Identification of a role for Janus kinase2 (JAK2) in skeletal muscle mitogenesis, myogenesis, and hypertrophy

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Identification of a role for Janus kinase2 (JAK2) in skeletal muscle mitogenesis, myogenensis, and hypertrophy

by

Symantha Ann Anderson

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Genetics

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Major Professor

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For the Major Program
I dedicate this thesis to my husband, Ross. Thank you for your love and support.

You gave me the courage to finish, cheered me on through the rough days, and celebrated with me when I succeeded. I could not have done it without you. I love you.
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## ACKNOWLEDGEMENTS

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Skeletal muscle is a dynamic tissue with tight developmental control and the ability to significantly remodel in the postnatal animal. Understanding the molecular mechanisms underlying skeletal muscle development and postnatal growth is critical to potential therapeutic manipulation of this tissue. Janus kinase 2 (JAK2) is a tyrosine kinase critical for growth of numerous cell types and is causative in many cancers, including leukemia. In this study we analyzed the role of JAK2 in skeletal muscle proliferation, myogenesis, and hypertrophy. We identified that kinase active JAK2 is necessary, and sufficient, to stimulate proliferation of C2C12 myoblasts. We also determined that incubation with a JAK2 inhibitor, AG490, inhibited myoblast migration and severely impacted the actin cytoskeleton of differentiating cells.

In addition, JAK2 was found to have a unique, protein-dependent role in skeletal muscle myogenesis. Over expression of JAK2 protein caused an increase in terminal differentiation, although myotubes in these cells appeared to be narrower, and less complex, than control cells. Strikingly, we observed a drastic change in JAK2 cellular localization during myogenesis. Although JAK2 is traditionally described as a kinase associated with receptors at the cell membrane, we found that JAK2 was predominantly nuclear localized in proliferating myoblasts, or cells treated with AG490. Treatment with AG490 prevented myoblast fusion and terminal differentiation. In contrast, multinucleated myotubes contained JAK2 in both the nucleus and in the cytoplasm. These results suggest that the role of JAK2 in
Previous work in our lab demonstrated that JAK2 expression increased following three days of work overload in skeletal muscle. To clarify the role for JAK2 in skeletal muscle hypertrophy, we analyzed activation of the JAK/STAT pathway in response to cyclic stretch. Use of mechanical stretch is a convenient \textit{in vitro} model to specifically analyze the response of muscle cells to this stimulus. We identified that our stretch protocol was sufficient to induce proliferation in C2C12 myoblasts, and that kinase active JAK2 was required for this increase. Cyclic stretch increased JAK2 activation in myoblasts, as well as stimulated a relocalization of phosphorylated JAK2 to the nuclear/perinuclear domain. We also determined that this activation was regulated by phosphatases, since treatment with a tyrosine phosphatase inhibitor extended JAK2 activation in stretched myoblasts. Stretch-activation of JAK2 appeared to be at least partially regulated by two distinct autocrine/paracrine mechanisms. Treatment with media collected from stretched cells was sufficient to increase JAK2 phosphorylation. This activation occurred in two distinct waves, one at five minutes that was not inhibited by a translation blocker, and one at one hour that did appear to be dependent on synthesis of new proteins. We also identified that STAT1 and STAT5 were phosphorylated, and accumulated in the nucleus, in response to short periods of cyclic stretch. These results indicate that the JAK/STAT pathway is stimulated in response to mechanical stretch in C2C12 myoblasts.
In conclusion, the results of this study identify a critical role for JAK2 in key skeletal muscle regulatory events. This data demonstrates that JAK2 is a novel regulatory protein, with diverse functions, in the control of this distinctive tissue. Collectively, this study is a major advance in the understanding of the classical JAK/STAT signaling cascade in skeletal muscle.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Control of both skeletal muscle embryonic development and postnatal growth is a delicate balance of proliferation, migration, apoptosis, and fusion. Sufficient proliferation must occur to supply an adequate number of mononucleated cells, while growth arrest, fusion, and terminal differentiation are required to form multinucleated myotubes and mature muscle. Until recently, our understanding of the controls of this complex process was confined to the interplay between growth factors and their regulators. Over the past 10 years and, in particular, the past five, our understanding of the intracellular signaling cascades that function downstream of these factors has greatly increased. Understanding of the roles these pathways play in skeletal muscle development, and the response of skeletal muscle to external stimulus in the adult animal, is critical to any potential therapeutic manipulation of skeletal muscle mass.

Skeletal muscle atrophy is associated with AIDS, aging, and disuse of skeletal muscle due to sedentary lifestyle, paralysis, cardiopulmonary disease, and other factors that limit movement. Accretion of skeletal muscle is desirable to a large segment of the human population for improved athletic ability, decreased fat, increased independence with age, and to combat muscle atrophy. In addition, the ability to manipulate muscle mass and composition of meat animals is of interest to those in the livestock industry, who are interested in increased muscle growth, and to consumers interested in improved meat quality. Clearly, our understanding of the
regulation of skeletal muscle, both before and after birth, is of public relevance and importance.

As mentioned previously, our understanding of the mechanisms involved in skeletal muscle myogenesis has greatly increased over the past few years. Improved ability to target individual signaling molecules, combined with a greater understanding of the components of signaling cascades, has significantly improved our capability to analyze these pathways. However, the analysis of signal transduction in skeletal muscle is still in its infancy when compared to other fields such as neurobiology, immunology, and oncology. Using information from these tissues is helpful in selecting potential candidates for discovery, but the composition of skeletal muscle, combined with skeletal muscle-specific signaling factors, ensures that the control of this tissue is unique. Therefore, research in this field is exciting, likely novel, but also constrained by the lack of in-depth analyses.

In this study we sought to understand the role of the JAK/STAT signaling cascade in skeletal muscle. In particular, this research focused on the role of JAK2 in skeletal muscle mitogenesis, myogenesis, and hypertrophy. Very little work has been done regarding the role of JAK2 in skeletal muscle and, to date, no studies have been undertaken to analyze a possible function for JAK2 in skeletal muscle myogenesis or hypertrophy. With this work we demonstrate that JAK2 is necessary and sufficient for proliferation of C2C12 myoblasts. We also show a unique, protein-dependent role for JAK2 in the control of myogenesis. Finally, we identify the JAK2/STAT signaling cascade as activated by mechanical stretch of C2C12 myoblasts. The results of this study establish that JAK2 is a critical signaling protein
in the control of skeletal muscle cells \textit{in vitro}. In addition, it identifies the JAK/STAT pathway as a potential therapeutic target for skeletal muscle manipulation.

\textbf{Thesis organization}

This dissertation is a presentation of the author's research in the alternative format. A general introduction and a review of the relevant literature are included in the first chapter. The second chapter contains a manuscript prepared for the Journal of Biological Chemistry. This paper describes a critical role for the protein tyrosine kinase, JAK2, in \textit{in vitro} skeletal muscle cell proliferation and terminal differentiation. The third chapter is a manuscript describing the activation of the JAK/STAT pathway in response to mechanical stretch of C2C12 myoblasts. This paper was also prepared for the Journal of Biological Chemistry. The final chapter contains the author's final conclusions regarding the novel roles for the JAK/STAT in light of the research described. This dissertation also contains three appendices. The first (A) is a description of the role of high glucose in C2C12 proliferation, migration, and terminal differentiation, as well as the ability of high glucose to activate JAK2. Appendix B contains a short description of the affect of TNF\(\alpha\) treatment on the localization of phospho-JAK2 in C2C12 myoblasts. The final appendix (C) describes a novel technique utilized to detect proteins via immunofluorescence in cells plated on silastic membranes.
Review of Relevant Literature

Myogenesis

I. Overview

The ability of skeletal muscle to differentiate into multinucleated cells is a critical and unique characteristic required during development and restructuring events such as those that occur during muscle hypertrophy and regeneration. Many advances have been made in understanding myogenesis. In particular, the study of muscle development has been greatly enhanced by the discovery of the myogenic regulatory factor (MRF) family of transcription factors, MyoD (1), Myf5 (2), MRF4 (3) and myogenin (4). Control of these factors during embryogenesis is largely by secreted morphogens including sonic hedgehog (Shh) (5,6), Wnts (7), and bone morphogen protein4 (BMP4) (8,9). Other key regulators of embryonic skeletal muscle growth include myostatin (10-12), a TGF-β superfamily member, and follistatin, an inhibitor of TGF-β family members (13).

Postnatal skeletal muscle also has the potential to grow in response to stimulus such as damage and increased load. Signaling pathways involved in adult muscle growth are similar and postnatal control of myogenesis by the MRFs is primarily orchestrated by growth factors such as the fibroblast growth factors (FGFs) (14,15), hepatocyte growth factor (HGF) (16,17), and insulin-like growth factor-1 (IGF-1) (18-20). Defects in skeletal muscle myogenesis are involved in a number of important human diseases. Impaired muscle differentiation has been implicated as a possible cause of the disease process seen in Duchenne muscular dystrophy
DMD is an X-linked disease, identified by severe muscle wasting and dystrophin deficiency (22).

To begin the process of terminal differentiation, proliferating myoblasts must first exit the cell cycle. Cells transition from G₁ to G₀ phase and begin the process of fusion into multinucleated myotubes and expression of muscle-specific proteins (23). A large number of proteins interact with the MRFs and regulate cell cycle progression including p21, retinoblastoma (pRb), cyclin D1, and cyclin-dependent kinase 4 (cdk4) (24,25). Positive controllers of proliferating cells, and continuance of the cell cycle from G₁ to S phase, include cyclins and their target proteins, the cdks (26,27). Inhibitors of the cell cycle, and promoters of terminal differentiation, include cyclin kinase inhibitors (CKIs) like p21, p27, and p57 (28,29), and Rb proteins. Rb proteins are tightly controlled via phosphorylation by cyclin dependent kinases like cdk4, and interaction with the E2F transcription factors (30-32).

Once myogenic cells exit the cell cycle and enter G₀ phase they fuse into multinucleated myotubes. Studies of myoblast fusion have largely been concentrated in Drosophila models (33). Genes including loner, dumbfounded, roughest, sticks and stones, and, most recently, the small GTPase ARF6, have been identified as involved in fly myoblast fusion (33-35). Fused muscle cells are irreversibly committed and typically cannot undergo proliferation. However, one factor that has been identified as capable of reversing this commitment is Msx1, a protein involved in regeneration in amphibians. Expression of this homeobox-containing transcriptional factor can cause dedifferentiation and cleavage of
myotubes, resulting in cells capable of proliferating and re-differentiating into a variety of cell types (36).

II. Role of MRF transcription factors

Unique to skeletal muscle development is the MRF family of transcription factors. Muscle regulatory factors were first described by their ability to induce non-muscle cells to undergo myogenic conversion (2, 4, 37-39). The first MRF family member identified was MyoD by Davis et al. in 1987 (1). Identification of this novel beta helix loop helix (bHLH) family member greatly increased our understanding of skeletal muscle myogenesis and led to the identification of three other family members: Myf5, MRF4, and myogenin. These bHLH factors have been shown to bind to E box sites (CANNTG) in the DNA of target genes (40). Accordingly, MRFs heterodimerize with E proteins, which are non-myogenic, ubiquitously expressed bHLH proteins that function as transcriptional regulators (41, 42). E boxes are found in a number of genes critical for the development of mature skeletal muscle including myostatin (12), myocyte-specific enhancer binding factor 2 (MEF2) (43), muscle creatine kinase (44), and dystrophin (45). MRF genes also contain E boxes. MyoD has the ability to regulate its own transcription and the expression of myogenin, which is co-regulated by MEF2 and MyoD (46). In addition, MRFs are modified by, and regulate, proteins involved in the cell cycle to control proliferation of skeletal muscle cells (24, 47-52).

During early skeletal muscle development, expression of Myf5 is observed in the presomitic mesoderm (53). Following segmentation of the somites, Myf5
expression is increased in the medial wall of the developing mouse (54); while in avian somites MyoD is upregulated in the same region (55). However, normal migration of cells takes place without the expression of Myf5 (56), suggesting that the early process of determination of limb muscle occurs prior to MRF signaling. In the murine somite, expression of Myf5 occurs at day E8.0, myogenin at day E8.5, MRF4 at day E9.0, and MyoD at day 10.5 (54,57-59). This temporal pattern of expression illustrates the key role MRF proteins play in early muscle cell determination and mesoderm development.

Once in the limb, Myf5 is the first MRF identified and is present in proliferating myoblasts (60). Another marker of proliferating myoblasts is Pax3, a paired box homeotic gene, that is upstream of MyoD (61) and is required for normal migration (62) and skeletal muscle development (63,64). Following Myf5 expression, the next MRFs detected in post-mitotic myocytes are MyoD and Myogenin (60,65). Expression of the final MRF, MRF4, is restricted to multinucleated myotubes and is the primary MRF in adult muscle; in mice expression in the limb occurs two days after MyoD and Myogenin transcripts are first identified (58). In cell culture, expression of the MRFs mimics the timing observed in vivo with Myf5 found in proliferating myoblasts, MyoD and Myogenin in myocytes and differentiated cells, and MRF4 the final MRF expressed (66).

Due to their critical role in the control of skeletal muscle development, the MRFs are tightly regulated (67). Negative regulators of the MRFs include Id, which inhibits terminal differentiation (68). Id is a HLH protein incapable of binding DNA (69) that inhibits the MRFs by sequestering E2A gene products, E12 and E47 (68),
and is upregulated by growth factors (70). Another important negative regulator is Twist, a bHLH protein that is capable of inhibiting terminal differentiation (71). Twist functions in a similar fashion as Id and sequesters E proteins to prevent dimerization with MRFs (72). An additional inhibitor that blocks myogenesis via complexing and inactivating E proteins is MyoR, which functions as a transcriptional repressor (73). Finally, Mist1 is a protein that heterodimerizes with MyoD and acts as a dominant-negative protein to repress myotube formation (74).

The nuanced functions, and potential redundancies, of MRF proteins have been largely identified using knockout mouse models. Mice deficient for MyoD have normal skeletal muscle development and an increase in expression of Myf5 (75). However, these proteins are clearly not entirely redundant as mice null for Myf5 die immediately following birth due to rib defects. These mice have retarded myotomal cell development in the somite, but newborn mice have a normal skeletal muscle phenotype (76). Although these factors appear to be largely redundant in skeletal muscle development, studies have demonstrated that MyoD and Myf5 are expressed in different regions of the developing myogenic tissue (77). In addition, Myf5 is primarily required for the development of epaxial muscle and MyoD for hypaxial muscle (78). Research has indicated that Myf5 cannot regulate differentiation by itself in the absence of MyoD, MRF4, or myogenin (79,80). Strikingly, mice that lack both MyoD and Myf5 do not survive because they do not develop skeletal muscle or even myoblasts (81,82). However, a new study has demonstrated this lack of skeletal muscle is highly dependent on whether MRF4, a gene linked with Myf5, is disturbed during gene targeting. If it is not impaired, MRF4
can act as a muscle determination factor and direct early myogenesis in the absence of Myf5 (83). In the absence of MRF4, a partial redundancy with myogenin becomes apparent as expression of this MRF is sharply upregulated. It is of note that, as in Myf5 null mice, MRF4 null mice display rib abnormalities (84), although this is largely influenced by the method used to eliminate MRF4 expression (85).

Targeted mutations of myogenin result in severe anomalies in skeletal muscle, and prenatal death, of null mice. This dramatic phenotype appeared to be largely due to defects in differentiation and secondary myotube formation (86). This finding corresponds with earlier work indicating a role for myogenin in the later stages of terminal differentiation. Double mutants of both MRF4 and myogenin have a phenotype very similar to myogenin null mice (87) as did MyoD/myogenin and Myf5/myogenin knockouts (88). These results demonstrate the non-redundant functions of these MRFs and the critical requirement for myogenin in the final stages of skeletal muscle development. In addition, double mutants of MyoD and MRF4 display a severe impairment of late myogenesis similar to the phenotype observed in myogenin knockouts, although myogenin was expressed in these cells (87). These results demonstrate that myogenin, while an absolutely critical factor for normal muscle development, is not sufficient to maintain the skeletal muscle program without expression of other MRFs. However, another study found that myogenin temporally expressed from the Myf5 locus can substitute, at least to an extent, for either Myf5 or MyoD in early myogenesis. These mice have reduced skeletal muscle, and die after birth, but do have a greatly increased skeletal muscle mass as compared to other Myf5:MyoD (-/-) mice (89). Lessons from these mice identify the
detailed cascade of events regulated by these factors. Clearly, MRFs display both unique, and overlapping, roles in their finite control of skeletal muscle myogenesis.

III. Intracellular signaling cascades involved in skeletal muscle myogenesis

The fate of a myoblast is under constant control. Signals compete to maintain proliferation or induce cell cycle exit, fusion and terminal differentiation. Transduction of these signals is critical to embryonic skeletal muscle development and the activation, proliferation, and fusion of satellite cells during postnatal skeletal muscle growth. Initially, it became clear that competing signaling pathways were critical in the molecular switch between a proliferating and differentiated state. For example, IGF-1 is capable of stimulating both mitogenesis through stimulation of cell cycle progression, and MRF suppression and myogenesis through an increase of MRF expression and fusion of mononucleated cells (90). The first pathways identified in IGF-regulation of myogenesis were mitogen-activated protein kinase (MAPK) kinase (MEK) and phosphatidylinositol-3 kinase (PI3-K) (91). This study identified a role for the MAPK pathway in promoting proliferation and preventing differentiation, and PI3-K in stimulating terminal differentiation.

The PI3-K pathway has been established as critical for skeletal muscle myogenesis (92,93). Inhibition of PI3-K blocks markers of cell cycle arrest and terminal differentiation (93,94) and expression of a constitutively-active (CA) PI3-K is sufficient to augment terminal differentiation (94). Downstream of PI3-K, the serine-threonine kinase, Akt, is also able to regulate myogenesis. Expression of a dominant-negative (DN) Akt inhibits formation of multinucleated myotubes and
decreases expression of markers of terminal differentiation. In addition, expression of a CA Akt increased myogenic differentiation and was able to rescue the affect of PI3-K inhibition on myogenesis (95). The PI3-K/Akt pathway has been identified to enhance viability of myoblasts (96), promote MyoD/MEF2 binding (97), and to increase transcription of myogenin (97-99) and Cdk5 (100). However, the affect of this cascade on myogenesis is not entirely sufficient to stimulate myogenic differentiation. Activation of the PI3-K/Akt cascade by IGF-1 must be accompanied by a decrease in ERK1/2 phosphorylation to influence myogenesis (101), indicating that this pathway is negatively regulated by MEK/ERK MAPK. In addition, the p38 MAPK pathway is required for terminal differentiation (92,102-104) and promotes differentiation via arrest of the cell cycle and inhibition of the Raf/MEK/ERK cascade (104). p38 MAPK is sufficient to induce expression of myogenin, myosin light chain, and MyoD (102) and is involved in cross-talk with the PI3-K/Akt pathway (92). Finally, it is clear that the role of these signaling proteins is complex as PI3-K/Akt stimulates cell cycle progression (105), and phosphorylation by p38 inhibits E47, a key dimerization protein for the MRFs (106).

Several GTPases and their downstream targets are important in regulation of terminal differentiation. Initially, oncogenic Ras was determined to inhibit myogenesis (107,108). A downstream target of Ras, Raf, affects myogenesis via the Raf/MAPK signaling pathway (109-111). In addition, Rac1 inhibits myogenesis and promotes proliferation downstream of TGFβ through activation of the C-jun N-terminal kinase (JNK) (112,113). However, RhoA, a GTPase involved in control of the actin cytoskeleton, promotes terminal differentiation (112,114), although this
regulation is complex (113). The MEK/ERK MAPK signaling cascade promotes proliferative signaling in response to IGF-1 (115) and FGF2 (115-118). Stimulation of ERK1/2 is required for cell cycle progression (116) and inhibition of MEK/ERK augments myoblast fusion and expression of markers of terminal differentiation (118). Clearly, activation of this signaling cascade is a critical component of myoblast proliferation during skeletal muscle growth.

Another signaling pathway identified in the control of myogenic differentiation is the nuclear factor-kappa B (NF-κB) cascade. Research on this transcription factor has indicated a complex role for NF-κB in regulation. NF-κB stimulates proliferation, and inhibits myogenesis (119,120), by increasing transcription of Cyclin D1 (119). However, a number of studies indicate a role for NF-κB in promotion of differentiation downstream of IGF-II (121,122), TNF-α (123,124), insulin (125,126), and IL-6 (127). Other signaling molecules involved in myogenesis are calcineurin (128) and NFAT (129,130) which promote terminal differentiation, and Src kinases. Srcs promote proliferation, inhibit differentiation (131), and prevent apoptosis of myotubes (132). This research demonstrates the importance of intracellular signaling cascades in regulation of skeletal muscle myogenesis. In the future, it will be important to discover cross-talk between established pathways, and identify novel signaling factors involved in this complex process.

