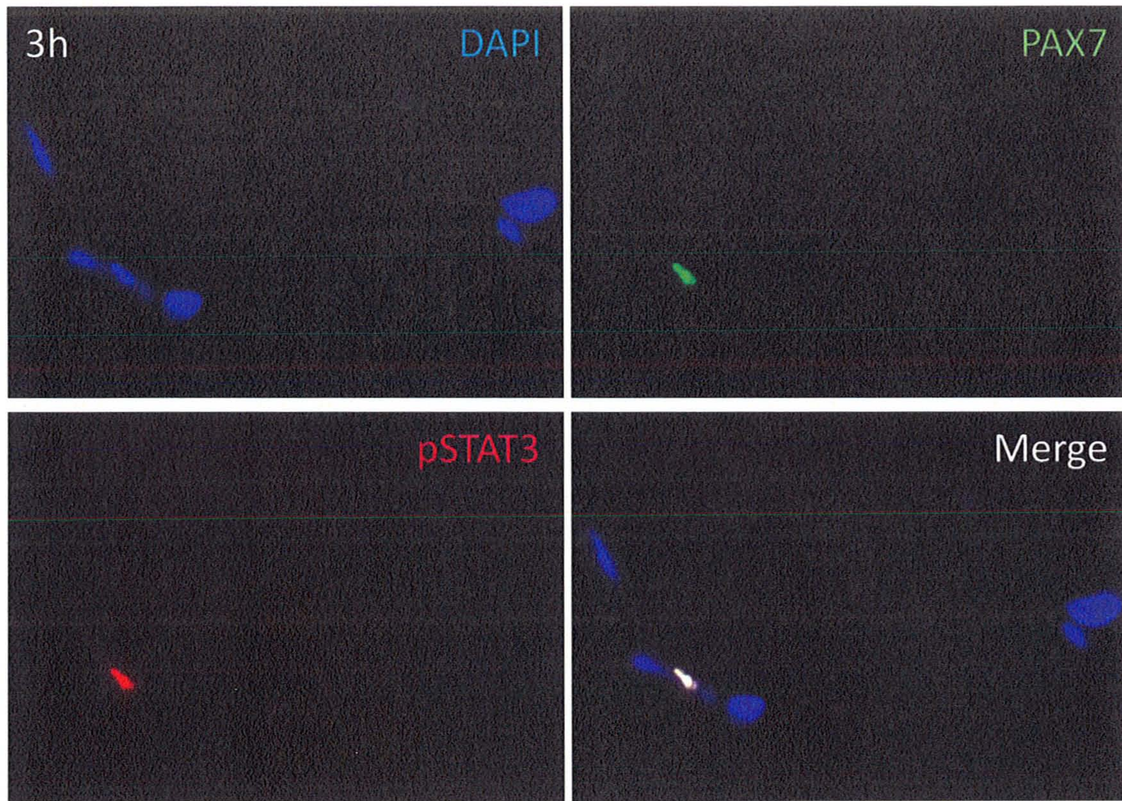

Figure ii: Triple Immunofluorescent stain of p-STAT3, PAX7 and DAPI at 100X magnification. Note the virtually identical staining pattern observed for PAX7 and DAPI as seen with p-STAT3 and the colour change observed in the merged image.

In addition, the computer software (NIS-Elements Advanced Research) has a feature that allows for the detection of co-localization versus random staining on different planes, through mixing of colours of the individual fluorophore (E.g. red PAX7 and green p-STAT3 become a yellow or white colour) as seen in figure ii. Finally, the significant changes seen in the IL-6, p-STAT3 and cMYC positive SC populations would seem to be much more indicative of an actual signalling cascade occurring resulting in proliferation of SCs than immunohistochemical artefact occurring simultaneously with each separate

measure. Although the possibility exists that the co-localization of IL-6, p-STAT3, and cMYC may not be nuclear, the high nuclear to cytoplasmic ratio that characterises SCs precludes against this. In addition, these proteins show the same staining pattern as PAX7 which is almost exclusively in the nucleus again providing evidence that they are indeed nuclear.

It was also somewhat surprising that a significant change was not observed in the p-JAK2 western blots. It was speculated that the peak in phosphorylation may have occurred sometime between 3 and 24 hours but this is unlikely as one would expect JAK2 phosphorylation to occur simultaneously to the increases seen with p-STAT3 in the cytoplasmic fraction, which was significantly increased 24 hour post MLC. Another possible explanation may be due to the rate at which JAK2 phosphorylates STAT3. Finally, it may be that during the nuclear and cytoplasmic fractionation process, a proportion of p-JAK2 became bound to the transmembrane receptor subunit GP130, remained bound and were in the cytoskeletal fraction that was not analysed, preventing us from observing a change in JAK2.

c. Future Directions

While we have described the role of the IL-6/JAK2/STAT3 signalling cascade on propagating human SC proliferation, there are still many questions that remain to be answered. The following provides areas where future research may further this work and bring us to a greater understanding of the regulation of the SC.

In the current work we described the presence of IL-6 in the SC population after MLC. While there was an increase in the IL-6$^+$/PAX7$^+$ SC population, we did not definitively prove that SCs produced and released IL-6 *in vivo* to act in an autocrine signalling cascade. It is possible that IL-6 released from the fiber can be sequestered by the SC and used to initiate migration or begin its proliferative response. As more SCs are needed to repair the damage, the SCs themselves begin to produce IL-6, leading to increased proliferation through the IL-6 induced STAT3 signalling cascade as shown in this work. This dual role for IL-6 may be seen with the increase in IL-6$^+$/PAX7$^+$ cells before the significant increase seen in the PAX7$^+$ cell population. Two potential methods to discern whether SCs produce IL-6 include *in situ* hybridization whereby SCs are tagged and then IL-6 mRNA species can be probed for specifically in the SCs. Alternatively, single cell PCR could be employed whereby SCs are sorted from a muscle biopsy and then analysed for IL-6 mRNA. Both of these methods could allow for the determination of whether IL-6 is being produced by SCs *in vivo.*

The process of muscle repair in response to damage is clearly quite complex. The interaction between the SC and other cell types such as macrophages may be an important regulator of the SC response to muscle damage. *In vitro*, damaged myofibers release factors, such as IL-6, which attract macrophages to the sites of injury [6]. Macrophages are responsible for phagocytosing cellular debris allowing for more efficient migration of SCs [7, 8]. Macrophages that infiltrate the muscle might also chemotactically attract SCs to the site of injury [9]. SCs then begin to release their own IL-6 [10]. Once released by SCs, IL-6 could induce the proliferation [1] of the SC pool

as well as induce apoptosis of neutrophils and macrophages [11]. While not proven *in vivo*, this series of events may help to explain the how the repair of the damaged myofibers is achieved. Work to uncover if this system is acting *in vivo* would be a welcome addition to the literature.

d. Perspectives

While many questions were answered in this study, many more remain. We believe that we have shown that an IL-6/STAT3/JAK2 signalling cascade is active in human SCs following a bout of damaging lengthening contraction, ultimately propagating the proliferation of SCs. Although we have shed considerable light on the regulation of human SC, we must continue to screen other potential regulators of SC. For example, other IL-6 family members such as oncostatin M, IL-11, cardiotrophin-1, ciliary neurotrophic factor and cardiotrophin-like cytokine should be studied for their regulation of SCs as all of these factors signal through the GP130 receptor subunit. These different areas of study will allow for a greater understanding of the SC response with the potential to culminate in the application of such knowledge in treatments for muscle-related diseases.

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