Hypertrophy in Skeletal Muscle

I. Overview of muscle hypertrophy
Postnatal growth of skeletal muscle is predominantly due to hypertrophy of cells in response to physical stress. Hypertrophy of skeletal muscle causes an increase in size of muscle cells. This size increase occurs without augmenting the number of cells (hyperplasia). On the cellular level, hypertrophy is marked by increased transport of amino acids (133,134), increased RNA synthesis (135) and an increase in total protein. This increase is facilitated by an increase in synthesis and a decrease in degradation of protein (136,137). Little is known about the intracellular signaling pathways that regulate skeletal muscle hypertrophy. Pathways implicated to be involved in transducing signals during hypertrophy include PI3K/Akt, a pathway downstream of IGF-1 (138) and sufficient to induce hypertrophy \textit{in vivo} (139), and calcineurin/NFAT (140,141), although the involvement of this pathway is controversial (142). A major negative regulator of muscle hypertrophy is the TGF-\beta superfamily member, Myostatin. Mice null for myostatin have greatly increased skeletal muscle hypertrophy (11). However, more research is needed into these, and other, signaling pathways to allow future manipulation to counteract muscle atrophy \textit{in vivo}.

II. Mechanical stretch as a model system \textit{in vitro}

Mechanical stretch \textit{in vitro} has long been described as a model to delineate the mechanism of skeletal muscle hypertrophy (143). Use of an \textit{in vitro} cyclic stretch model can stimulate skeletal muscle growth (144), secretion of IGF-1 (145), activation of satellite cells (146) and increased protein accretion (147), and mimics \textit{in vivo} stretch-stimulated gene expression (148-151). Cyclic mechanical stretch
models have been utilized in vitro to examine skeletal muscle hypertrophy. These models have identified mechanical stretch to inhibit myogenesis (152,153) and to activate MAPK (154,155), Akt (156), NF-κB (153,157), GTP-binding proteins (153,158), and FAK (153) signaling proteins. These models have also identified HGF and NOS as activators of satellite cells (146,159,160).

Mechanical stretch has been used in a number of other cell types to analyze the response of cells to mechanical stimulus. In particular, the mechanisms involved in cardiac hypertrophy have been extensively studied using in vitro stretch (161-165). In addition, the pathways involved in the response of vascular smooth muscle cells to stress have been examined using mechanical models (166-168). Finally, in vitro mechanical stimulation has been utilized to examine cell migration (168-172) and actin cytoskeleton rearrangement in response to stress (172-174). Use of in vitro models has the advantage of isolation of cell type and simplicity of system. This makes these models ideal for initial studies of the signaling pathways involved in stretch-induced responses, although results will clearly need to be confirmed, and re-examined, in vivo.

III. Satellite cells

Currently, it is believed that the majority of growth observed in skeletal muscle hypertrophy is facilitated by a population of cells located between the sarcolemma and basal lamina. These satellite cells represent a reserve population of quiescent mononucleated cells committed to the myogenic lineage (175). They were originally identified as the source of nuclei for postnatal growth of skeletal muscle (176).
Satellite cells are a distinctive adult stem cell population distinguished by expression of Pax7 (177), c-Met (178), myocyte nuclear factor (179), syndecan-3, and syndecan-4 (180). Although the exact mechanism of satellite cell origin is unknown, it is clear that the paired homeobox transcription factor, Pax7, is required for their development. Mice that lack Pax7 have normal embryonic skeletal muscle growth but are completely devoid of satellite cells (177).

Satellite cells are activated out of their quiescent state in response to hypertrophy-stimulating events (176,181-183). These stimulating events include work overload (181,182), denervation (184), and synergistic ablation (183,185). In addition, muscle injury stimulates satellite cell activation (186). Potential satellite cell-activating molecules include HGF (160), leukemia inhibitory factor (LIF) (187,188), IGF-I (189), and interleukin-4 (IL-4) (190). Muscle regeneration stimulates migration of activated satellite cells from both local and distant sources to the injured muscle (191,192). This migration is controlled primarily by HGF, which has shown to be a chemoattractant for satellite cells both in vitro (193) and in vivo (194). Activated satellite cells have an increased ratio of cytoplasmic to nuclear volume as well as an expanded amount of intracellular organelles (191). Expression of MRFs MyoD and Myf5 is restricted to activated, proliferating cells (185). Growth factors shown to stimulate satellite cell proliferation include HGF and FGFs 1, 2, 4, 6, and 9 (195). Following activation and proliferation, satellite cells either exit from the cell cycle and differentiate into new or existing fibers (178), or, as a self-renewal mechanism, return to a quiescent state (196,197). Studies have demonstrated that satellite cells are present in two distinct populations, one self-renewing and
proliferative for extended periods while the second population is capable of quickly fusing into myotubes (196).

Muscle disease and aging impair the ability of satellite cells to increase and sustain adult skeletal muscle. For example, Duchenne muscular dystrophy (DMD), a widespread and debilitating muscle disease, is largely caused by the inability of muscle stem cells to appropriately preserve skeletal muscle mass in afflicted individuals (198). Aging also limits the capacity of satellite cells to counteract the effects of muscle atrophy and to maintain skeletal muscle mass (199). Aged muscle has a reduced capacity to recover from exercise and injury (200,201). Clearly, the ability to manipulate satellite cell activation, and subsequent proliferation, has far-reaching consequences in the fight to combat muscle atrophy and disease.

**IV. Adult stem cells and skeletal muscle**

Satellite cells are not the only population of stem cells with the capacity to increase postnatal skeletal muscle mass. A separate population of cells had been hypothesized, as irradiation of skeletal muscle did not completely ablate compensatory hypertrophy (202). Recent studies have identified stem cells that are hematopoietic in source located in skeletal muscle (203,204). Cells with a similar marker phenotype have also been observed in clonal skeletal muscle cell lines (205). These cells have the ability to differentiate into myotubes both in vitro (206) and in vivo (207). These cells are both located in skeletal muscle (203,204) and able to migrate from transplanted bone marrow (207). These studies indicate the
possibility that transplantation of these cells could be used to treat human diseases like DMD (208). Hematopoietic stem cells (HSCs) found in skeletal muscle both have the ability to differentiate into muscle (209) and to repopulate irradiated bone marrow with adult blood cells (203). These cells are capable of proliferating and becoming myogenic when activated in injured muscle (210,211). HSCs express unique markers of stem cells, CD45 and Sca1, that are not seen in satellite cells (212) and exclude HO33258 dye (212,213).

It is of note that a very recent study has indicated a greatly reduced role for HSCs in postnatal muscle growth (214). Sherwood et al. described that the myogenic capacity of cells derived from the bone marrow is severely restricted. Although these cells relocate to the same area as satellite cells, they do not appear to be able to terminally differentiate. However, co-culture of these cells with myogenic cells did cause bone marrow-derived cells to occasionally express myosin heavy chain, a marker of terminal differentiation. These results agree with other studies that have identified a key regulator of satellite cells, Pax7, to be necessary and sufficient for adult stem cells to adopt a myogenic lineage (215) and that co-culture with myoblasts can induce induction of HSCs into myofibers (212). Clearly, these results challenge the ability of bone marrow-derived stem cells to contribute significantly to adult skeletal muscle mass.

Overview of the JAK/STAT Signaling Pathway
I. Overview of JAK/STAT in signaling

The Janus kinase and signal transducer and activator of transcription (JAK/STAT) signaling pathway is a classic cell surface to nuclear signaling pathway. Traditionally, the pathway is thought to be activated by a cell ligand, often a cytokine such as IL-6, binding to its respective receptor. The JAK proteins are associated with the intracellular domains of these receptors, which lack intrinsic kinase ability. Once the ligand is bound, the associated JAKs are juxtaposed and auto- or trans-phosphorylate. Activated JAKs then phosphorylate their associated receptor, which provides docking sites for latent STAT proteins primarily found in the cytoplasm of unstimulated cells. Once bound, STATs are phosphorylated on specific tyrosine residues required for activation. The activated STATS then homo- or heterodimerize, via reciprocal interactions of phosphorylated tyrosine residues and SH2 domains, and the resulting complexes are able to translocate to the nucleus and bind to specific DNA motifs. This outlines a pathway that, in response to signaling from a wide variety of factors, is able to quickly influence gene transcription. Due to the biological relevance of this pathway it has been extensively reviewed in the literature (216-221).

II. Overview of JAK2

Janus kinase 2 (JAK2) is a receptor-associated kinase and a member of the Janus family of tyrosine kinases. JAKs derive the name "Janus" from the Roman myth of a two-faced god representing doors, gates and beginnings. In mammalian cells, this family consists of four proteins: JAK2, JAK2, JAK3, and Tyk2 (222). JAKs
are classically described as a member of the JAK/STAT cell membrane to nuclear signaling cascade (216).

JAK2 is a 120 kDa protein consisting of seven conserved Janus homology (JH) domains and was first described in 1991 (223). Although the crystal structure of JAK2 has not yet been determined, three-dimensional models have characterized the structure of the protein to include a FERM, Src Homology 2 (SH2), kinase-like, and kinase domain (224). Functionally, the kinase domain (JH1) and pseudo-kinase domain (JH2) are of particular interest. The catalytically inactive pseudo-kinase (JH2) domain inhibits JAK2 activity and removal of this domain renders the mutant protein constitutively active (225,226). Recent research has identified a specific tyrosine residue in the JH2 domain that, when phosphorylated, inhibits JAK2-dependent signaling (227,228). Mutations of this residue, Tyr\textsubscript{570}, result in a constitutively active JAK2 protein (228). In contrast, phosphorylation of a specific tyrosine residue, Tyr\textsubscript{221}, results in increased JAK2 signaling (227). In addition, a key glutamic acid residue (E1046) is required for angiotensin II (Ang-II) signaling via JAK2. Mutations of this residue greatly inhibited JAK2 activation and the ability of JAK2 to phosphorylate STAT1 (229). Research has also identified a pair of key tyrosine residues in the activation loop of the kinase domain (Y1007 and Y1008). Mutations in both these residues, or just Y1007, blocks kinase activity of JAK2 (230). This research clearly demonstrates that JAK2 is a complex molecule capable of regulation of, and by, many factors and biological events.

JAK2 is often described as an activator of proliferation in a number of cell types including vascular smooth muscle cells (231,232), pulmonary epithelial cells...
(233), skeletal muscle satellite cells (234), and mammary epithelium (235). In addition, JAK2 is involved in several critical processes including erythropoiesis (236), mammary epithelium differentiation (235) and apoptosis (237), as well as being implicated in a number of cancers. These cancers include acute lymphoblastic leukemia (238), lymphoma metastasis and invasion (239), myeloid leukemia (240), and breast cancer (241). Clearly, JAK2 is responsible for signaling in a wide range of biological events. Creation of a JAK2-deficient mouse resulted in embryonic lethality at 12.5 days due to a lack of erythropoiesis (236,242). Unfortunately, this early death from severe anemia limited analysis of other tissues in these mice. However, recent generation of a conditional JAK2 knockout mouse will allow targeted inhibition, and more in depth studies, of the role of JAK2 in specific tissues (243).

**III. Requirements of JAK2 for receptor signaling**

Signaling via the JAK/STAT pathway often begins with the binding of a cytokine to their corresponding receptor. These receptors lack intrinsic kinase activity and consequently require a physically associated kinase for activation of their downstream signaling cascades. Upon binding of the cytokine the receptors undergo a conformational change and aggregate, juxtaposing the JAK proteins. This oligomerization triggers auto- or trans-phosphorylation of the receptor-associated JAKs. This tyrosine phosphorylation activates the JAKs, creates binding sites on the phosphorylated receptor subunits, and begins the phosphorylation signaling cascade (244).
Shortly after the identification of JAK2, it was found to be associated with the erythropoietin (EPO) receptor and required for EPO signaling (245). After this identification, JAK2 has been determined to be required for multiple cytokine signaling events. Type I cytokines primarily utilize JAK2, although a few Type II cytokines, including Interferon λ (IFN λ), signal through JAK2. JAK2 is associated with signaling via heterodimeric Type I cytokine receptors including: Interleukin (IL)-3, IL-5, IL-6, IL-11, IL-12, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF), ciliary neurotrophic factor (CNTF), LIF, cardiotrophin-1 (CT-1), and Leptin (218). In addition, JAK2 is the primary Janus kinase associated with cytokine signaling through homodimeric receptors such as those for EPO (246), Thrombopoietin (TPO), Prolactin, and GH (218,247). Development of JAK2 knockout mice identified the unique, critical role of JAK2 in the signaling of many cytokines including EPO, IL-3, GM-CSF, IL-5, TPO, and IFNλ (242). In addition to its involvement in cytokine signaling, JAK2 is also associated with several seven transmembrane G-protein coupled receptors including the angiotensin 1 (AT1) receptor (248,249) and the α₁ adrenergic receptor (250).

As the number of receptors requiring JAK2 for signaling grows, it is becoming apparent that JAK2 is a highly structured protein with specific residues and regions required for receptor association. The portion of JAK2 most commonly utilized for binding and activation of receptors is the amino (NH₂) portion. This region is required for binding and activation of GM-CSF (251), GH (252), AT1 (253), and EPO receptors (244,245,254). A requirement for this region is not surprising as it contains both the pseudo-kinase (JH2) and kinase (JH1) domains. Requirement for
a catalytically active kinase domain is essential to activate the above receptors, as well as necessary for association with others including the AT1 receptor (255,256). Specifically, mutation of glutamic acid residue 1046 (E1046) reduces Ang-II signaling by both inhibiting JAK activity and the ability of JAK2 to associate with the AT1 receptor (229). However, although the JH1 domain is important, the JH2 domain is also critical for signaling via cytokine receptors and, although it has no intrinsic catalytic activity, is still necessary for signaling to occur (225). Clearly, these results demonstrate the role of JAK2 at the level of the receptor is far broader than simple activation of associated proteins. It is quite likely that JAK2 is a key bridge molecule for proteins such as STAT1 (256) and c-Src (253). Finally, JAK2 has been identified as playing a major role in receptor processing, particularly in processing of the EPO receptor (254), but a role has also been suggested in regulation of the GH receptor (257,258).

IV. Location of JAK2 in the cell

Classically, JAK proteins are thought to be predominantly located at the cell membrane, bound to receptors, ready for activation and subsequent activation of downstream targets (216,242). However, this traditional role has been challenged by studies that have identified JAK proteins in the nucleus of a number of cell types, including CHO (259), fibrosarcoma (260), liver (261), islet, and skeletal myoblasts (262), as well as during early oocyte development (263). Although a specific motif has only been described for Tyk2 (260), putative nuclear localization signals (NLS) have been identified for JAK1 and JAK2 (259). However, other groups have
questioned the specificity of antibodies used and, using alternative strategies, found JAK1 and JAK2 to be largely located in the cytoplasm or at the cell membrane (257,264). It is doubtful that the location of JAK proteins in the nucleus is purely an artifact, however. A number of studies have utilized western blotting techniques on nuclear extracts to identify JAK2 in this fraction (259,261,262), and an antibody used to locate JAK2 in the nucleus has been shown to be specific in skeletal muscle (265).

Several recent studies have identified novel roles for JAK2, suggesting a wealth of alternative functions. JAK2 is required for processing of the EPO receptor (254) and is involved in the regulation of p97, a AAA protein required for endoplasmic reticulum (ER) assembly (266). JAK2 protein has also been localized in the neuro-muscular junctions of skeletal muscle (265). In addition, another critical signaling protein with similar functions, ERK3, can be nuclear localized (267). These results contest the conventional roles of signaling kinases and suggest new functions for these proteins, potentially dependent on their cellular localization.

VI. Negative regulation of JAK2

Constituitive activation of JAK2 has been associated with oncogenesis. This aberrant JAK2 phosphorylation is often due to a disruption of negative control of kinase activity. Thus, the JAKs are tightly regulated by a number of proteins including traditional phosphatases and specific binding proteins (268). The best characterized phosphatase to inhibit JAK2 is SHP-1, a SH2-containing cytoplasmic
tyrosine phosphatase (269). Mice lacking SHP-1 (motheaten) have aberrant JAK activation and an excess of hematopoietic cell production (270). SHP-1 binds directly with, and dephosphorylates, JAK2 (271). Another phosphatase recently demonstrated to inhibit JAK2 is protein-tyrosine phosphatase 1B (PTP1B) (272). PTP1B is able to bind JAK2 and regulates JAK2 via dephosphorylation of key tyrosine residues (Y^{1007/1008}) required for activity (273). Ovarian cancer cells, which have impaired p53, a tumor suppressor gene and an activator of SHP-1 activity, display JAK2 hyperphosphorylation, which is reversed by expression of wt p53 (274).

Another potent inhibitor of the JAKs is the suppressors of cytokine signaling (SOCS) family of proteins (275). SOCS-1 inhibits JAK2 via direct interaction between the JH1 domain of JAK2 and the kinase inactivation domain of SOCS-1 (276,277). Accordingly with a negative regulatory role, SOCS-1 selectively binds kinase-activated JAK2 (277). This is of interest since a specific small peptide inhibitor of JAK2, with significant homology to the SOCS-1 inhibitory domain, has the ability to bind both active and inactive JAK2 (278). Recently, SOCS-2 has been suggested to inhibit GH-dependent activation of JAK2 (279), an affect of particular interest as SOCS-2 knock-out mice display an extreme, high-growth phenotype (280). Finally, SOCS-3 is able to bind the JH1 domain of JAK2 (281) and inhibits leptin-induced phosphorylation of JAK2 (282). In the future it is anticipated that further research on this family of cytokine inhibitors will increase our understanding
of negative regulation of the JAK/STAT pathway and potentially lead to therapeutic targets in a number of important diseases.

Although the tyrosine kinase inhibitor tyrphostin AG490 has long been described as a JAK2 inhibitor, recent studies have questioned its specificity (283). Originally indicated as a treatment for acute lymphoblastic leukaemia (238), AG490 is widely utilized to inhibit JAK2 both in vivo (284-286) and in vitro (287-290). Clearly, the description of AG490 as a JAK2-specific inhibitor is problematic. Research has demonstrated AG490 inhibits a variety of molecules including JAK3 (291-293), Cdk2 (294,295), epidermal growth factor receptor (296), and c-src (297). Even more significant is the broad effect AG490 can have on critical cell processes such as cell cycle progression (294,295), serum-stimulated growth and DNA synthesis (296), and actin cytoskeleton formation (298). This evidence suggests care must be used when AG490 is used as a JAK2 inhibitor and underscores the need for a second inhibitory approach.

VII. Alternative downstream targets

Although JAK2 traditionally is thought to signal through the STAT transcription factors, additional downstream factors have been identified. This includes the signal transducing adaptor molecules (STAMs). Currently, two STAM proteins have been identified, STAM1 and STAM2. Both proteins associate with JAK2 and JAK3 (299-302). The JAK/STAM pathway is triggered by upstream signaling factors such as IL-2, IL-3, IL-4, IL-7, GM-CSF, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (300-302). Originally, the search for an alternate downstream
JAK signaling protein began when no evidence was found for STAT5 to be involved in c-myc transcription following IL-2, IL-3, and GM-CSF signaling (300). Stimulation by IL-2 led to the identification of a 70 kDa protein that was heavily tyrosine phosphorylated. Purification of this protein led to the discovery of STAM, an adapter molecule containing two major domains (300,301). STAM2 was individually identified based on mass spectrometry proteomic (299) and cDNA homology genomic approaches (302). The first STAM domain is an essential src homology 3 (SH3) region located approximately in the center of the protein. The second is the immunoreceptor tyrosine-based activation motif (ITAM) domain located in the carboxy-terminal. These domains are both required for c-myc induction (300-302). However, only deletion of the ITAM domain resulted in a decrease in tyrosine phosphorylation of STAM and blocked JAK2 and JAK3 association (300). Both STAM proteins have a similar structure and share 50.1-55% homology (302). Murine knock-out models of STAM1 mice displayed retarded growth, premature mortality, and abnormal hippocampal development (303). Clearly, these proteins play an important role in the JAK2 signaling cascades and their discovery illustrates the complexity of signal transduction pathways outside of the traditionally studied, linear JAK/STAT paradigm.

VIII. Role of JAK2 in muscle signaling

Although a role for the JAK/STAT pathway has not yet been identified in skeletal muscle hypertrophy, it is activated in cardiac hypertrophy (163,304-306). JAK2 is activated by mechanical stretch in cardiomyocytes (163) and overload in the
heart (306). It is also required for vascular smooth muscle cell (VSMC) proliferation (307,308). JAK2 is activated by known cardiac hypertrophic agonists such as Ang-II (306,309), LIF (310), Cardiotrophin-I (311), and IL-6 (287). Although little has been done to demonstrate a requirement for JAK2 in skeletal muscle, studies have demonstrated a necessity for JAK2 in LIF-induced satellite cell proliferation (188). In addition, JAK2 is activated in EPO-induced myoblast proliferation (312), and in response to leptin in skeletal muscle in vivo (313).

Growth hormone (GH) is a potent regulator of a number of critical growth pathways, including myogenesis, and regulates expression of IGF-I (90). JAK2 is associated with the GH receptor (GHR) and is activated upon GH binding to the GHR (247). In C2C12 myoblasts and myotubes, administration of GH causes a rapid increase in the phosphorylation of JAK2, STAT5A/B (314), and STAT3 (315). C2C12 myoblasts that overexpress GHR have inhibited myogenesis, and increased JAK2 activation, in response to autocrine GH (316). In addition, JAK2 is required for growth hormone (GH)-induced actin cytoskeletal reorganization (317) and migration (241). JAK2 also phosphorylates SH2B-ß (318), a Rac-binding protein required for the membrane ruffling and cytoskeletal rearrangement observed following treatment with GH (318,319). Finally, JAK2 physically interacts with focal adhesion kinase (FAK), a protein found in focal contacts and involved in cell migration, and Paxillin, a target of FAK also found in focal adhesions, when stimulated with GH (320).

VIV. Growth factors in myogenesis and JAK2
Many growth factors have been identified to influence skeletal muscle cell proliferation. Insulin-like growth factor (IGF-I) stimulates proliferation in skeletal muscle stem cells (321), satellite cells (322) (321), and C2C12 myoblasts (323). Both insulin and IGF-1 are sufficient to induce myofiber hypertrophy \textit{in vitro} (324). IGF-I inhibits differentiation and promotes proliferation in C2C12 skeletal myoblasts cultured in low serum media (325). IGF-I increases phosphorylation of JAK2 in pancreatic \( \beta \)-cells (326) and JAK2 and STAT3 in 293T cells (327). Dominant-negative JAK2 is able to block IGF-I induced activation of STAT3 in 293T cells (327). These studies indicate a potential role for JAK2 in IGF-I mediated skeletal muscle proliferation.

Hepatocyte growth factor (HGF) is a potent activator of satellite cell proliferation that stimulates these cells to enter the cell cycle (328). It also inhibits differentiation and promotes proliferation in turkey satellite cells (329). The HGF receptor, c-met, is a unique marker for satellite cells (178,328) and HGF can act synergistically with FGF2, 4, 6 and 9 to increase satellite cell proliferation (330). HGF activates the JAK2/STAT5 pathway during pancreatic \( \beta \)-cell proliferation and IGF-I further increased this effect (326). Fibroblast growth factor (FGF) is a strong mitogen for satellite cells. FGF1, 2, 4, 6 and 9 all significantly increase proliferation of satellite cells (330). FGF2 stimulation increases phosphorylation of STAT3 in proliferating myoblasts and interacts cooperatively with LIF in this activation (331).

Angiotensin II (Ang-II) has important roles in skeletal muscle hypertrophy, cardiac hypertrophy, and vascular smooth muscle cell (VSMC) proliferation. Specifically, Ang-II signaling through the AT\(_1\)R contributes to overload-induced
cardiac hypertrophy (332), and Ang-II is released from mechanically stretched cardiac myocytes (333). Although the role of Ang-II has been well characterized in cardiac hypertrophy, its role in skeletal muscle hypertrophy is less defined. Inhibition of Ang-II using ACE inhibitors reduces overload-induced skeletal muscle hypertrophy in vivo. This inhibition could be partially rescued by local perfusion of exogenous Ang-II (334). In the same study, blocking the AT$_1$R resulted in a decrease in overload-induced hypertrophy that could not be rescued by the addition of Ang-II. Ang-II and the AT$_1$R also have a critical role in the proliferation of VSMC (335).

A critical role for Ang-II signaling in cardiac and smooth muscle has been well described, but the role of Ang-II signaling in skeletal muscle cell proliferation has not been clarified. In C2C12 myoblasts, Ang-II treatment increases protein synthesis (336,337). However, these studies disagree on the impact of Ang-II signaling on C2C12 proliferation. Puri et al. found that treatment with Ang-II (10$^{-5}$ M) for 24 hours in quiescent (0.1% FCS) C2C12 cells caused a significant increase in DNA synthesis. In contrast, Hliang et al. found that quiescent C2C12 cells stimulated with 100 nM AngII for 24 hours did not significantly increase proliferation. Ang-II phosphorylates JAK2 through the AT$_1$R in VSMCs in vitro (338) and in vivo (339). Activated JAK2 forms a complex with the AT$_1$R in VSMCs (340). Ang-II also induces the activation of STAT1, STAT2 and STAT3 (232) in VSMCs and STAT1, STAT3, and STAT5A/B in mesangial cells (341). AG490, a JAK2 inhibitor, blocks Ang-II induced proliferation in VSMCs (232).
Interleukin-6 (IL-6) is a potent activator of proliferation and is implicated in a number of cancers including multiple myeloma (342-344), Hodgkin's disease (345), and in renal cancer cells (346). The JAK2 inhibitor, AG490, inhibits proliferation in Hodgkin's disease (345), multiple myeloma (343) and in renal cancer cells (346). IL-6 activates the STAT proteins through its association with JAK2. Specifically, STAT3 is constitutively activated in IL-6-induced proliferation of multiple myeloma cells (343) and renal cancer cells (346). Moreover, in these cell lines, AG490 inhibited STAT3 activation and repressed IL-6-induced proliferation. In addition, IL-6-like cytokines increase proliferation of skeletal muscle cells in vitro. IL-6 and LIF both increase proliferation of cultured myoblasts (347). Leukemia inhibitory factor (LIF), increases skeletal muscle satellite cell proliferation and activates JAK2 in vitro. This increase in proliferation, and accompanying STAT3 activation, were blocked by the addition of AG490 (234).

IL-6-induced activation of JAK2 is mediated by the receptor-associated signal transducer, glycoprotein 130 (gp130) (348). gp130 plays a critical role in proliferation in a number of cell types. Notably, gp130 and activated STAT3 are required to preserve embryonic stem cells in an undifferentiated state (349). An acidic domain present on gp130 is necessary for cell proliferation in Cos-7 cells (350). In addition, STAT3 is required for LIF-induced, gp130-mediated cardiac myocyte hypertrophy (351).

These studies illustrate the complexity of the JAK tyrosine kinases. As research of this intriguing family of signaling proteins proceeds in the future, it is likely that our current assumptions about their major roles will be challenged. In
addition, our understanding of the role of JAK2 signaling for normal, and aberrant, function in many tissues will likely grow. In skeletal muscle in particular, a wealth of information in other tissues suggests a key role for this protein in moderating, and influencing, cell growth and differentiation. Future work will, no doubt, clarify and expand the role of JAK2 in skeletal muscle development and postnatal growth.

X. Overview of STAT proteins

STATs are latent cytoplasmic proteins; phosphorylation of a tyrosine residue is necessary for activity. Cytokines and growth factors can activate STATs and this activation controls the expression of SIF (sis inducing factor) target genes. Seven STATs have been identified (STAT 1-4, 5a, 5b, and 6). Activation of the STATs is classically described through phosphorylation by the JAK family of tyrosine kinases. STATs are activated by phosphorylation of tyrosine residues in the SHP2 domain. Interestingly, a constitutively active STAT3 fusion protein is able to maintain pluripotency in murine embryonic stem cells (349). Other modes of activation include activation by cytokine receptors with inherent kinase ability (RTK), such as the platelet-derived growth factor (PDGF) receptor (352) and by recruitment of a non-receptor tyrosine kinase (NRTK) like Src (353). Negative cytoplasmic regulation of STAT signaling includes protein-tyrosine phosphatase 1B (PTP1B) (354,355), suppressor of cytokine signaling (SOCS) proteins (356), and src homology phosphatases (SHPs) (357,358).

Following STAT activation and subsequent dimerization through the SHP2 domain, STAT proteins translocate to the nucleus. STAT1 and STAT2 contain an
arginine-lysine-rich region located in the DNA-binding domain. Mutations in this domain produce mutants that can be phosphorylated and dimerize, but are unable to translocate into the nucleus (359). In addition, dimers require two active arginine-lysine rich regions to translocate (359). To initialize facilitated transport into the nucleus, tyrosine phosphorylated STAT1/STAT2 and STAT1/STAT1 dimers bind to importin-α5 (360-362). The binding of STAT dimers to importin-α5 appears to be mediated through the arginine-lysine nuclear localization sequence (NLS) (360,361). Only dimers that contained two functional arginine-lysine rich regions were able to bind to importin-α5. STAT/importin-α5 complex consists of a STAT dimer and two importin-α5 proteins (360). One important exception to these prior studies is the identification of the nuclear translocation of unphosphorylated STAT1 that proceeds regardless of mutations in the NLS (363). Finally, STAT1 translocation is dependent on the GTPase activity of Ran (362). Ran GTPase is a key protein in the disassembly of importin-α5/target protein complexes in the nucleus (364).

Binding of inhibitory proteins can negatively regulate STATs in the nucleus and inhibit their ability to activate transcription. These inhibitory proteins include PIAS1 and 3 which inhibit STAT1 and 3 respectively (365,366), and truncated STAT proteins which as dominant-negative inhibitors (367). Protein tyrosine phosphatases also negative regulate STAT activity. STAT1 is tyrosine dephosphorylated in the nucleus before being exported to the cytoplasm (368). Several phosphatases have been suggested for this role. T-cell protein tyrosine phosphatase (TCPTP) dephosphorylates STAT1 (369), STAT5A, and STAT5B (370). Arginine methylation of STAT1 has been suggested to increase dephosphorylation by TCPTP, and PIAS1
is required to mediate this effect (371). SHP2 has also been identified as a nuclear phosphatase of STAT1 that dephosphorylates tyrosine and serine residues and inhibits STAT1 transcription (372). Interestingly, serine phosphorylation of STATs by glycogen synthase kinase-3 (GSK-3) increases nuclear export of STATs in

*Dictyostelium* (373). Finally, studies have demonstrated STAT1 to have nuclear export signals in the coiled-coil and DNA binding domains required for translocation into the nucleus (374,375).

**XI. STAT proteins and skeletal muscle**

Like research of their upstream JAK activators, study of the role of STATs in muscle is primarily in smooth and cardiac tissues. STATs have been well described as activated during hypertrophy in cardiac muscle. In rat cardiomyocytes, mechanical stretch activates STAT1 and STAT3 (376), and pressure overload in the rat heart caused the rapid phosphorylation of STAT1, STAT2, and STAT3 (377). STAT1, 2, and 3 are also activated by a potent regulators of cardiac hypertrophy: Ang-II (309,378) and LIF (310,379). IGF-1, another potent regulator of cardiac hypertrophy (380), activates STAT1 and STAT3 (381). In VSMCs, STAT1 and STAT3 are required for proliferation in response to Ang-II (307). STAT3 is phosphorylated by stimulation with Ang-II, IL-6, and PDGF in smooth muscle cells (287,382,383) and is required for vascular remodeling *in vivo* (308). These results clearly establish a critical role for signaling via the JAK/STAT pathway in cardiac and smooth muscle.
However the role of this pathway in skeletal muscle is less clear, although the STATs are slightly better described than their activating kinases. STAT1, 3, and 5 are activated in proliferating myoblasts in response to upstream factors: EPO, FGF, GH, and LIF (312,384,385). STAT3 is phosphorylated during LIF-induced satellite cell proliferation (234), and satellite cell activation and subsequent proliferation during muscle regeneration (386). In addition, STAT3 is in the skeletal muscle of mice stimulated with leptin injection (313). Combined, these results demonstrate a role for the STAT proteins in control of skeletal muscle myogenesis and in mediating skeletal muscle hypertrophy.

The aims of this study were: 1) To identify if JAK2 was required for skeletal muscle myoblast proliferation in vitro, 2) To determine if JAK2 was necessary for skeletal muscle myogenesis in vitro, and 3) To examine the response of the JAK/STAT pathway to in vitro cyclic stretch. This work provides the foundation for a critical role for JAK/STAT signaling pathway in skeletal muscle and indicates future direction for analysis of this intracellular signaling cascade.

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CHAPTER 2. JANUS KINASE 2 (JAK2) IS REQUIRED FOR PROLIFERATION AND MYOGENESIS IN C2C12 MYOBLASTS: IMPLICATION OF A KEY ROLE FOR NUCLEAR JAK2 IN THE REGULATION OF SKELETAL MUSCLE TERMINAL DIFFERENTIATION

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SUMMARY

Janus kinase (JAK) 2 is typically described as a receptor-associated signaling protein that activates signaling cascades at the level of the cell membrane. In this report, we show that JAK2 has distinct roles and protein localization in proliferating skeletal myoblasts and differentiating myotubes. We utilized pharmacological and genetic approaches to demonstrate that catalytically active JAK2 is required for proliferation in C2C12 myoblasts. Whereas, expression of a constitutively active JAK2 (TEL-JAK2) increases proliferation of myoblasts. Our research indicates a novel role for JAK2 in skeletal muscle myogenesis, as over-expression of JAK2
protein enhances myogenesis. Strikingly, we found that, in proliferating myoblasts, JAK2 protein is largely confined to the nucleus, while in terminally differentiated, multi-nucleated myotubes, JAK2 is distributed throughout the cell. We also show that inhibition of JAK2 activity in myoblasts with AG490 is transient as cells recover rapidly and proliferate normally when returned to normal growth media. In contrast, differentiating myoblasts treated with JAK2 inhibitor have an attenuated recovery, and only partially differentiate upon removal of AG490. Further analysis revealed that treatment with AG490 has a profound impact on the actin cytoskeletal structure of terminally differentiated cells. Cells treated with AG490 during myogenesis appear to retain the cellular localization of JAK2, and actin cytoskeleton, of an undifferentiated myoblast. These results represent the first characterization of a necessary and sufficient requirement for JAK2 in skeletal muscle myoblast proliferation and suggest a novel role for JAK2 in skeletal muscle myogenesis.

INTRODUCTION

Adult skeletal muscle contains a unique population of muscle stem cells called satellite cells that are found between the basal lamina and cell membrane of adult skeletal muscle (1). During embryonic development and postnatal hypertrophy, skeletal myoblasts and satellite cells proliferate and ultimately differentiate into multinucleated cells. These cells exist in a quiescent state unless activated to proliferate and fuse to existing muscle fibers. Satellite cells are the predominant source of additional nuclei in postnatal skeletal muscle growth (2) and are required
for skeletal muscle hypertrophy (3). Growth factors such as hepatocyte growth factor (HGF)/Scatter factor, fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), and cytokines like interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) are known to stimulate proliferation of immature muscle cells (4-8). The later events of myogenesis are largely orchestrated by the myogenic regulatory factors (MRFs): MyoD, Myf5, Myogenin, and MRF4. This helix-loop-helix family of transcription factors works in concert with other factors that include myocyte enhancer factor 2 (MEF2) and cell-cycle regulators like p21 and pRb (9-12). Muscle regulatory factors are characterized by their ability to induce non-muscle cells to undergo a myogenic transformation (13,14). However, the precise upstream mechanism(s) that drives the transition of a proliferating myoblast to its terminally differentiated fate is largely unknown.

The Janus kinase (JAK) family of tyrosine kinases consists of four family members: JAK1, JAK2, JAK3, and Tyk2. Classically, JAKs have been described as part of the JAK/STAT (signal transducers and activators of transcription) intracellular cell membrane to nuclear signaling cascade (15,16). Wilks et al. first identified Janus kinase 2 (JAK2) in 1991. JAK2 is implicated as a causative factor in a number of cancers and is often described as an activator of proliferation in a number of cell types including vascular smooth muscle cells (17,18), pulmonary epithelial cells (19), skeletal muscle satellite cells (20), and mammary epithelium (21). Initial studies in skeletal muscle have shown JAK2 to be required for LIF-induced increases in satellite cell proliferation (20), and JAK2 is phosphorylated during erythropoietin-stimulated proliferation in C2C12 myoblasts (22). Our previous
research indicated that JAK2 mRNA expression increased after three days of work overload in rat skeletal muscle (23). Studies have demonstrated that STAT proteins may be involved in skeletal muscle cell proliferation as STAT1, 3, and 5A are activated during myoblast proliferation (5,22). STAT3 is phosphorylated in leukemia inhibitory factor (LIF)-stimulated satellite cell proliferation (20) and during satellite cell activation and subsequent proliferation during muscle regeneration (24). Another Janus kinase, JAK3, has also been implicated in growth hormone (GH) signaling in skeletal muscle (25).

In the past ten years, several studies have shown that Janus kinases are located in the nucleus (26-30). Although a specific motif has only been described for Tyk2 (28), potential nuclear localization signals (NLS) have been described for both JAK1 and JAK2 (29). However, other groups have questioned these results and found JAK2 to be largely located in the cytoplasm or at the cell membrane (31,32). Traditionally, JAKs are thought of as receptor-associated kinases signaling at the level of the cell membrane (33,34). However, JAK2 is also required for receptor processing (35) ER assembly (36). This research demonstrates that JAK2 is involved in many critical cell processes beyond the established JAK/STAT pathway.

This study was conducted to investigate the role of JAK2 in skeletal myoblast proliferation and terminal differentiation. Our findings demonstrate that JAK2 is both necessary and sufficient to promote C2C12 myoblast proliferation. In addition, we present evidence that JAK2 plays a critical, and novel, role in skeletal muscle myogenesis. Our evidence suggests that this novel role may, in part, depend on the location of JAK2 protein in the cell.
MATERIALS AND METHODS

Cell Culture - C2C12 murine skeletal muscle myoblasts (American Type Culture Collection, Manassas, VA) were grown at 37°C in a humidified atmosphere of 5% CO₂ in growth medium (GM) that contained DMEM, 10% fetal bovine serum, 1.0 mg/mL penicillin/streptomycin and 0.3 mg/ml L-glutamine. Unless noted, cell culture reagents were purchased from Gibco-BRL Life Technology (Carlsbad, California). In terminal differentiation experiments, cells were cultured in restricted-serum differentiation medium (DM), which consisted of DMEM supplemented with 2% horse serum, 10 μg/ml insulin, 1.0 mg/mL penicillin/streptomycin and 0.3 mg/ml L-glutamine. Differentiation media was replaced every 48 hours throughout the time course of experiments. Transfection media for creation of the stable cell lines was identical to growth media, except it did not contain antibiotics. Cells were plated on 35-mm tissue culture dishes (BD Biosciences, Lexington, KY) at 1250 cells/cm² for proliferation assays, 20,000 cells/cm² for differentiation assays, and 5000 cells/cm² for nuclear and cytoplasmic/membrane extracts. For immunofluorescence experiments, culture dishes were coated with bovine fibronectin before plating cells. In proliferation and differentiation experiments, AG490 (Calbiochem, San Diego, California) was used as a JAK2 inhibitor and WHI-P131 (Calbiochem, San Diego, California) was used to inhibit JAK3 kinase activity. During the AG490 and WHI-P131 experiments, all control cells, and rescue cells in normal media, contained an identical amount of DMSO; the vehicle for AG490 and WHI-P131. Unless noted, all
chemicals were purchased from Fisher Scientific (Pittsburgh, Pennsylvania) or Sigma (St. Louis, Missouri).

**Stable Cell Lines** - To create dominant-negative (DN)-JAK2, wild-type (WT)-JAK2, and pCINeo (control) stable cell lines, the following plasmids were used: WT-JAK2, JAK2-DN (37), and pCINeo, which was the vector that WT-JAK2 and JAK2-DN cDNAs were cloned into. JAK2-DN has a K882E substitution in the wild type sequence. C2C12 cells were plated at 1250 cells/cm$^2$ on 10-cm tissue culture dishes (BD Biosciences, Lexington, KY). Cells were transfected with Fugene 6 (Roche, Basel, Switzerland) and 500 ng of linearized WT-JAK2 (WT), JAK2-DN (DN), or pCINeo DNA. To create the TEL-JAK2 (5-12) and pcDNA3 (control) stable cell lines, the plasmids pcDNA3-TEL-JAK2 (5-12) and pcDNA3 were used (38). Cells were transfected as described for DN-JAK2 stable cells. After 18 hours of incubation in transfection media, cells were changed to growth media that contained 400 μg/mL G418. Subclones that expressed the genes of interest were maintained by continual selection with G418. JAK2 over expression was confirmed by western blot analysis.

**Wounding Assay** - C2C12 myoblasts were plated to confluence (20,000 cells/cm$^2$) on a fibronectin-coated 35 mm cell culture dishes with a line marked on the bottom. Just prior to wounding, vehicle, 2 μM, or 20 μM AG490 was added to the cells. The confluent monolayer of cells was wounded the day after plating using a plastic, sterile pipette tip. Specific areas of the wound, above and below the marked line, were photographed throughout the experiments with a Nikon digital camera (DXM 1200). The percentage of wound coverage was determined from these photographs.
by comparing the wound at the start of the time course (T0) and after eight hours (T8).

**Differentiation Assay** - Cells were photographed throughout experiments with a Nikon digital camera (DXM 1200). At the time of collection, cells were washed three times with phosphate buffered saline (PBS), and 0.5 M glycylglycine buffer, pH 6.75, was added to the plates. Cells collected throughout the duration of the experiments (0 and 72 hours) were frozen at -80°C until completion of the time course. Cells collected at the conclusion of the experiments were frozen at -80°C for a minimum of one hour before all cells were scraped from the plates. After collection, cells were sonicated for 15 seconds using 50% cycling setting and centrifuged at 20,000 X g for one minute to pellet cell debris. The supernatant was collected and creatine kinase (CK) activity was determined as described by the manufacturer's protocol (Sigma, St. Louis, MO). Total protein was quantified with the Bradford protein assay (Biorad, Hercules, CA). Creatine kinase units were reported corrected for total protein in nanograms.

**Proliferation Assay** - Myoblasts were incubated with 10 μM BrdU and dCTP for the final 12 hours of the proliferation assay time course. Cells were washed twice with PBS, fixed for 10 minutes in 2% formaldehyde and incubated twice for five minutes in PBS. To denature genomic DNA, 4M hydrochloric acid (HCl) was added to the cells for 15 minutes and plates were washed twice with PBS. Cells were treated with 1.0% IGEPAL for four minutes and washed twice with PBS that contained 0.1% Tween-20 (PBS-T). Blocking buffer (PBS-T + 1.0% bovine serum albumin) with a 1:125 dilution of a primary anti-mouse BrdU antibody (Developmental Studies
Hybridoma Bank, Iowa City, IA) was incubated on the plates overnight at 4°C. Plates were washed and blocking buffer with a 1:250 dilution of secondary anti-mouse IgG, FITC-conjugated antibodies (Sigma, St. Louis, MO) was added to the plates for one hour. Plates were washed and incubated with 1.0 μg/mL Hoeschst 33258 (HO33258) in PBS-T for 10 minutes. Total and proliferated nuclei were quantified by visualization of HO33258 and BrdU-positive nuclei, respectively, with a Nikon fluorescent microscope. To determine the percentage of proliferated cells, we divided FITC-positive nuclei by the total number of nuclei.

**Immunoblots** - The expression of myosin heavy chain (MHC) was determined by Western blot analysis. Cells were washed twice with PBS and scraped from plates following addition of lysis buffer, pH 7.4 (39), which contained 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 10 mM Na3VO4, 5 mM EDTA, 1.0 mM Na4P2O7, 1.0% Triton X-100, 10% glycerol, 0.1% SDS, 1.0% Deoxycholic acid, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. The lysate was then centrifuged for ten minutes at 20,000 X g, and supernatant was removed for analysis. Total protein was quantified with the detergent compatible (DC) protein assay (Biorad, Hercules, CA). Protein (12.5 μg) was subjected to SDS-PAGE (6.5% resolving and 5% stacking gel) and transferred to Westran PVDF membrane (Schleicher & Schuell BioScience, Keene, NH). To inhibit non-specific antibody binding, the membrane was blocked with 5% non-fat dry milk and then incubated with undiluted MF20 anti-MHC antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) for an additional hour. A 1:10,000 dilution of secondary goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma, St.
Louis, MO) in blocking buffer was used. Myosin heavy chain protein was visualized with the enhanced chemiluminescence (ECL) kit from Amersham (Piscataway, NJ). Nuclear extracts were prepared as previously described (40). Cytoplasmic and membrane fractions were extracted using a harvest buffer containing 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 100 mM NaF, 1 mM PMSF, 4 µg/ml aprotinin, and 4 µg/ml leupeptin. Cells were incubated on ice for five minutes and nuclei were removed by centrifugation for five minutes at 1000 x g. 500 µg of extract were separated on an 8% SDS-PAGE stacking gel and western blotting proceeded as described above. Membrane was blocked with 5% BSA and JAK2 protein was detected with a 1:1000 dilution of HR-758 primary antibody and 1:5000 dilution of donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham, Piscataway, NJ). Detected protein was visualized using ECL plus (Amersham, Piscataway, NJ) on a charged-coupled device (CCD) camera FluroChem™ 8800 using FluroChem™ IS-800 software (Alpha Innotech, San Leandro, CA).

Immunofluorescence - C2C12 myoblasts were fixed after 24 hours (proliferating cells) or after 72 hours (differentiating cells) of culture. Cells were fixed for 30 minutes (1% formaldehyde and 0.5% sucrose in PBS), rinsed, and incubated with detergent for five minutes (1% IGEPAL). Following detergent treatment, cells were blocked for 20 minutes (5% goat serum, 0.4% BSA, and 0.2% Triton X-100 in PBS). Cells were then incubated overnight with a 1:500 dilution of rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) at 4°C. For actin-only staining, this incubation also contained 1.0 µg/mL Hoeschst 33258. JAK2 protein was localized
using the polyclonal antibodies C-20 (Santa Cruz, Santa Cruz, CA), directed against amino acids 1100-1200, HR-758 (Santa Cruz, Santa Cruz, CA), directed against amino acids 700-800, and p1007/1008 JAK2 (Biosource, Camarillo, California). Tyrosine phosphorylated (pTyr) proteins were detected with 4G10 antibody (Upstate Biotechnology, Waltham, MA). Cells were incubated with 1:500 phalloidin and 1:500 JAK2 or pTyr primary antibody dilutions overnight at 4°C. Coverslips were then rinsed and incubated overnight at 4°C with a 1:500 dilution of anti-rabbit (JAK2) or anti-mouse (pTyr) secondary antibodies (Molecular Probes, Eugene, OR) and 1.0 μg/mL Hoeschst 33258. Slides were visualized with a Leica DMIREE2 (Leica, Bannockburn, IL) with at 40x magnification and photographed using OpenLab software (Improvision Inc., Lexington, MA).

Statistical Analysis - In all experiments, C2C12 cells were randomly assigned to treatments. Experimental results are representative of a minimum of two replications of each experiment. Data was analyzed by one-way ANOVA and LS means contrasts. Statistical significance was determined as p ≤ 0.05.

RESULTS

Inhibition of JAK2 blocks myoblast proliferation in C2C12 cells - In previous studies, we demonstrated that JAK2 expression increased after three days of work overload in rat skeletal muscle (23). Thus, we decided to analyze the role of JAK2 in myoblast proliferation and terminal differentiation. To examine if JAK2 signaling is required for C2C12 myoblast proliferation, myoblasts were incubated with 0.2, 2, or
20 μM AG490 (Figure 1A). In a separate experiment, we found that 20 μM AG490 completely abolished the phosphorylation of JAK2 at amino acids 1007 and 1008 (Figure 6C). C2C12 cells incubated with AG490 had a dose-dependent decrease in myoblast BrdU incorporation (Figure 1B; p ≤ 0.0001). Cells treated with 20 μM AG490 had almost no BrdU incorporation (2.3%) as compared to control cells (66%; Figure 1B). These results suggest that JAK2 activity is required for C2C12 myoblast proliferation.

To specifically examine the affect of JAK2 on proliferation, the rate of BrdU incorporation was quantified in stable C2C12 lines that expressed dominant-negative (DN) or wild-type (WT) JAK2. Myoblasts that expressed DN-JAK2 had decreased levels of BrdU positive cells as compared to cells that expressed wild-type JAK2 or control cells (p ≤ 0.0001; Figure 1C). Although over-expressed WT-JAK2 has been shown to be constitutively active in other cell types (41,42), we did not observe an affect of WT-JAK2 over-expression on C2C212 myoblast proliferation. In addition, cells transiently transfected with WT- and DN-JAK2 display similar results (data not shown). These results indicate over-expression of a dominant-negative JAK2 attenuates proliferation in C2C12 myoblasts.

Constitutively active JAK2 increases proliferation in C2C12 myoblasts - To determine if JAK2 could increase proliferation, a constitutively active JAK2 construct was expressed in C2C12 cells. This JAK2 construct consisted of a fusion protein between the ets transcription factor, TEL, and JAK2 (38,43,44). TEL-JAK2 myoblasts had a significantly higher percentage of myoblasts that proliferated as
compared to control cells (p ≤ 0.01; Figure 1D). These results indicate that expression of this constitutively active form of JAK2 was sufficient to increase BrdU incorporation in C2C12 cells.

**JAK2 is nuclear localized in proliferating myoblasts** - To further explore JAK2 protein in proliferating myoblasts, we utilized immunofluorescence techniques to localize JAK2 in the cell. In C2C12 cells, JAK2 was tightly localized to the nucleus. Treatment with AG490 did not appear to change JAK2 localization in proliferating cells (Figure 2A). To confirm the presence of JAK2 in the nucleus of myoblasts, cell fractions were collected and analyzed. JAK2 protein was detected in the nuclear extract of myoblasts, with dramatically lower amounts of JAK2 in the cytoplasmic/membrane fraction (Figure 2B). Although total JAK2 appeared to be predominantly located in the nucleus, phospho-JAK2 (pJAK2\(^{Y1007/1008}\)) was located throughout the cell: in the nucleus, the cytoplasm, and at the cell membrane (Figure 2B).

**C2C12 myoblasts previously treated with AG490 rapidly recover and proliferate in normal growth media** - To determine if transient treatment with AG490 had a long-term impact on mitogenesis, C2C12 myoblasts were incubated with 20 μM AG490 for 24 hours, then washed and incubated in normal GM (Figure 3A). As in our previous experiment, treatment with AG490 for 24 or 48 hours dramatically decreased the number of BrdU positive myoblasts (p ≤ 0.0001). After removal of AG490, there were a dramatically higher percentage of cells that had incorporated BrdU, as compared to myoblasts continuously treated with AG490 for 24 hours (Figure 3B; p ≤ 0.0001). The recovered cells had a similar number of BrdU positive
cells as 48-hour control cells (Figure 3B; p > 0.05). These results indicate that C2C12 myoblasts can rapidly recover from pharmacological inhibition of JAK2 and proliferate at a similar level to control cells.

**Inhibition of JAK2 with AG490 blocks terminal differentiation in C2C12 cells** - To investigate if JAK2 had a potential role in terminal differentiation, cells were cultured for 72 hours in differentiation media (DM) that contained 0, 0.2, 2, or 20 μM AG490. Creatine kinase (CK) activity, a marker of terminal differentiation in skeletal muscle, was significantly lower in 2 μM (p ≤ 0.05) and 20 μM (p ≤ 0.0001) AG490 treated cells (Figure 4A). These results indicate that, like proliferation, terminal differentiation was inhibited by AG490. However, higher concentrations (2 and 20 μM) of AG490 were required to inhibit myogenesis as compared to myoblast proliferation, where very low concentrations (0.2 μM) were inhibitory.

To determine if, after a certain period of differentiation, treatment with AG490 was no longer effective, C2C12 cells were incubated in the presence of 20 μM AG490 for either 72 hours, or the final 48 or 24 hours of differentiation. Control cells were cultured in DM for 72 hours (Figure 4B). Myotube formation was completely blocked in cells incubated with AG490 for the entire time course of differentiation (72 hours) or the final 48 hours, and severely restricted in cells where JAK2 activity was inhibited for the final 24 hours of differentiation (Figure 4C). Creatine kinase activity was significantly decreased in cells treated with AG490 as compared to control cells (Figure 4D; p ≤ 0.0001). In addition, when treated with AG490 for the final 24 hours of differentiation, immature myotubes (AG24) had significantly higher levels of CK activity (p ≤ 0.001) over cells continuously incubated with AG490 (AG72) and cells
incubated with AG490 for the final 48 hours of differentiation (AG48) (Figure 4D). These results indicate that AG490 can inhibit terminal differentiation in C2C12 myoblasts, which could indicate a role for JAK2 in skeletal muscle myogenesis. 

Inhibition of JAK2 blocks MHC protein expression

- Like CK, myosin heavy chain (MHC) was utilized as a muscle-specific indicator of terminal differentiation. C2C12 myoblasts were cultured as previously described, with AG490 added to the media for the final 72, 48, and 24 hours of differentiation (Figure 4B). Myosin heavy chain was readily detected in control cells. In addition, MHC protein was detected at low levels in cells incubated with AG490 for the final 24 hours (AG24) of differentiation. However, MHC protein was not detected in cells continuously incubated with AG490 (AG72) or myoblasts incubated with AG490 for the final 48 (AG48) hours of differentiation (Figure 4E). These results correlate with our morphological and biochemical data and together indicate that treatment with AG490, regardless of time of addition, inhibits terminal differentiation. 

AG490 inhibits myoblast fusion and myofibril formation in C2C12 cells - A detrimental affect of high concentrations of AG490 on the actin cytoskeleton (45) and reorganization of myofilaments in cardiomyocytes (46) have been reported. Furthermore, an intact cytoskeleton is required for terminal differentiation (47). In addition, in a number of cell types, phosphorylation by JAK2 is critical in regulation of the cytoskeletal (48-50). Based on this research, we investigated the impact of AG490 on the cytoskeleton of proliferating and differentiating C2C12 cells. Prior studies analyzing the effects of AG490 on the cytoskeleton have utilized very high concentrations (400 μM), which result in almost a complete ablation of intact stress
fibers (45). However, we found that 200 uM AG490 was highly toxic to C2C12 myoblasts (unpublished results). In the current experiments, we sought to visualize the affect of AG490 on the actin cytoskeleton. Exposure of proliferating C2C12 myoblasts to 2 and 20 µM AG490 for 24 hours had little observable affect on the cells, and intact stress fibers were clearly visible (Figure 5A and B). However, a qualitative difference noted was an increase in actin at the sacrolemma (Figure 5C).

However, in differentiating cells, incubation for 72 hours with 20 µM AG490 resulted in a severe phenotype. These cultures contained only large, mononucleated cells with an apparent increase in sarcolemma actin (Figure 5F). Control cells incubated with DMSO, and cells incubated with 2 µM AG490, appeared morphologically normal following differentiation. In these cultures, large myotubes were present in abundance, and long actin filaments were visible in the myotubes (Figure 5D and E). In contrast, C2C12 myoblasts treated with 20 µM AG490 had no myotubes. Furthermore, these cells appeared to have actin morphology similar to proliferating myoblasts. Intact stress fibers were evident and, as noted above, (in 20 µM treated cells) an abundance of actin was located at the plasma membrane (Figure 5F). Clearly, AG490 has an impact on the ability of C2C12 myoblasts to fuse and form myotubes and actin distribution.

**JAK2 protein localization changes during terminal differentiation** - To further investigate JAK2 in skeletal muscle myogenesis, we used immunofluorescence techniques to localize JAK2 protein in differentiating C2C12 cells. To our surprise, we found large differences in cytoplasmic and nuclear distributions of JAK2 during terminal differentiation. Early in differentiation (T0h and T24h), JAK2 protein was
located almost exclusively in the nucleus. However, in mature (T72h) myotubes and, to a lesser extent, immature myotubes (T48h), JAK2 was detected in both the nucleus and the cytoplasm (Figure 6A). In cells treated with 20 µM AG490, a concentration that completely inhibits JAK2 kinase activity as detected by phospho-JAK2 (pJAK2) antibody (Fig. 6C), we found a severe attenuation in terminal differentiation. However, at a lower concentration (2 µM), cells were capable of fusion and JAK2 protein was located in the nucleus and cytoplasm of multinucleated myotubes (Figure 6B). These cells were phenotypically similar to controls. In 20 µM AG490-treated cells, the concentration where differentiation is blocked and no JAK2 kinase activity can be detected, JAK2 was tightly confined to the nucleus (Figure 6B). We also confirmed the presence of JAK2 protein in the nucleus using a second antibody (C-20; data not shown).

Using the phospho-JAK2 (pJAK2) antibody, we found pJAK2 to be primarily cytoplasmic in differentiated cells (Figure 6C). We also confirmed that treatment of cells with 20 µM AG490 inhibited JAK2 phosphorylation (Figure 6C). In contrast, an abundance of other phospho-tyrosine proteins were clearly visible in differentiated cells, even with AG490 treatment, as detected by a general phosphotyrosine antibody (4G10; Figure 6D).

C2C12 cells previously treated with AG490 partially recover and terminally differentiate - We next sought to determine if transient treatment with AG490 had a long-term impact on myogenesis, or if cells cultured in DM that contained AG490 could recover and differentiate (Figure 7A). As expected, cells continuously incubated with AG490 had significantly lower levels of CK activity as compared to
controls (Figure 7B; p ≤ 0.0001), even after 120 hours in differentiation media. After 48 hours with AG490 treatment and 72 hours following refeeding with normal DM, CK activity of rescued cells was significantly increased over cells continuously incubated with AG490. However, the CK activity remained significantly lower than in control cells (Figure 7B; p ≤ 0.0001). These results indicate that C2C12 myoblasts can fuse and differentiate after removal of AG490 and pharmacological inhibition of JAK2 may interfere with ability of C2C12 myoblasts to differentiate (Figure 7B).

C2C12 cells stably transfected with DN-JAK2, WT-JAK2 and TEL-JAK2 exhibit enhanced terminal differentiation - To determine if the effects of AG490 were the result of inhibition of JAK2 kinase activity, we quantified terminal differentiation in stable JAK2 C2C12 lines. Interestingly, cells that over-express any form of JAK2 had significantly higher levels of CK activity than control cells (Figure 8A; p ≤ 0.05). Strikingly, DN-JAK2 C2C12 cells had the greatest increase in CK activity (Figure 8A; p ≤ 0.0001). In addition, cells that over-express a constitutively active (CA) JAK2 (TEL-JAK2) had significantly higher levels of CK activity over control cells (Figure 8B; p ≤ 0.001). These results suggest a protein-dependent role for JAK2 in the promotion of terminal differentiation in C2C12 cells. It should be noted, we did not observe a different JAK2 protein localization phenotype in differentiated JAK2-over-expressing cells as compared to control cells (data not shown).

C2C12 cells over-expressing DN- and WT-JAK2 have unusual myotube morphology - We sought to determine if cells that over-express WT- or DN-JAK2 had any perturbations in their actin cytoskeleton similar to those observed in cells treated with AG490. After 72 hours of differentiation, control cells expressing vector alone
had formed large myotubes comparable to those typically found in normal, untransfected C2C12 cells. Stable WT- or DN-JAK2 cell cultures also contained myotubes, but by qualitative analysis, the myotubes present appeared to be much narrower than control cells (Figure 8C).

*C2C12 myoblasts treated with AG490 display impaired migration* - In addition, we observed an impaired ability of cells treated with AG490 to migrate normally. Cells treated with 20 μM AG490 had a severely impaired ability (Figure 9A) to close a wound in the cell monolayer as compared to control cells and cells treated with 2 μM AG490 (Figure 9B; p ≤ 0.00001). In contrast, cells over-expressing either WT- or DN-JAK2 were able to migrate normally (Figure 9C). Neither WT- or DN-JAK2 had a significantly different amount of wound closure when compared to cells expressing the plasmid backbone alone (Figure 9D; p ≥ 0.05).

*Pharmacological inhibition of JAK3 with WHI-P131 does not inhibit myoblast proliferation or terminal differentiation* - Since research has demonstrated that AG490 can inhibit JAK3 (39,51,52), we examined the necessity of JAK3 activity for myoblast proliferation and terminal differentiation using the JAK3 pharmacological inhibitor, WHI-P131. To examine if JAK3 is involved in proliferation, C2C12 myoblasts were treated with 20 μM WHI-P131. The percentage of treated cells that were BrdU positive was not different from the percentage of control cells that were BrdU positive (Figure 10A; p>0.05). C2C12 cells were also allowed to differentiate in the presence of 20 μM WHI-P131 to determine if JAK3 is required for terminal differentiation. Myoblasts treated with JAK3 inhibitor had levels of CK activity similar
to control cells (Figure 10B; p>0.05). These results indicate that JAK3 activity is not required for myoblast proliferation or terminal differentiation in C2C12 cells.

DISCUSSION

The role of JAK2 in skeletal muscle myoblast proliferation - Previous studies have shown that AG490 can block LIF-induced proliferation and JAK2/STAT activation in satellite cells (20). In addition, Erythropoietin (EPO), a potent activator of JAK2 signaling, stimulates phosphorylation of JAK2 and proliferation of C2C12 myoblasts (22). In a previous study, we found that JAK2 transcripts increase following three days of work overload in rat skeletal muscle (23). This increased expression corresponds to the time of satellite cell proliferation, fusion to the existing fiber, and terminal differentiation in response to overload. In this current work, pharmacological inhibition of JAK2 with AG490 decreased proliferation of C2C12 myoblasts. Proliferation of C2C12 cells was sensitive to AG490 treatment, as even very low concentrations significantly inhibited BrdU incorporation. However, incubation of cells with AG490 did not appear to have a permanent impact on proliferation, since cells could recover quickly from treatment and proliferate when returned to normal growth media. In addition, myoblasts that stably over-expressed dominant-negative JAK2 exhibited a significant decrease in the percentage of BrdU positive cells. Results from both pharmacological inhibition and dominant-negative JAK2 expression experiments indicated that JAK2 kinase activity is necessary for proliferation of C2C12 myoblasts. Furthermore, these experiments provide evidence
that kinase-active JAK2 is both required for normal proliferation and expression of TEL-JAK2 is sufficient to increase proliferation in myoblasts.

Previously, JAK2 has been shown to play a role in growth hormone (GH)-induced actin cytoskeletal reorganization (53) and migration (54). JAK2 phosphorylates SH2B-β (49), a protein required for membrane ruffling and cytoskeletal rearrangement induced by growth hormone (49,55). In addition, AG490 significantly reduces GH-induced cell spreading of mammary cancer cells (54) and JAK2 appears to physically interact with focal adhesion kinase (FAK), a protein found in focal contacts and involved in cell migration, when stimulated with GH (56). In our experiments, treatment with AG490 appears to have a less-severe impact on the morphology of proliferating myoblasts. In contrast to differentiating cells, proliferating myoblasts appeared to have normal cytoskeletal structure, even after treatment with AG490 or over-expression of JAK2 constructs. However, as in differentiating cells treated with 20 µM AG490, there was an increase in cortical actin in these cells. Also of interest, cells treated with 20 µM AG490 had greatly attenuated cell migration following wounding, as compared to control cells. This increase of cortical actin, and impairment of migration, is of note, particularly if JAK2 is playing a role in the cytoskeletal changes that accompany skeletal muscle myogenesis.

Upstream activators of JAK2 include a number of growth factors implicated in the proliferation of several cell types. Notably, Angiotensin II (Ang-II) through the Angiotensin-1 receptor (A1R) (57-60); interleukin-6 through the IL-6/gp130 complex (61,62); insulin-like growth factor-1 (IGF-1) (63,64); and hepatocyte growth factor
(HGF) (63) all activate JAK2. Interleukin-6 (8), LIF (8,20), IGF-1 (65-67), and HGF (6,7,68) act as potent mitogens of skeletal myoblasts. Thus, JAK2 may regulate myoblast proliferation by mediation of signaling from these growth factors.

The Role of JAK2 in Skeletal Muscle Myogenesis - Until now, a role for JAK2 in skeletal muscle terminal differentiation has only been suggested in a study of the potent JAK2 activator, EPO that demonstrated addition of EPO inhibits terminal differentiation of myoblasts (22). In our work, treatment with AG490 blocked terminal differentiation in C2C12 myoblasts, as evidenced by decreased creatine kinase (CK) activity and myosin heavy chain (MHC) protein levels.

In the past ten years, several studies have been published describing JAK2 localization in the nucleus of cultured cells (27,29,30) and during distinct stages of early oocyte development (26). In addition, both JAK1 and Tyk2 can be nuclear localized (28,29). However, the specificity of commercially available JAK antibodies for immunofluorescence has been called into question by other studies (31,32). Although minimal work has been done in the area of JAK2 localization via immunofluorescence, many of the JAK2-nuclear studies have also confirmed the presence of JAK2 in the nucleus using western blotting techniques (27,29). In addition, a new study used blocking peptides to show the specificity of the JAK2 HR-758 antibody in skeletal muscle fibers (69). These latter results indicate that identification of nuclear JAK2 is more than merely an artifact of immunofluorescence work.

Understanding the potential role JAKs may play in the nucleus is a substantial challenge, given that the majority of work to date describes the primary roles of JAKs
as translocating signals in the cytoplasm and at the cell membrane. Exploring a nuclear role for JAK2 will likely result in the discovery of a novel function for this tyrosine kinase. One of our most striking observations was the location of JAK2 throughout the cell in differentiated myotubes. Since JAK2 is tightly nuclear localized in proliferating cells and cells treated with AG490, it is tempting to speculate that this relocalization is important for the role of JAK2 in myogenesis. Of particular interest was the observation that cytoplasmic/membrane-associated JAK2 was primarily observed in mature, multi-nucleated myotubes. Since cells treated with AG490 displayed a marked impairment of fusion and migration, and a tightly nuclear JAK2 localization, this is a critical observation. This observation may explain the delayed recovery from AG490 treatment seen in differentiating C2C12 cells, but not in proliferating cells. These experiments suggest that JAK2 has a protein-dependent role in myogenesis and a kinase-dependent role in proliferating cells. Interestingly, one of the studies identifying JAK2 in the nucleus only indicated a change in phospho-JAK2 nuclear localization following stimulus, while total JAK2 in the nucleus remained constant (29). In our work, pJAK2 appeared to be located throughout the cell in proliferating myoblasts; at the membrane, in the cytoplasm, and in the nucleus. In terminally differentiated cells, pJAK2 appeared to be located throughout the cells with potentially a slight increase in the cytoplasm. Clearly, more research is needed to further understand the phenomenon of nuclear localized JAK2 and the potential role it may play in skeletal muscle myogenesis.

Unlike proliferating myoblasts treated with AG490, CK activity was only inhibited by 2 μM and 20 μM concentrations of AG490 during terminal differentiation.
In addition, our immunofluorescence experiments confirmed that 20 μM AG490 abolishes JAK2 kinase activity. Treatment with AG490 had a severe impact on the cytoskeleton of differentiating myoblasts. Cells treated with AG490 did not recover quickly, but they can terminally differentiate when changed to normal media. Although recovered cells did show an increase in CK activity when compared to cells continuously incubated with AG490, the levels of activity were markedly lower than control cells. This is in direct contrast to the rapid return to normal phenotype observed in proliferating cells incubated with AG490. This delay in recovery to a terminal differentiation phenotype may be due to the severe effect AG490 has on JAK2 localization, and the actin cytoskeleton, of terminally differentiated cells. Cells treated with 20 μM AG490 appear to have not undergone the cytoskeletal rearrangements of alignment and fusion that typically occur during terminal differentiation. In fact, treated cells primarily retained the nuclear concentration of JAK2, and cytoskeletal characteristics, observed in proliferating myoblasts. The rapid recovery from AG490 treatment observed in proliferating cells, and conspicuously absent in terminally differentiating cells, may influenced by the difference in location of JAK2 protein and the impact of AG490 on the actin cytoskeleton.

Terminal differentiation was increased in C2C12 cells that stably over-expressed dominant-negative or wild-type JAK2. Moreover, terminal differentiation was greater in cells that over-expressed dominant-negative JAK2 than in cells that over-express wild-type JAK2. A constitutively-active JAK2 also increased terminal differentiation in C2C12 cells. These results provide evidence for a potential kinase-
independent, protein-concentration dependent role for JAK2 in skeletal muscle terminal differentiation. They also indicate that an increase in JAK2 protein concentrations in C2C12 myoblasts correlated with an increase in CK activity, a marker of terminal differentiation. Previously, JAK2 has been shown to have kinase-independent activities. For example, the N-terminus of JAK2 is required for expression of the EPO receptor on the cell surface (70). In another study, differences were noted between cells treated with AG490 and those expressing a kinase-negative JAK2 protein, which suggests a kinase-independent role for JAK2 in luteinized granulosa cells (71). It is possible that JAK2 protein itself plays a role in targeting cellular localization or complex formation of proteins that are critical to terminal differentiation of myoblasts. Also notable, cells over-expressing JAK2 retain the ability to align and fuse into mature myotubes and migrate normally when the cell monolayer is wounded. While the actin cytoskeleton and the location of JAK2 in these cells were, for the most part, normal, they did have some differences in morphology. Primarily, these cells did not form the large, branched myotubes seen in control cells or cells expressing the plasmid backbone alone. WT-JAK2 or DN-JAK2 cells displayed long, narrow myotubes that were very distinctive when compared to control cells (Figure 8C).

Although the tyrosine kinase inhibitor AG490 has long been described as a JAK2 inhibitor, recent studies have questioned its specificity (72). Originally indicated as a treatment for acute lymphoblastic leukemia (73), AG490 is widely utilized to inhibit JAK2 both in vivo (74-76) and in vitro (77-80). However, research has demonstrated AG490 inhibits, to varying degrees, a number of other molecules
including JAK3 (39,51,52), Cdk2 (81,82), epidermal growth factor receptor (83), and c-src (84). Since JAK3 can be inhibited by AG490 treatment (52,58), and previous research has indicated that JAK3 is required for growth hormone (GH) induced STAT3 activation in C2C12 myoblasts (25), we considered the possibility that interference of JAK3 may be involved in the inhibition of terminal differentiation by AG490. However, our experiments indicated that JAK3 activity is not required for terminal differentiation or myoblast proliferation in C2C12 cells.

The results of this study demonstrate that JAK2 has distinct roles in myoblast proliferation and terminal differentiation. The combination of the increased AG490 dose requirements to inhibit terminal differentiation, the abnormal phenotype and increased CK activity of JAK2 stable cell lines, and the change in JAK2 localization during myogenesis, suggest a novel, protein-dependent role for JAK2 in mediating myogenesis, potentially independent of kinase activity. Our results indicate that, unlike proliferating myoblasts, myoblasts are capable of terminally differentiating when exposed to low levels of AG490. However, cells treated with 20 µM AG490 cannot quickly recover and terminally differentiate, which is in contrast to the results in proliferating myoblasts. This point is underscored by the vast differences observed between the JAK2 location in proliferating myoblasts and terminally differentiated myotubes. Since JAK2 is found in the nucleus of mononucleated cells, but in both the nucleus and the cytoplasm of mature, multi-nucleated myotubes, we hypothesize that the location of JAK2 influences myogenesis. These results strongly suggest a critical role for JAK2 protein in normal myogenesis of C2C12 myoblasts.
Myogenesis is an intricate interplay of pathways that determine the ultimate fate of an individual myoblast. Even under proliferative conditions, high cell density can induce a fraction of cells to fuse and differentiate into myotubes (85). Similarly, under reduced serum, myogenic conditions, a small population of C2C12 cells do not fuse and form a subset of cells described as “reserve cells” (86). These diverse cell populations within skeletal muscle cell culture rely on unique signaling events within cells cultured in the same environment. Our research has identified a novel role for JAK2 as a protein that is critical for both terminal differentiation and myoblast proliferation. The determination that JAK2 protein enhances terminal differentiation, regardless of kinase activity, while kinase activity is necessary for proliferation, suggests a potential model for the split in the myoblast regulatory pathways. Examination and identification of factors involved in this key switch may lead us to an explanation of the role JAK2 plays during myogenesis. Ongoing research has also identified the MAPK (87-93) and PI3-K pathways (12,66,94-98) as having unique, and critical, roles in the diverse processes of myogenesis and mitogenesis. Cross-talk between these pathways is potentially a factor in the ultimate fate of each individual myoblast.

We propose a model for JAK2 function in skeletal muscle that rests on the bifurcation of two signaling pathways. The role of JAK2 in myoblast proliferation is likely through a traditional, phosphorylation-driven signaling cascade, potentially resulting in activation of STAT factors. Our evidence suggests cells can recover quickly from AG490 treatment and proliferate readily. In addition, expression of a constitutively-active JAK2 fusion protein is sufficient to increase myoblast
proliferation. Accordingly, proliferation decreases in cells where JAK2 is inhibited through pharmacological and dominative-negative approaches. This research agrees with much of the established literature on the role of JAK2 in cell proliferation. In contrast, we propose that JAK2 has a unique, protein concentration- and location-dependent role in terminal differentiation. Our JAK2 immunofluorescence work provides evidence for this model, since it shows that JAK2 changes from a nuclear concentrated protein in mononucleated cells to localization in both the cytoplasm and the nucleus of myotubes. During differentiation, JAK2 may be a member of specific protein complexes, and its presence could be a rate-limiting factor for transmitting the signal for terminal differentiation. This involvement may link several signaling pathways, or create the protein complex profile required to stop the cell cycle and induce the genes required for terminal differentiation. It is also possible that this protein-dependent role of JAK2 is required for the actin cytoskeletal rearrangement observed during myogenesis. In the future, it will be of interest to further investigate the relationship of JAK2 localization, activation, and protein interactions in skeletal muscle myogenesis.

REFERENCES


FOOTNOTES

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The abbreviations used are: JAK, Janus kinase; DMEM, Dulbecco's modified eagle medium; WT, wild type; DN, dominant negative; DM, differentiation media; GM, growth media, CK, creatine kinase; BrdU, Bromodeoxyuridine; p, phospho; and MHC, myosin heavy chain.
FIGURE LEGENDS

Fig 1. Inhibition of JAK2 blocks myoblast proliferation in C2C12 cells. A, C2C12 myoblasts were incubated with 10-fold dilutions of AG490 for 36 hours. Solid gray lines indicate incubation with GM that contained AG490. Solid black lines indicate incubation with GM that contained vehicle (DMSO) only. Dashed lines indicate incubation with GM that contained BrdU (10 μM) and dCTP (10 μM) for the final 12 hours of culture. Cells were fixed as described in Materials and Methods. B, The percentage of proliferated cells was determined by dividing the number of FITC-positive nuclei by the total number of nuclei identified by HO33258. *Significantly different than control (0; p ≤ 0.0001). C, Proliferation was quantified in stable C2C12 cell lines that expressed dominant-negative JAK2 (DN-JAK2), wild type JAK2 (WT-JAK2), and plasmid backbone (control). Cells were cultured for 36 hours in normal GM, and BrdU (10 μM) and dCTP (10 μM) were added for the final 12 hours of culture. *Significantly different than controls (WT-JAK2 and control; p ≤ 0.0001). D, Rate of proliferation was quantified in stable C2C12 cell lines that expressed constitutively active JAK2 (TEL-JAK2 5-12) and plasmid backbone. Cells were cultured for 36 hours in normal GM, and BrdU (10 μM) and dCTP (10 μM) were added for the final twelve hours of culture. *Significantly different than control (pcDNA3; p ≤ 0.01).
Fig 2. JAK2 is nuclear localized in proliferating myoblasts. A, Proliferating cells were incubated in GM with 20 μM AG490 (AG) or DMSO (Co) for 24 hours. Filamentous actin was detected using rhodamine-conjugated phalloidin, total JAK2 was detected using the HR-758 antibody, and total nuclei were identified by HO33258. B, Nuclear (NE) and cytoplasmic/membrane (CE) extracts of proliferating C2C12 myoblasts were collected and detected with the anti-JAK2 HR-758 antibody. C, Proliferating cells were incubated in GM for 24 hours before detection. Filamentous actin was detected using rhodamine-conjugated phalloidin and total JAK2 was detected using phospho-specific p\textsuperscript{1007/1008}JAK2 antibody.

Fig 3. C2C12 myoblasts previously treated with AG490 rapidly recover and proliferate in normal growth media. A, Cells were initially incubated with either 20 μM AG490 (AG) or DMSO (Co) for 24 hours. After 24 hours, rescue and 48 hour control cells were washed, changed to fresh GM, and cultured an additional 24 hours. AG24 and Co24 cells were fixed and counted after 24 hours. Solid lines indicate incubation in media that contained vehicle (DMSO) only. Dotted lines indicate incubation with media that contained AG490. Dashed lines indicate incubation with GM that contained BrdU (10 μM) and dCTP (10 μM) for the final 12 hours of culture. Cells were fixed as described in Materials and Methods and counted at 24 or 48 hours. B, Brackets indicate statistical comparisons performed as described in results. The percentage of proliferated cells was determined by dividing the number of FITC-positive nuclei by the total number of nuclei identified by HO33258. *Indicates a statistical difference (p ≤ 0.0001).
**Fig 4.** Inhibition of JAK2 with AG490 blocks terminal differentiation in C2C12 cells. 

A, Cells were cultured in differentiation media (DM) for 72 hours. AG490 (0.2, 2, and 20 µM) was added to the cells at the start of differentiation. Control cells were cultured in DM that contained vehicle (DMSO). Creatine kinase (CK) units were determined as described by the manufacturer's protocol (Sigma) and standardized to total protein in nanograms. *Significantly different than control (Co; p < 0.05). B, Cells were cultured in DM for 72 hours. AG490 (20 µM) was added to the cells at the start of differentiation (AG72), after 24 hours in DM (AG48), and after 48 hours in DM (AG24). Solid lines indicate incubation in media that contained vehicle (DMSO) only. Dashed lines indicate incubation with media containing AG490. C, Cells were photographed at 0, 24, 48, and 72 hours after the addition of DM. Photographs are of representative fields. D, Creatine kinase (CK) units were determined as described previously. *Significantly different than control (Control; p ≤ 0.0001). E, Inhibition of JAK2 inhibits MHC protein in C2C12 skeletal muscle cells. Cells were cultured according to time course and treatments described in B. MHC protein levels were quantified using Mf-20 antibodies from Developmental Studies Hybridoma Bank.

**Fig 5.** AG490 inhibits myoblast fusion and myofibril formation in C2C12 cells. All cells were fixed as described in Materials and Methods. Proliferating cells were incubated in GM with DMSO (Control; A), 2 µM AG490 (B; 2 µM), 20 µM AG490 (20 µM; C) or for 24 hours. The filamentous actin was detected using rhodamine-
conjugated phalloidin and total nuclei were identified by HO33258 (photos displayed are an overlay of both). Cells were cultured in differentiation media (DM) for 72 hours. Differentiating cells were cultured in DM that contained DMSO (Control; D), 2 μM AG490 (2 μM; E) or 20 μM AG490 (20 μM; F) was added to the cells at the start of differentiation. Cells were detected as described in A.

Fig 6. JAK2 protein localization changes during terminal differentiation. All cells were differentiated for 72 hours unless stated otherwise. All control cells in AG490 experiments were treated with an equivalent volume of vehicle (DMSO). Cells were fixed as described in Materials and Methods; the actin cytoskeleton was detected with rhodamine-conjugated phalloidin and total nuclei were visualized using HO33258. A, Differentiating C2C12 cells were fixed after 0 (T0), 24 (T24), 48 (T48), and 72 (T72) hours of differentiation. Total JAK2 was detected using the HR-758 antibody. B, Cells were incubated with vehicle, 2 μM, or 20 μM AG490. Total JAK2 was detected using the HR-758 antibody. C, 20 μM AG490 was added to cells at the start of differentiation and control cells contained DMSO. pJAK2 was detected using the phospho-specific p^{1007/1008}JAK2 antibody. Note: cells treated with AG490 were photographed at 100x the exposure length as control cells. D, 20 μM AG490 was added to cells at the start of differentiation and control cells contained DMSO. Tyrosine phosphorylated proteins (pY) were detected using the 4G10 antibody.

Fig 7. C2C12 cells previously treated with AG490 partially recover and terminally differentiate. A, Control cells were incubated in DM for 72 hours. Continuous cells
had AG490 (20 μM) in the DM for up to 120 hours. Rescue cells were incubated with AG490 (20 μM) for 48 hours, then washed and changed to control DM. Solid lines indicate incubation in media that contained vehicle (DMSO) only. Dashed lines indicate incubation with media that contained AG490. Control cells were collected at 0, 24, 48, and 72 hours. Continuous AG490 cells were collected at 24, 48, 72, 96, and 120 hours. Rescue cells were collected at 72, 96, and 120 hours. B, Creatine kinase (CK) units were determined as described previously. *Indicates a statistical difference (p ≤ 0.0001).

Fig 8. Over-expression of JAK2 enhances terminal differentiation in C2C12 cells. A, Stable C2C12 cell lines that expressed dominant-negative JAK2 (DN-JAK2), wild type JAK2 (WT-JAK2), or plasmid backbone (Control) were cultured in differentiation media (DM) for 72 hours. The filamentous actin was detected using rhodamine phalloidin and total nuclei were identified by HO33258 (photos displayed are an overlay of both). B, Terminal differentiation was quantified in stable C2C12 cell lines that expressed dominant-negative JAK2 (DN-JAK2), wild type JAK2 (WT-JAK2), and plasmid backbone. *Indicates a statistical difference (p ≤ 0.05). C, Terminal differentiation was quantified in stable C2C12 cell lines that expressed a constitutively active JAK2 (TEL-JAK2 5-12) or plasmid backbone. Cells were cultured in differentiation media (DM) for 72 hours. Creatine kinase (CK) units were determined as previously described. *Significantly different than control (pcDNA3; p ≤ 0.001).
Fig 9. C2C12 myoblasts treated with AG490 display impaired migration. A, C2C12 were treated with DMSO (Control), 2 μM AG490 (2 μM), or 20 μM AG490 (20 μM) for eight hours after wounding. Cells were photographed at the start of the time course (T0) and eight hours after wounding (T8). B, After eight hours of treatment the percentage of the wound covered by migrating cells was determined from photographs. Cells treated with 20 μM AG490 (20 μM) had impaired migration (p < 0.00001) as compared to control cells (Control) and cells treated with 2 μM AG490 (2 μM). C, Cells stably over-expressing pCINeo backbone (Control), DN-JAK2, or WT-JAK2 were wounded and photographed at the start of the time course (T0) and eight hours after wounding (T8). D, Eight hours after wounding, the percentage of the wound covered by migrating cells was determined from photographs (p ≥ 0.05).

Fig 10. Pharmacological inhibition of JAK3 with WHI-P131 does not inhibit myoblast proliferation or terminal differentiation. A, C2C12 myoblasts were incubated in GM that contained either 20 μM WHI-P131 or control DMSO for 36 hours, and BrdU (10 μM) and dCTP (10 μM) for the final 12 hours of culture. Cells were fixed as described in Materials and Methods. The percentage of proliferated cells was determined by dividing the number of FITC-positive nuclei by the tota
I number of nuclei identified by HO33258. B, Terminal differentiation was quantified in C2C12 cells cultured for 72 hours in DM containing either 20 μM WHI-P131 or control DMSO. Creatine kinase (CK) units were determined as previously described.
Figure One
**Figure Two**

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**B**

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Figure Three

A

0 h 12 h 24 h 36 h 48 h

BrdU ↓ — — — — Co24

BrdU ↓ — — — — Co48

BrdU ↓ — — — — AG24

BrdU ↓ — — — — AG48

BrdU ↓ — — — — Rescue

B

* * * * *

% Proliferated

0 10 20 30 40 50 60 70

Co24 Co48 AG24 AG48 Rescue

Treatment
Figure Four

Panel A: Graph showing CK U/mg protein levels at different treatment times (0h, 24h, 48h, 72h) for Control, 0.2 uM, 2 uM, 20 uM treatments.

Panel B: Timeline with X's marking Control, AG72, AG48, AG24 treatments.

Panel C: Images showing control cells at 0h, 72h AG480, 48h AG480.

Panel D: Graph showing CK U/mg protein levels at different treatments (0, AG72, AG48, AG24, Control).

Panel E: Western blot analysis for MHC protein expression at different treatments.
Figure Five

Proliferating

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Differentiating

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### Figure Six

#### (A) Time Points

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#### (B) Concentration Levels

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#### (C) 72h Conditions

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#### (D) 72h Conditions

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Figure Seven

A

0 h 24 h 48 h 72 h 96 h 120 h

X X X X Control

X X X X X Continuous

X X X X Rescue

B

300

CK Ultrasensitive

Control

Rescue

Continuous

0 24 48 72 96 12

Time
Figure Eight

A

Cell Line

Control  WTJAK2  DNJAK2

B

C

CK U/mg protein

Control  WT-JAK2  DN-JAK2

Control  TJ 5-12

Cell Line

Cell Line
Figure Nine

A

Control 2 uM 20 uM

T0

T8

B

% Wound Closure

Control 2 uM 20 uM

Treatment

***

C

Control DN-JAK2 WT-JAK2

T0

T8

D

% Wound Closure

Control DN-JAK2 WT-JAK2

Cell Line

***
Figure Ten

A

![Bar chart showing % Proliferated for Control and WHI-P131 treatments.]

B

![Bar chart showing CK U/mg protein for Control and WHI-P131 treatments.]

CHAPTER 3. IDENTIFICATION OF A ROLE FOR
THE JAK/STAT PATHWAY IN CYCLIC STRETCH
OF C2C12 MYOBLASTS

A paper prepared for submission to the American Journal of Physiology Cell Physiology
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ABSTRACT

Mechanical stretch of cultured cells is a critical tool to study pathways involved in skeletal muscle hypertrophy in vivo. Regulation of cell proliferation in response to mechanical stress is influenced by a number of distinct signaling pathways. In this study, we demonstrate that the JAK/STAT signaling cascade is activated in response to cyclic stretch in C2C12 myoblasts and that JAK2 is required for stretch induced myoblast proliferation. Stimulation of myoblasts with cyclic stretch was sufficient to increase proliferation of myoblasts. Treatment with AG490, a JAK2 inhibitor, or transfection with dominant-negative JAK2, blocked this increase. We also observed that short periods of cyclic stretch activated JAK2, as identified by immunofluorescent localization of JAK2 phosphorylated at the Yp1007/1008 residues. This activation resulted in a large increase of phosphorylated JAK2 protein in the nuclear/perinuclear domain. Treatment with vanadate, a tyrosine phosphatase
inhibitor, was sufficient to maintain high JAK2 activation. We also determined that JAK2 activation occurred as a result of autocrine/paracrine signaling as transfer of conditioned media from cells stretched for five minutes and one hour increased activation of JAK2 in static cells. Activation at the later time point required translation as treatment cycloheximide during stretch blocked JAK2 phosphorylation. In addition, STAT1 and STAT5 are activated and rapidly translocate to the nucleus in response to short periods of cyclic stretch. These results demonstrate that the JAK/STAT pathway is activated in response to cyclic stretch in C2C12 myoblasts.

**INTRODUCTION**

Satellite cells are a population of cells located between the sarcolemma and basal lamina of skeletal muscle. These cells have been identified as the major source of additional nuclei during the growth of postnatal skeletal muscle (59) and represent a cell population committed to the myogenic lineage that exist in a quiescent state unless activated (56). One of the primary activators of satellite cells are events stimulating muscle hypertrophy (15, 59, 83, 85). Expression of myogenic regulatory factors (MRFs), MyoD, Myf5, Myogenin, and MRF4, are required for terminal differentiation of developing skeletal muscle. Potential activators of satellite cells include hepatocyte growth factor (HGF) (89), LIF (7, 86), IGF-I (2), and interleukin-4 (24).

Mechanical stretch has long been used as a model to study skeletal muscle hypertrophy in vitro (92). These models have identified mechanical stretch as an
inhibitor of myogenesis (3, 43) and identified upstream activators of satellite cells (5, 89, 90). However, the majority of research in the area of mechanical stretch has been focused in cardiac muscle. The mechanisms involved in cardiac hypertrophy have been extensively studied using in vitro stretch models (28, 41, 67, 79, 80). In addition, pathways activated in response to mechanical stress of vascular smooth muscle cells in vitro have been examined (26, 49, 50).

The Janus family of tyrosine kinases was first described in the early 1990s (94) and four family members have been identified: JAK1, JAK2, JAK3, and Tyk2. Traditionally, the JAKs have been identified as receptor-associated kinases capable of activating the signal transducers and activators of transcription (STAT) factors (1, 36). Although the JAK/STAT pathway has not yet been directly implicated in skeletal muscle hypertrophy, it is activated during cardiac hypertrophy (40, 44, 66, 67). JAK2 is phosphorylated in response to mechanical stretch in cardiomyocytes (67) and overload in the heart (66), and is required for vascular smooth muscle cell proliferation (53, 84). STAT proteins are also activated during cardiac hypertrophy. In cardiomyocytes, in vitro mechanical stretch activates STAT1 and STAT3 (69) and pressure overload of the heart in vivo caused the rapid phosphorylation of STAT1, STAT2 and STAT3 (68).

Although the requirement for JAK2 in skeletal muscle is not as well examined, studies have demonstrated that JAK2 and STAT3 are phosphorylated during LIF-induced satellite cell proliferation (86), JAK2, STAT1, 3, and STAT5A are phosphorylated during EPO-induced myoblast proliferation (64), and growth hormone (GH) stimulation of myoblasts activates JAK2, STAT3, and STAT5 (19).
Active JAK2 is also required for ephrin-A1-induced acetylcholinesterase expression in C2C12 myoblasts (45). In addition, STAT3 is activated in proliferating satellite cells following crush injury, during muscle regeneration (31).

In this study, we sought to determine if the JAK2/STAT pathway was activated during cyclic mechanical stretch of C2C12 myoblasts, and if JAK2 was required for stretch-induced proliferation. Our research demonstrates that active JAK2 is required for stretch-induced proliferation in myoblasts and that JAK2, STAT1, and STAT5 are activated by cyclic stretch. The STAT proteins are phosphorylated and rapidly translocate to the nucleus following mechanical stretch and the concentration of active JAK2 greatly increases near the perinuclear/nuclear region. Activation of JAK2 appears to occur via an autocrine/paracrine mechanism as conditioned media from stretched cells was sufficient to cause activation and increased concentration of nuclear-region phosphorylated JAK2. This concentration of activated JAK2 appears to be regulated by protein tyrosine phosphatases as treatment with active sodium orthovanadate, a tyrosine phosphatase inhibitor, maintained this stretch-induced localization. Our results clearly indicate activation of the JAK/STAT pathway in mechanical stretch and demonstrate a requirement for JAK2 in stretch-induced proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture
C2C12 skeletal muscle myoblasts (American Type Culture Collection, Manassas, VA) were grown in a 5% CO₂ humidified atmosphere at 37°C. Proliferating myoblasts were grown in growth media (GM) that consisted of DMEM, 10% fetal bovine serum (FBS; Gibco-BRL Life Technology, Carlsbad, California), 1.0 mg/mL penicillin/streptomycin (Gibco-BRL Life Technology, Carlsbad, California) and 0.3 mg/ml L-glutamine (Gibco-BRL Life Technology, Carlsbad, California). Transfection media for transient proliferation assays was the same as the GM except it did not contain antibiotics. For proliferation experiments, cells were transiently transfected with WT-JAK2, dominant-negative JAK2 (DN-JAK2; (81)), and eGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 24 hours prior to stretch. AG490 (Calbiochem, San Diego, California) was used to inhibit JAK2 kinase activity in proliferation assays and all control cells for these experiments contained an identical amount of DMSO (Fisher, Pittsburgh, PA); the vehicle for AG490. For stretch experiments, cells were plated onto Bioflex six-well collagen-coated plates (Flexcell, McKeesport, PA). When utilized for immunofluorescence (IF), these plates were also coated with bovine fibronectin (Sigma, St. Louis, Missouri). For static IF, cells were plated onto 12-well plates with glass coverslips coated with bovine fibronectin. Myoblasts were plated at 1250 cells/cm² for IF analysis and 5000 cells/cm² for cell extracts. For stretch experiments, cells were equibiaxially stretched using a Flexcell FX-3000 apparatus (Flexcell, McKeesport, PA). Cells were stretched without posts using a cyclical stretching program of four seconds of 20% sine stretch followed by 10 seconds of rest. To inhibit tyrosine phosphatase activity, cells were incubated with 1 mM Na₃VO₄ for three hours prior to stretch. To prepare conditioned media
(CM), stretch cells were plated at 5000 cells/cm$^2$ on collagen-coated Bioflex plates. To block translation, cells were incubated with 10 μg/mL cyclohexamide (Sigma, St. Louis, Missouri) for three hours prior to stretch. Control cells were incubated with vehicle (EtOH). Following stretch, media was immediately collected and transferred to recipient (non-stretched) cells plated at 1250 cells/cm$^2$ on fibronectin-coated coverslips. Recipient cells were incubated as described above with CM for ten minutes.

**Immunofluorescence**

Following stimulation, cells were rinsed with phosphate buffered saline (PBS) and then fixed for 30 minutes (1% Formaldehyde and 0.5% Sucrose in PBS). For phosphoJAK2 (pJAK2) IF PBS-V (1 mM Na$_3$VO$_4$) was used for all rinses. Following fixation, cells were rinsed and then incubated with detergent for five minutes (1% IGEPAL). Cells were then blocked for 20 minutes (5% goat serum, 0.4% BSA, and 0.2% Triton X-100 in PBS) before overnight incubation in PBS with the appropriate antibodies. Bioflex membranes were removed from the plate at this stage by cutting with a razorblade. Membranes were then incubated in the same manner as glass coverslips. Primary antibodies used for JAK2 were HR-758 (Santa Cruz, Santa Cruz, CA) and pJAK2$^{1007/1008}$ (Biosource, Camarillo, California). STAT antibodies used were M-22 for STAT1 (Santa Cruz, Santa Cruz, CA) and C-17 (Santa Cruz, Santa Cruz, CA) for STAT5. All primary antibodies were used at a 1:500 dilution.

For cytoskeletal detection, cells were incubated with a 1:500 dilution of rhodamine-
conjugated phalloidin (Molecular Probes, Eugene, OR). Cells were then rinsed and incubated overnight with 1:500 dilution of anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were visualized by incubation in 1.0 μg/mL Hoeschst 33258 (HO33258; Sigma, St. Louis, MO). Coverslips were mounted using VectraShield media (Vector Lab, Burlingame, CA). Bioflex membranes were placed cell side down on the glass slides on top of mounting media. To improve visualization, additional VectraShield was applied to the top (non-cell side) of the membrane and an additional glass coverslip was placed over the media. All membranes and slips were sealed using clear nail polish. Slides were visualized with a Leica DMIRE2 (Leica, Bannockburn, IL) with at 40x magnification and photographed using OpenLab software (Improvision Inc., Lexington, MA).

**Immunoblots**

For total cell extracts, myoblasts were stimulated and then rinsed with ice-cold PBS-V. Ice-cold lysis buffer at pH 7.4 (35) containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl (Fisher, Pittsburgh, PA), 50 mM NaF (Fisher, Pittsburgh, PA), 10 mM Na$_3$VO$_4$ (Sigma, St. Louis, MO), 5 mM EDTA (Fisher, Pittsburgh, PA), 1.0 mM Na$_4$P$_2$O$_7$ (Fisher, Pittsburgh, PA), 1.0% Triton X-100 (Sigma, St. Louis, MO), 10% glycerol (Fisher, Pittsburgh, PA), 0.1% SDS (Fisher, Pittsburgh, PA), 1.0% Deoxycholic acid (Sigma, St. Louis, MO), 1.0 mM phenylmethysulfonyl fluoride (Sigma, St. Louis, MO), 10 μg/mL aprotinin (Sigma, St. Louis, MO), and 10 μg/mL leupeptin (Sigma, St. Louis, MO) was added to the wells. Cells were incubated on ice for one hour,
scraped, and collected. Lysates were centrifuged for 1000 x g at 4°C to pellet cellular debris. For immunoprecipitation, 1 mg of protein lysate was incubated overnight with 1 μg/ml of appropriate primary antibody. Antibody/protein complexes were collected by incubating with prepared Pansorbin (Merck Biosciences, Nottingham, UK) for two hours at 4°C. Nuclear extracts were prepared as previously described (6). Samples were separated on an 8% SDS stacking gel and transferred to a Westran PVDF membrane (Schleicher & Schuell BioScience, Keene, N.H.). Protein was visualized using 1:1000 dilutions of respective primary antibodies. Primary antibodies for total protein are the same as described above. In addition, 4G10 antibody (Upstate Biotech, Waltham, MA) was utilized for detection of tyrosine phosphorylation. Primary antibodies were detected using 1:5000 dilutions of anti-mouse or anti-rabbit secondary antibodies (Amersham, Piscataway, NJ). Detected proteins were visualized using ECLplus (Amersham, Piscataway, NJ) with a charged-coupled device (CCD) camera FluroChem™ 8800 (Alpha Innotech, San Leandro, CA) using FluroChem™ IS-800 software (Alpha Innotech, San Leandro, CA).

**Proliferation Assay**

Proliferation was assayed using BrdU incorporation. Prior to stretching, 10 μM BrdU and dCTP was added to the media. Cells were stretched for 12 hours and then fixed and detected as described above in immunofluorescence. An additional 15 minute incubation with 4M HCl was added to denature the DNA prior to primary antibody
incubation. Transfected cells were identified by either visualization of eGFP or over-expression of JAK2 protein as detected by primary antibody as described above. Cells positive for eGFP or over-expressed JAK2 were counted for proliferation. Proliferating cells were identified using an anti-BrdU antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) and total nuclei were detected using HO33258.

**Statistical Analysis**

Cells were randomly assigned to treatments in all experiments and the data described is from a minimum of two separate replicates. For proliferation assays, the percentage of proliferating cells was analyzed by one-way ANOVA and LS means contrasts. Significance was determined as \( p \leq 0.05 \).

**RESULTS**

*Stretch stimulates proliferation of C2C12 myoblasts*

Mechanical stretch activates satellite cells *in vitro* (90) and the activation and subsequent proliferation of satellite cells *in vivo* is a critical component of postnatal skeletal muscle growth (59). Cyclic stretch also inhibits myogenesis *in vitro* (43). We first sought to determine if mechanical stretch induced C2C12 myoblasts to proliferate. Following 12 hours of stretch, C2C12 cells had an increased level of
proliferation (p ≤ 0.00001; Figure 1). This result indicates that our stretch program
does increase proliferation of C2C12 myoblasts, making this protocol useful as a tool
to explore factors involved.

**JAK2 is activated by stretch in C2C12 myoblasts**

In previous studies, we demonstrated an increase in JAK2 expression following
three days of work overload in rat skeletal muscle (13). Prior work has identified that
JAK2 is activated in response to stretch in cardiac myocytes (67). To analyze if
JAK2 is activated in response to cyclic stretch of skeletal muscle cells, we utilized a
phospho-specific JAK2 antibody directed against the phosphorylated tyrosine
1007/1008 residues. Since these domains appear to be autophosphorylation sites,
and phosphorylation at tyrosine 1007 is critical for JAK2 kinase activity (18),
detection of JAK2 with this antibody indicates an active JAK2. We found
phosphorylation of JAK2 increased after five and fifteen minutes of stretch.
Qualitatively, the intensity of JAK2 fluorescence increased substantially after five
minutes of stretch. Semi-quantitatively, control cells were photographed at 100x the
exposure length of cells stretched for five minutes. In non-stretched, control cells,
pJAK2 was found throughout the cell; at the membrane, in the nucleus, and in the
cytoplasm (Figure 2A). Strikingly, in stretched cells the increase in active JAK2
appeared to be primarily located near the nucleus. This phenomenon was intensely
visible in cells stretched for five minutes. In cells stretched for fifteen minutes, active
JAK2 was still increased and located near the nucleus. However, the strength of this
signal was greatly reduced as compared to five-minute stretched cells. These
results indicate that mechanical cyclic stretch activates JAK2.

Activated JAK2 is present in membrane ruffles in myoblasts

We also observed several unique JAK2 localization patterns in proliferating
myoblasts. Primarily, we noticed phospho-JAK2 (pJAK2) at the membrane ruffles of
C2C12 cells (Figure 2B). In our cells, we found pJAK2 at the edges of filopodia and
ruffles, in particular at the edges of actin filaments in these regions (Figure 2B).
Previously, JAK2 has been shown to phosphorylate SH2B-1 (62), a protein required
for growth hormone (GH)-induced membrane ruffling and cytoskeletal
rearrangement (23, 62). Although we could not determine if pJAK2 at the ruffles and
extensions increased during stretch due to difficulty with over-exposure, we did
notice an increase, in particular, of filopodia with stretch (Figure 5C). This
observation indicates that pJAK2 is present at the end of actin filaments at the cell
membrane and suggests that increased cell ruffling and filopodia formation occurs
during stretch of C2C12 myoblasts.

Nuclear-region active JAK2 is maintained by treatment with Sodium
Orthovanadate

As mentioned above, pJAK2 greatly increases following five minutes of stretch but
by fifteen minutes the signal began to decline (Figure 2A and 3A). We utilized
sodium orthovanadate, a tyrosine phosphatase inhibitor, to attempt to maintain active JAK2. As in the previous experiment, cells were stretched for five and fifteen minutes. When cells were stretched for fifteen minutes and treated with sodium orthovanadate JAK2 intensity, and location near the nucleus, was maintained (Figure 3B). Treatment with sodium orthovanadate had no apparent affect on cells stretched for five minutes (Figure 3B). This result suggests that a tyrosine phosphatase is responsible for the decrease in JAK2 activity, and location near the nucleus, after fifteen minutes of stretch.

**JAK2 is activated in static myoblasts treated with conditioned media collected from stretched C2C12 cells**

To determine if stretched myoblasts were generating factors that would influence JAK2 signaling in neighboring cells, we collected conditioned media (CM) from stretched cells. Transfer of conditioned media has been used previously to identify signaling pathways activated by mechanical stretch in cardiac myocytes (79). Cells were stretched for five minutes, fifteen minutes, and one hour. Following stretch, CM was collected and immediately transferred to static cells. Cells were stimulated with CM for 10 minutes before fixation and detection. We found that media from cells stretched for five minutes and one hour were capable of activating JAK2 in non-stretch myoblasts (Figure 4A). Again, we found the same nuclear/perinuclear localization of activated JAK2 in static cells treated with CM. In particular, JAK2 intensity and tight localization near the nucleus was observed in cells treated with
CM from cells stretched for one hour. The affect of CM on JAK2 activation appeared to occur in two waves, one at five minutes and another at one hour (Figure 4A).

Because of the appearance of two stages of activation, we next sought to establish if the effect of CM on static cells required protein translation in response to stretch. C2C12 myoblasts were treated with cyclohexamide, an inhibitor of translation in eukaryotic cells, for three hours prior to stretch. Cells were stretched and media transferred as described above. Treatment with cyclohexamide had no effect on the CM from cells stretched for five minutes as cells treated with this media showed a large increase of active JAK2 near the nucleus (Figure 4B). However, incubation with cyclohexamide did block the effect of media from cells stretched for one hour since incubation with cyclohexamide-treated CM had no observable increase in active JAK2 (Figure 4B). Thus, cells stretched for one hour appear to generate a JAK2-activating factor that is translated in response to stretch. By contrast, the molecule secreted by cells stretched for five minutes does not appear to be dependent on translation.

**Cellular localization of total JAK2 following stretch of C2C12 myoblasts**

In a previous study (S.A. Anderson, unpublished observations), we found that total JAK2 protein was primarily located in the nucleus of mononucleated C2C12 myoblasts. In this study, we wanted to determine if total JAK2 protein localization was impacted by stretch. Although the majority of JAK2 was still located in the nucleus, we did note the appearance of large, punctuate regions of high JAK2
intensity and an increase in JAK2 location in the cytoplasm following stretch (Figure 5A). Small vesicle-like regions of high JAK2 intensity appeared primarily in the cytoplasm of the cell (Figure 5B). As described above, stretched cells had a qualitative increase in the number, and size, of filopodia observed. It is of note we observed high JAK2-intensity vesicles in these extensions (Figure 5C).

**STAT1 and STAT5 are tyrosine phosphorylated in response to stretch**

In order to form dimers and re-locate to the nucleus, STAT proteins must be tyrosine phosphorylated. To determine if STAT proteins are phosphorylated in response to stretch, we examined STAT immunoprecipitates for tyrosine phosphorylation. We observed that both STAT1 and STAT5 were phosphorylated in response to stretch (Figure 6A and 6B). Western blot analysis found that STAT1 had a stepped increase in tyrosine phosphorylation, with the highest levels observed after fifteen minutes of stretch (Figure 6A). Maximum STAT5 tyrosine phosphorylation was observed after five minutes of stretch (Figure 6B). In contrast, STAT3 tyrosine phosphorylation was not affected by cyclic stretch (data not shown). These results indicate that STAT proteins are activated in response to stretch in C2C12 myoblasts.

**STAT1 and STAT5 relocate to the nucleus in stretched myoblasts**

We examined the response of STAT signaling proteins to mechanical stretch in C2C12 myoblasts. In control cells, we found STAT proteins to be distributed
throughout the cell. However, we found nuclear concentration of STAT1 and STAT5 increased in response to stretch (Figure 6C and 7). Using western blotting techniques, STAT1 had the highest level of nuclear localization after fifteen minutes of stretch (Figure 6C), which agrees with our immunofluorescence data (Figure 7A). In contrast, the nuclear concentration of STAT5 peaked after five minutes of stretch and had largely returned to control whole-cell distribution after fifteen minutes of stretch. This effect was observed in both nuclear extracts (Figure 6C) and whole cell immunofluorescence of total STAT5 (Figure 7B). Analysis of STAT3 did not show any response to stretch (data not shown). These results indicate that STAT1 and STAT5 concentrate in the nucleus in response to stretch in C2C12 myoblasts.

**Inhibition of JAK2 blocks stretch-induced proliferation of C2C12 myoblasts**

Cyclic stretch of C2C12 myoblasts stimulates proliferation (p ≤ 0.00001; Figure 1). We next sought to determine if JAK2 was required for stretch-induced activation of C2C12 myoblasts. Cells were stretched in media containing 20 μM tyrphosin AG490, a pharmacological JAK2 inhibitor. Our previous data demonstrated that this concentration of AG490 blocks JAK2 activation in C2C12 cells (S.A. Anderson, unpublished observations). Incubation with 20 μM AG490 blocks stretch-induced proliferation (p ≤ 0.0001; Figure 8A) and, surprisingly, the proliferation of AG490/stretched cells was lower than that of AG490/non-stretched cells (p ≤ 0.05).

We next transiently transfected cells with either eGFP, wild-type (WT) JAK2, or dominant negative (DN) JAK2. Cells transfected with GFP were activated by
cyclic stretch ($p \leq 0.01$; Figure 8B). However, transfection with DN-JAK2 suppressed proliferation in non-stretch cells as compared to WT-JAK2 and GFP transfected cells ($p \leq 0.0001$; Figure 8B). These results indicate that transfection of DN-JAK2 blocked stretch-induced proliferation. Other groups have reported WT-JAK2 to be constitutively active (76, 77). However, we have not found WT-JAK2 sufficient to increase proliferation in C2C12 cells ($p > 0.05$). These results indicate that active JAK2 is required for mechanical stretch-induced increases in proliferation.

**DISCUSSION**

Mechanical stretch of skeletal muscle *in vitro* has been identified to mimic *in vivo* hypertrophy (92), as evidenced by increased skeletal muscle growth (93), induced release of HGF (89) and IGF-1 (71), increased total protein in stretched muscle (20, 93), activation of satellite cells (90, 95, 96), and modulation of gene expression (12, 27, 91). Studies in a variety of cell types have identified many signaling pathways involved in the biological responses to mechanical stretch *in vitro* including nitric oxide (NO) (89, 91, 97), MAPK (26, 49, 54, 55), PKC (49, 50), Akt (82), NF-κB (42, 43), GTPases (14, 43), and FAK (43).

Previous studies have shown the JAK/STAT pathway to be activated by mechanical stretch in cardiomyocytes and pressure overload in the heart (66, 67). An earlier study by our group identified that JAK2 expression in skeletal muscle increases in response to work overload *in vivo* (13). Limited studies on the role of JAK2 in skeletal muscle have demonstrated JAK2 to be required for LIF-induced
satellite cell proliferation (86) and identified JAK2 as activated in EPO-induced myoblast proliferation (63). IGF-1, a known stimulator of skeletal muscle hypertrophy (2), activates JAK2 phosphorylation (22). However, until now, the role of the JAK/STAT pathway in skeletal muscle hypertrophy has not been examined.

In this study, we demonstrate that active JAK2 is required for stretch-induced proliferation of C2C12 myoblasts. Since our initial results demonstrated that our cyclic stretch protocol stimulates proliferation in C2C12 myoblasts, we sought to determine if JAK2 was required in this process. We first demonstrate this requirement using AG490, a JAK2 inhibitor (57). We also confirmed this inhibition using transient transfection of DN-JAK2, a construct we have previously identified to inhibit normal myoblast proliferation (S.A. Anderson, unpublished observations). These results demonstrate a need for kinase active JAK2 in stretch-induced stimulation. A requirement for JAK2 during this process clearly helps underscore the critical role JAK2 plays in the proliferation of skeletal muscle myoblasts, under a variety of conditions.

Our results also indicate that mechanical stretch causes an increase in the amount of kinase-active JAK2 in C2C12 myoblasts. Surprisingly, immunofluorescent analysis also identified a change in phospho-JAK2 (pJAK2) location in response to stretch. We observed a rapid increase in pJAK2 observed in a circular or semi-circular pattern, near the nucleus. This localization is curious, since activated JAK2 is typically thought to reside at the plasma membrane, interacting with receptors. A similar pattern has been described previously, in cancer cells transfected with GH (33). However, this study did not use a pJAK2
antibody and instead identified JAK2 activation by analyzing co-localization of a total JAK2 antibody and a phospho-tyrosine antibody. In our work, we had the advantage of using an antibody specifically directed against activated JAK2. Our results demonstrate that JAK2 is activated in response to stretch. In addition, the unusual patterning of activated JAK2 suggests a novel, non-receptor, function of this protein in response to stretch. Moreover, when combined with the requirement for active JAK2 in stretch-induced myoblast proliferation, these results demonstrate that JAK2 is a critical signaling protein in the biological response of cells to mechanical stretch.

The location of pJAK2 in the nuclear/perinuclear region is of particular interest due to our, and others, identification of nuclear JAK localization (52, 72, 73). Although the nuclear localization of JAK2 is controversial (8, 60), we have found JAK2 in the nucleus using a number of antibodies for immunofluorescence as well as by nuclear extract/western blotting techniques (S.A. Anderson, unpublished observations). It is of note that while we did observe a slight increase in cytoplasmic JAK2 in response to stretch, it was not as overt as the change in localization that we previously observed during myogenesis (S.A. Anderson, unpublished observations). An unusual alteration in the location of total JAK2 in response to stretch was the appearance of punctuate, potentially vesicular, pattern of JAK2 observed in the cytoplasm, and filopodia, of stretched cells. This appearance suggests a potential transport of JAK2 in response to stretch in myoblasts.

In non-stretched cells, we observed pJAK2 at the edges of the cell membrane, in particular at the end of F-actin filaments found in filopodia and ruffles.
Traditionally, JAK2 is thought to associate with receptors located at the cell membrane (1), although our research has found the majority of total JAK2 protein to be in the nucleus of myoblasts (S.A. Anderson, unpublished observations). However, recent research supports a role for JAK2 in actin cytoskeleton regulation. JAK2 interacts with SH2-Bβ, a protein that regulates actin rearrangement (17, 23, 62), and focal adhesion kinase (FAK), a protein found in focal adhesions and involved in regulating cell motility (78, 88). Our previous work demonstrated that inhibition of JAK2 activity with AG490 greatly reduced myoblast migration and that long-term treatment with AG490 during differentiation had a severe impact on the actin cytoskeleton and blocked myotube formation (S.A. Anderson, unpublished observations). Myoblasts and satellite cells are highly migratory (9, 16, 58) and motility of these cells to the limb bud is required for normal muscle development (10). Combined, these results suggest that active JAK2 may be involved in regulating the actin cytoskeleton in C2C12 myoblasts.

Our experiments indicate that JAK2 is quickly activated within five minutes of cyclic stretch. However, this activation is transient and both increased JAK2 activation, and location of pJAK2 in the nuclear region, is reduced following 15 minutes of stretch. Phosphorylation of JAK2 is highly regulated as expression of constitutively active forms of the protein can result in cancer (11, 70) and resistance to apoptosis (75). Negative regulators of JAKs include suppressors of cytokine signaling (SOCS) proteins (4) and protein tyrosine phosphatases including protein tyrosine phosphatase 1B (21, 61) and SH2-containing phosphatase (SHP-1) (30). In our system, we utilized sodium orthovanadate, a tyrosine phosphatase inhibitor, to
maintain JAK2 activation in stretched myoblasts. Treatment with vanadate has previously been shown to reverse JAK2 inactivation (37, 46, 74). We were able to maintain JAK2 activation for fifteen minutes, as compared to control cells, which displayed peak JAK2 activation five minutes after stretch. It is of particular interest that phosphatase inhibition also maintained JAK2 localization in the nuclear/perinuclear region. Previous research has indicated that SHP-1 translocates to the nucleus when stimulated by growth hormone (GH), a potent activator of JAK2. This work suggests possible regulation of JAK2 by SHP-1 in the nucleus (73). Our results demonstrate maintenance of JAK2 activation, and localization by orthovanadate, which suggests a role for tyrosine phosphatases in regulation of stretch-induced signaling by JAK2. The indication that phosphatases are involved in the regulation of the stretch-induced response highlights the transient, and complex, nature of the signaling involved in this event.

We also demonstrated that JAK2 is activated by conditioned media (CM) transferred from stretched to static cells. Mechanical stretch has been shown to increase several key signaling molecules including NOS (89, 91), TNF-α (65), IGF-1 (65, 71, 87), HGF (89), Angiotensin II (80), and IL-6 (29, 34, 38, 65). Many of these molecules are activators of the JAK2 signaling pathway. Intriguingly, we found a dual time point activation of JAK2, one at five minutes of stretch and one following one hour of stretch. As in stretched cells, the primary increase in pJAK2 appeared to concentrate in the nuclear/perinuclear region. These two distinct events suggest activation of JAK2 by two different sets of upstream factors. This point is further supported by our results demonstrating that incubation with the translation inhibitor,
cycloheximide, only blocked CM-induced JAK2 activation after one hour of stretch. Cells stretched for five minutes in the presence of cycloheximide were still able to activate JAK2 phosphorylation and translocation, as compared to control cells. This selective inhibition demonstrates that activation of JAK2 occurs via two discrete extracellular molecule profiles. These factors are present in the media of cells stretched for five minutes and capable of activating JAK2 in static cells. However, the influence of these factors is quite transient, as their affect is nullified by the fifteen minute time point. The observation that cycloheximide is only able to inhibit JAK2 activation by media collected from cells stretched for one hour suggests a translation-dependent, secondary wave of factors produced in response to stretch-induced signaling. These results demonstrate a cyclic stretch-regulated, autocrine/paracrine induction of JAK2 in C2C12 myoblasts.

Although activation of the JAK proteins has an effect on a wide variety of cell functions, these kinases primarily influence gene transcription through the activation of the STAT transcription factors (47, 48, 51). We were able to demonstrate that cyclic stretch also activates STAT1 and STAT5, but not STAT3. This was a surprising result, as STAT3 is activated by mechanical stretch in cardiomyocytes (67) and LIF and GH signaling in skeletal muscle (19, 32, 86). STAT3 is also regarded as a protein critical for growth-stimulation in a wide variety of cell types and tissues (25). In addition, STAT3 is activated in proliferating satellite cells but not in differentiated cells in regenerating rat skeletal muscle (31). It is of note that research in pressure overload and angiotensin II stimulus demonstrated that STAT3 was primarily activated at later time points (39, 66). It is possible that we did not stretch
the cells for a sufficient length of time to visualize STAT3 activation. We did identify that STAT1 was up-regulated in response to cyclic stretch, which mirrors results observed in mechanical stretch of cardiomyocytes and pressure overload in the heart (66, 67). STAT1 also translocates to the nucleus in a similar time frame. In addition, STAT5 is rapidly activated, and nuclear translocated, in response to stretch, with the peak of activation occurring after five minutes of stimulation. This activation, and corresponding nuclear accumulation, demonstrates that STATs are activated in response to cyclic stretch. Finally, these results demonstrate that the JAK/STAT pathway is capable of transducing a mechanical, extracellular signal to the nucleus, and potentially influencing gene transcription in C2C12 myoblasts.

Combined, these results show that the JAK/STAT pathway is activated by mechanical stretch in skeletal muscle myoblasts. In addition, this pathway is critical for stretch-induced myoblast proliferation, since kinase-active JAK2 is required for the increase observed following stretch. Identification of regulatory pathways involved in normal, and mechanically stimulated, proliferation of skeletal muscle cells is critical to identify potential targets for manipulation in the treatment of muscle wasting diseases. Our research identifies the JAK/STAT pathway as a novel regulator of mechanical stretch signaling in C2C12 myoblasts. Since JAK2 is activated by many factors identified as stimulators of skeletal muscle hypertrophy in vivo, our results highly suggest a role for this pathway in the regulation of skeletal muscle mass in response to stretch.

ACKNOWLEDGEMENTS
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94. **Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, and Ziemiecki A.** Two novel protein-tyrosine kinases, each with a second phosphotransferase-related


**FIGURES**

Figure 1. Stretch stimulates proliferation of C2C12 myoblasts. Cells were cyclically stretched (4 second 20% sine stretch followed by 10 seconds of rest) for 12 hours and 10 mM BrdU and dCTP were added to cells at the start of stretch. Following stretch, cells were fixed and detected as described in materials and methods. Cells that had proliferated were identified with an anti-BrdU antibody and total nuclei were visualized using HO33258. The percentage of cells that had proliferated was calculated by dividing the number of BrdU positive cells by the total number of nuclei. Control cells had a significantly higher number of proliferated cells in response to stretch (*p ≤ 0.00001*).
Figure 2. JAK2 is activated by stretch in C2C12 myoblasts. (A) Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five and fifteen minutes. Following stretch, cells were fixed and detected as described in materials and methods. Phospho-JAK2 (pJAK2) was detected using pJAK2\textsubscript{1007/1008} antibody, filamentous actin was detected using rhodamine-conjugated phalloidin, and nuclei were detected using HO33258. Control cells and cells stretched for fifteen minutes were photographed at the same exposure. Myoblasts stretched for five minutes were photographed at 1/100 the exposure length of control and fifteen minute stretched cells. (B) Phospho-JAK2 is present in the ruffles and at the end of actin filaments of C2C12 myoblasts. Photographs are of cells stretched for fifteen minutes.

Figure 3. Nuclear-region phospho-JAK2 is maintained by treatment with sodium orthovanadate. Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five and fifteen minutes. Cells were treated with 10 \textmu M activated sodium orthovanadate for three hours prior to stretch to inhibit tyrosine phosphatase activity. Following stretch, cells were fixed and detected for pJAK2 as described above. No stretch and fifteen minute stretched cells were photographed at the same exposure. Myoblasts stretched for five minutes (without vanadate) and five and fifteen minutes (with vanadate) were photographed at 1/100 the exposure length of above treatments.
Figure 4. Conditioned media from stretched myoblasts activates JAK2. Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five minutes, fifteen minutes, and one hour. Conditioned media (CM) was collected from stretched cells and static cells were treated for ten minutes and then fixed and detected for pJAK2 as described above. Translation was inhibited by treatment with cyclohexamide (CH). Cells were incubated with cyclohexamide or vehicle (Co) for three hours prior to stretch. Control no stretch and fifteen minute CM-treated cells, and no stretch, fifteen minute, and one hour CM from CH-treated cells were photographed at the same exposure. Myoblasts treated with CM from control, five-minute and one-hour stretched, and CM from CH-treated cells stretched for five minutes were photographed at 1/100 the exposure length of above treatments.

Figure 5. Cellular localization of total JAK2 following stretch of C2C12 myoblasts. (A) Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five minutes, fifteen minutes, and one hour. Following stretch, cells were fixed and total JAK2 was detected using the HR-758 antibody. (B) Total JAK2 is visible in bright, punctuate pattern in response to stretch in C2C12 myoblasts. Photograph is of a myoblast stretched for fifteen minutes. (C) Total JAK2 is visible in bright, punctuate pattern in the filopodia of stretched cells. Photograph is of cells stretched for one hour.
Figure 6. STATs are activated by stretch in C2C12 myoblasts. Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five and fifteen minutes. (A) STAT1 and (B) STAT5 were immunoprecipitated from cell extracts of cells cyclically stretched for five and fifteen minutes. Immunoprecipitates were detected using phospho-tyrosine (pTyr) 4G10 antibody and primary STAT antibodies as described in materials and methods. (C) Nuclear extracts (NE) were prepared from cells cyclically stretched for five and fifteen minutes as described in materials and methods.

Figure 7. Stretch induces STAT nuclear accumulation in C2C12 myoblasts. Myoblasts were cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for five and fifteen minutes. Following stretch, cells were fixed and total STAT1 (A) and STAT5 (B) were detected as described in materials and methods and nuclei were detected using HO33258 (HO).

Figure 8. Inhibition of JAK2 blocks stretch-induced proliferation of C2C12 myoblasts. Stretched cells (S) were compared to non-stretched cells (NS) within treatments. (A) 20 μM AG490 (AG) or vehicle (Control) and 10 μM BrdU and dCTP were added to cells at the start of stretch. Cells were then cyclically stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for 12 hours. Following stretch, cells were fixed and detected as described in materials and methods. Proliferating cells were identified using an anti-BrdU antibody and total nuclei were quantified using HO33258. The percentage of cells that had proliferated was
calculated by dividing the number of BrdU positive cells by the total number of
nuclei. Control cells had a significantly higher number of proliferated cells in
response to stretch (p ≤ 0.00001). AG490-treated cells had a decrease in
proliferated cells in response to stretch (p ≤ 0.05). (B) C2C12 cells were transiently
transfected with eGFP (GFP), WT-JAK2 (WT), or DN-JAK2 (DN). At the start of
stretch, 10 μM BrdU and dCTP was added to the cells. Cells were then cyclically
stretched (4 seconds of 20% sine stretch followed by 10 seconds of rest) for 12
hours. Following stretch, cells were fixed and detected as described in materials
and methods. Transfected cells were identified by either JAK2-overexpression or
eGFP visualization. Proliferating cells were identified using an anti-BrdU antibody
and total nuclei were quantified using HO33258. The percentage of cells that had
proliferated was calculated as described above. GFP transfected cells had an
increased number of proliferated cells in response to stretch (p ≤ 0.01). The
proliferation rate of DN-JAK2 transfected cells did not increase in proliferation in
response to stretch (p > 0.05) but was greatly decreased reduced as compared to
GFP or WT-JAK2 transfected cells (p ≤ 0.0001).
Figure One

![Graph showing comparison between No Stretch and Stretch treatments. The graph displays percentage proliferation with error bars and a significant difference indicated by an asterisk.](image)
Figure Two

A

Overlay  |  phosphoJAK2

No Stretch | No stretch

5 min. Stretch | 5 min. stretch

15 min. Stretch | 15 min. stretch

B
Figure Three

A

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B

Orthovanadate
Figure Four

A

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Figure Five

A

No Stretch

Five Minutes

Fifteen Minutes

One hour

B

C
Figure Six

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Figure Seven

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Figure Eight

A

% Proliferating

Control NS  Control S  AG NS  AG S

Treatment

B

% Proliferating

GFP NS  GFP S  DN NS  DN S  WT NS  WT S

Treatment
CHAPTER 4. GENERAL CONCLUSIONS

Understanding the signaling pathways involved in skeletal muscle development and postnatal growth is critical to treatment of diseases such as DMD and atrophy. In this work we have identified a role for the tyrosine kinase, JAK2, in skeletal muscle cell proliferation and terminal differentiation. In addition, we have demonstrated, for the first time, activation of the JAK/STAT pathway by cyclic stretch in C2C12 myoblasts. This activation of JAK/STAT suggests a role for this pathway in skeletal muscle hypertrophy.

In our first study, we identify two unique roles for JAK2 in skeletal muscle myogenesis. We first sought to ascertain if JAK2 was required for normal proliferation of myoblasts *in vitro*. Using two approaches, over-expression of JAK2 proteins and use of a chemical JAK2 inhibitor, AG490, we were able to demonstrate that active JAK2 is required for proliferation of C2C12 myoblasts. This result is significant since myoblast proliferation is a critical process for normal skeletal muscle development. It is also suggestive of a role for JAK2 in satellite cell proliferation, an important component of postnatal skeletal muscle growth. In addition, we were able to establish that treatment of cells with AG490 did not appear to significantly impair the cytoskeletal of myoblasts, although we did observe a slight increase in cortical actin in treated cells. This impact on myoblast structure is significant, as we were also able to show that C2C12 myoblasts were able to quickly recover from treatment with AG490 and proliferate in response to a return to normal growth media. The role
of JAK2 in myoblast proliferation dovetailed with other studies detailing the necessity for kinase-active JAK2 in normal, and aberrant, growth of a number of cell types.

However, a role for JAK2 in terminal differentiation has not previously been well described in skeletal muscle. We were surprised to find a complex function of JAK2 in terminal differentiation. It is well known that myogenesis is a complex and unique process, since skeletal muscle develops large, complicated multinucleated cells and requires expression of a number of muscle-specific factors in this process. As such, our understanding of the major players in this process including growth factors, muscle regulatory factors, and cell cycle regulators, is fairly well described. However, the role of intracellular cascades involved in the transduction of these signals is less well known. To identify the role of JAK2 in myogenesis we again utilized two approaches, over-expression of JAK2 proteins and inhibition of JAK2 with AG490. Surprisingly, we found a large disparity in results based on which of these approaches we employed. When we utilized AG490 to inhibit JAK2, we discovered a severe impact on the terminal differentiation, actin cytoskeleton, and myotube formation, of C2C12 cells. Like proliferation, treatment with AG490 blocked myogenesis. Inhibition of terminal differentiation was analyzed via myosin heavy chain protein accumulation and creatine kinase activity. JAK2 inhibition with AG490 greatly decreased both of these biochemical markers. Myoblasts differentiated in the presence of AG490 had no cell fusion and resulting myotube formation. However, unlike proliferating myoblasts, we observed that this inhibition was much more severe. Cells treated with AG490 could not quickly recover and terminally differentiate when returned to normal media, as compared to control cells. A partial
recovery was possible, and cells did have increased CK activity when compared to cells continuously treated with AG490. However, this greatly reduced CK activity and the immature myotubes observed, indicated an attenuated, incomplete rescue of differentiation.

This result indicated a severe effect of JAK2 inhibition with AG490 on the ability of myoblasts to undergo proper myogenesis. In order to understand a possible influence of AG490 on the cytoskeletal structure of terminally differentiating cells, we analyzed the F-actin structure of these cells. It cannot be overstated that the effect of AG490 on treated, differentiating myoblasts was extreme. Cells treated with AG490 appeared as oversized, mononucleated cells with an increase in cortical actin. These cells were similar in structure to proliferating myoblasts but displayed a large increase in cortical actin and unusual actin structures, as well as a large increase in cell size. However, they did form stress fibers and, to most observers, would appear quite similar to normal myoblasts. This effect on the cytoskeleton also appeared to greatly inhibit the ability of these cells to migrate normally. Cells treated with AG490 were essentially unable to migrate across a wound in the myoblast monolayer. It is clear that the influence of AG490 on C2C12 myogenesis is at least partially due to this tremendous impact on the actin cytoskeleton.

We also examined the affect of stable over-expression of JAK2 proteins in C2C12 myoblasts. We were surprised to discover that over-expression of these proteins greatly increased creatine kinase (CK) activity. This result was consistent and very repeatable, despite the type of JAK2 construct utilized. We did find that cells over-expressing DN-JAK2 displayed the greatest increase in CK activity.
These results suggested a protein dependent, kinase independent, role for JAK2 in myogenesis. Of note, underscoring a potential role for JAK2 in regulation of the cytoskeleton, over-expression of WT- and DN-JAK2 resulted in unusual myotube formation. The myotubes formed in these cells were much narrower, and less complex than control cells. However, these cells were able to migrate normally, indicating that the effect of JAK2 over-expression was substantially less severe than treatment with AG490.

By far, the key result of this study was the observation of nuclear-localized JAK2 in C2C12 myoblasts. This outcome was completely unexpected but developed into a compelling model for the complex role of JAK2 in myogenesis. We observed, using both immunofluorescence and western blotting techniques, that the vast majority of JAK2 protein is located in the nucleus of proliferating myoblasts. This nuclear localization is unchanged, even in cells treated with AG490. When we examined differentiating cells, we found a large difference in JAK2 localization throughout myogenesis. This exciting observation noted that, while JAK2 remains in the nucleus of mononucleated cells, JAK2 protein was visible in the cytoplasm only of fused, multinucleated myotubes. Treatment with AG490 during stimulation of terminal differentiation, which prevents myoblast fusion, maintains tightly nuclear JAK2. When we examined location of phospho-JAK2 we did not notice a large change in localization. Thus, these results demonstrate that total JAK2 protein only appears in the cytoplasm of cells undergoing the final stages of terminal differentiation.
This result is important for a number of reasons. First, it suggests that JAK2 protein, independent of kinase activity, is playing a critical role in myogenesis. Second, the location of JAK2 only in the cytoplasm of fused cells, combined with our identification of a role for JAK2 in actin filament regulation, suggests regulation of the cytoskeleton as a potential function. Finally, it raises important questions regarding the use of sequestration in specific regions of the cells as a way to regulate JAK2 and suggests that interactions of the JAK2 protein, and not just kinase activity, are an important function. Examination of this potential role, dependent on localization and interactions of the protein, may elucidate the precise mechanism of JAK2 regulation of myogenesis.

This observation laid the groundwork for our model for the function of JAK2 in skeletal muscle myoblast proliferation and terminal differentiation. We believe that, in proliferating cells, JAK2 plays a traditional, kinase dependent role. This role may involve control of the cell cytoskeleton as well as transducing the effects of growth factors to other signaling proteins. Alternatively, we propose that JAK2 plays a novel, protein-dependent role in myogenesis. JAK2 may function to regulate myoblast fusion, or may accumulate in the cytoplasm as a result of fusion. Clearly, identification of the mechanism of function requires substantially more research. In the future it will be exciting to identify new interacting factors of JAK2 in the nucleus and cytoplasm, as well as possible regulation of an import/export mechanism and/or increased translation of JAK2 in the control of myogenesis.

In our second study we sought to identify a potential role for the JAK/STAT pathway in mechanical stretch of C2C12 myoblasts. Postnatal growth of skeletal
muscle is primarily due to proliferation of satellite cells. The response of these cells is a critical component of skeletal muscle hypertrophy, and the associated increase in skeletal muscle mass. Mechanical stretch is utilized in vitro to study the mechanisms involved in skeletal muscle cell responses to this stimulation. Using a computerized stretch apparatus we were able to identify that the JAK/STAT pathway is activated by cyclic stretch in vitro.

Since we were utilizing cyclic stretch in vitro to mimic the responses of skeletal muscle in vivo we first sought to determine if C2C12 myoblasts proliferate when stretched. We were able to show that these cells do proliferate in response to cyclic stretch, which identified that we had a system suitable for analysis of signaling pathways activated in response to this stimulus. Initially, we wanted to determine if JAK2 was required for stretch-stimulated proliferation. Utilizing both AG490 and transient transfection of JAK2 constructs, we were able to demonstrate that kinase-active JAK2 is required for proliferation in response to cyclic stretch. This requirement, in combination with results from our first study, establishes an important role for JAK2 in both normal and stretch-induced proliferation. These results suggest that JAK2 is involved in both normal skeletal muscle development and postnatal skeletal muscle growth.

We examined JAK2 activation in response to stretch using immunofluorescence based-techniques. Unlike total JAK2, phosphoJAK2 (pJAK2) is distributed throughout the cell. It is of note that the antibody we utilize to examine phosphorylated JAK2 is specifically directed against residues critical for kinase function located in the activation loop, as described in the second study. We also
noted that, in control cells, pJAK2 is located throughout the cell. In particular, we noted pJAK2 at the ends of the actin filaments, in the ruffles and filopodia. This is of interest because, in the first study, we noted that treatment with a JAK2 inhibitor, AG490, impacted cell migration and actin distribution.

We identified that JAK2 is highly activated by stretch in C2C12 myoblasts. A surprising observation was the location of activated JAK2. Unstimulated cells had pJAK2 distribution throughout the cell; in the cytoplasm, at the membrane, and in the nucleus. The increase in activated JAK2 observed in stretched cells is largely concentrated in the nuclear/perinuclear region. This concentration could be a result of movement of activated JAK2 to this domain or activation of JAK2 already located near the nucleus. This distinction is of particular interest as work described in our first study described the majority of JAK2 protein located in the nucleus.

We also described a role for tyrosine phosphatases in the regulation of JAK2 in response to stretch. Activation of JAK2 peaks five minutes following stretch and levels of phosphorylated JAK2 return to near-normal levels fifteen minutes after stretch. Treatment with a tyrosine phosphatase inhibitor, sodium orthovanadate, maintained both the intensity, and localization, of activated JAK2 for fifteen minutes. This maintenance indicates that JAK2 phosphorylation is quickly regulated by tyrosine phosphatases.

We were also able to demonstrate an autocrine/paracrine control of JAK2 by stretched cells, dependent upon translation of new proteins. Treatment with media collected from cells stretched for five minutes and one hour were able to stimulate JAK2 activation and relocalization in unstretched cells; similar to the phenotype
observed above. Since we did not observe an activation of JAK2 at an intermediary time point (fifteen minutes) these results suggested two waves of JAK2 stimulation. Using a translation inhibitor, cyclohexamide, we were able to inhibit the second activation of JAK2, at one hour. This inhibitor had no influence on JAK2 activation from media stretched for five minutes indicating that activation at this short time point was translation-independent. Identification of an autocrine/paracrine control indicates that stretched cells secrete factors able to activate JAK2 in response to stretch. It also suggests that mechanical stretch directly influences translation of new proteins that are capable of phosphorylating JAK2. Combined with a requirement for active JAK2 in stretch-induced proliferation, these results indicate that activation of JAK2 is a critical component of the cellular response to mechanical stretch.

Finally, we identified STAT1 and STAT5 to be activated by cyclic stretch in C2C12 myoblasts as demonstrated by an increase in tyrosine phosphorylation. In addition, we observed an increase in total STAT1 and STAT5 protein in the nucleus, observed both by immunofluorescent detection and western blot analysis of nuclear extracts. The discovery that STATs are activated is significant because activation, and nuclear translocation of these transcription factors, suggests that cyclic stretch is influencing gene transcription. These results indicate that the JAK/STAT pathway is activated in skeletal muscle myoblasts in response to cyclic stretch.

The results of these two studies suggest both novel and traditional roles of the JAK/STAT pathway in skeletal muscle. Our research represents a substantial
advance in our understanding of a role for JAK2 in skeletal muscle cell proliferation, and the first characterization of a role for JAK2 in myogenesis. Clearly, this work identifies JAK2 is critical for normal muscle responses to biological stimulus for proliferation, terminal differentiation, and mechanical responses. However, since this work was performed in a clonal cell culture line, it will be critical to confirm, and expand, our knowledge of this pathway in primary satellite cells and an in vivo environment. Future work will also hopefully clarify upstream signaling molecules that regulate these roles, identify potential gene targets of the STAT factors, as well as expand upon a possible function for nuclear-localized JAK2. In particular, an increased understanding of nuclear JAK2 is important to identification of a potential novel role for this kinase. Future identification of interacting factors such as phosphatases, transcription factors, kinases, and other regulatory proteins, would greatly expand, and likely challenge, our current knowledge of the function of JAK2. At the conclusion of these studies it is clear that our understanding of signaling pathways involved in skeletal muscle has expanded. It also accentuates the complexities of this unique tissue and illustrates the necessity of an increased understanding of the signals that regulate it.
APPENDIX A. ACTIVATION OF JAK2 BY HIGH GLUCOSE IN C2C12 MYOBLASTS

Symantha A. Anderson and James M. Reecy
Department of Animal Science, Iowa State University, Ames, IA 50011

SUMMARY

Signaling via increased glucose concentrations is causative of a number of human diseases. Understanding the mechanisms involved in cellular responses to high glucose is critical for understanding the normal, and diseased, signaling pathways activated by this important biological stimulus. Skeletal muscle is an insulin-sensitive tissue that has altered expression of glucose regulatory molecules during myogenesis. Studies have identified the activation of the JAK/STAT pathway in response to high glucose in a number of cell types, including mesangial and vascular smooth muscle cells. In this study we identify an inhibitory role for low glucose in normal C2C12 myoblast proliferation, migration, and terminal differentiation. We also demonstrate that JAK2 is activated, and changes cellular localization, in response to high glucose signaling. Finally, we identify a potential nuclear translocation of the STAT proteins in response to high glucose signaling.

INTRODUCTION
Skeletal muscle myogenesis is a complex cascade of events regulating proliferation, migration, and fusion of mononucleated muscle cells into multinucleated myotubes. Early control of proliferation is regulated by a host of growth factors including FGF, IGF-1, and HGF (1,2). HGF also has the ability to function as a chemoattractant to migrating cells in vivo (3). Sufficient proliferation and migration must occur in the developing embryo to ensure an appropriate population of myoblasts for mature skeletal muscle fiber formation. Following proliferation and migration, myoblasts exit the cell cycle, fuse, and differentiate into multinucleated myotubes. Regulation of this process is primarily controlled by the MRF family of transcription factors: MyoD, Myf5, Myogenin, and MRF4. These factors have the unique ability to induce non-muscle cells to take on a skeletal muscle fate (4-8).

Control of glucose uptake and transport is a critical function in maintaining homeostasis of glucose. In skeletal muscle, expression of genes involved in glucose transport is highly regulated during skeletal muscle development (9). The primary proteins involved in the facilitated diffusion of glucose in skeletal muscle are the glucose transporters: GLUT1 and GLUT4. GLUT1 has been demonstrated to be highly expressed in proliferating myoblasts and GLUT4 expression is observed in terminally differentiated myotubes (10-12). Regulation of GLUT1 expression is partially mediated by MyoD (12).

The Janus kinases have been implicated in the signaling stimulated by high glucose. This family of kinases consists of four members: JAK1, JAK2, JAK3, and Tyk2. Classically, they are associated with cytokine receptors lacking intrinsic kinase ability and are responsible for activating the signal transducer and activator of
transcription (STAT) family of transcription factors. Stimulation of this signaling cascade activates proliferation in a number of cell types. Specifically, JAK2 stimulates proliferation in vascular smooth muscle cells (VSMCs) (13,14), pulmonary epithelial cells (15), skeletal muscle satellite cells (16), mammary epithelium (17), and a number of leukemias (18-20). Treatment with high glucose activates JAK2 in mesangial cells (21,22) and VSMCs (23,24). In addition, high glucose increases phosphorylation of STAT1, 3, and 5 in mesangial cells (21,22), STAT1 and 3 in VSMCs (23), and STAT5 in skeletal muscle cells (25). It is also of interest that JAK2 is a substrate for leptin, a regulator of food intake and insulin signaling (26,27).

The goal of this study was to identify the role of high glucose in skeletal muscle myoblast proliferation, migration, and terminal differentiation, and to identify if the JAK/STAT pathway is activated in response to high glucose in C2C12 myoblasts. We determined that incubation in low glucose inhibited both proliferation and migration of myoblasts, as well as inhibited terminal differentiation. Finally, we identified that JAK2 is activated in response to high glucose stimulation and demonstrate a possible role for STAT1 and STAT3 in signaling in response to high glucose in C2C12 myoblasts.

EXPERIMENTAL PROCEDURES

Cell Culture
C2C12 skeletal muscle myoblasts (American Type Culture Collection, Manassas, VA) were cultured in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells were grown in either high (25 mM) or low glucose (5.5 mM) DMEM (Atlanta Biologicals, Lawrenceville, GA) containing 10% fetal bovine serum (FBS; Gibco-BRL Life Technology, Carlsbad, California), 1.0 mg/mL penicillin/streptomycin (Gibco-BRL Life Technology, Carlsbad, California) and 0.3 mg/ml L-glutamine (Gibco-BRL Life Technology, Carlsbad, California). In the terminal differentiation experiment, cells were cultured in low serum differentiation medium, which consisted of either high or low glucose DMEM containing 2% horse serum, 10 μg/ml insulin, 1.0 mg/mL penicillin/streptomycin (Gibco-BRL Life Technology, Carlsbad, CA) and 0.3 mg/ml L-glutamine (Gibco-BRL Life Technology, Carlsbad, CA). Cells were refreshed with new differentiation media every 48 hours. Cells were plated on 35-mm tissue culture dishes (BD Biosciences, Lexington, KY) at 20,000 cells/cm$^2$ for differentiation and migration assays and 5000 cells/cm$^2$ for cellular extracts. For immunofluorescence, myoblasts were plated at 1250 cells/cm$^2$ cells onto glass coverslips coated with bovine fibronectin (Sigma, St. Louis, MO) in 12-well plates.

**Wounding Assay**

C2C12 cells were plated to confluence (20,000 cells/cm$^2$) on fibronectin-coated 35 mm cell culture dishes. Dishes were marked with a line across the bottom of the plate. Cells were plated in low glucose (LG) and were either refreshed with new LG media or changed to high glucose (HG) media just prior to wounding. The cell
monolayer was wounded the day following plating with a sterile pipette tip. Along
the wound, above and below the marked line, cells were photographed during the
eight-hour time course with a Nikon digital camera (DXM 1200). To calculate the
percentage of wound coverage, confluence of cells across the wound were scored
by comparing the wound at the beginning of the time course (T0) and at completion
(T8).

**Immunofluorescence**

All cells were plated in LG media and rinsed and changed to HG media at the start
of each experiment. Following stimulation, cells were rinsed with phosphate
buffered saline containing sodium orthovanadate (PBS-V; 1 mM Na₃VO₄) and fixed
for 30 minutes (1% formaldehyde and 0.5% sucrose in PBS). After fixation, cells
were rinsed with PBS-V and incubated with 1% IGEPAL (Sigma, St. Louis, MO) for
five minutes. Cells were incubated in blocker for 20 minutes (5% goat serum, 0.4%
BSA, and 0.2% Triton X-100 in PBS) before overnight incubation in PBS with the
appropriate antibodies. The primary phospho-antibody used for JAK2 was
pJAK21007/1008 (Biosource, Camarillo, California). STAT antibodies used were M-22
for STAT1 (Santa Cruz, Santa Cruz, CA) and C-20 for STAT3 (Santa Cruz, Santa
Cruz, CA). All primary antibodies were used at a 1:500 dilution. Following
incubation with the primary antibody, cells were rinsed and incubated overnight with
1:500 dilutions of anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR).
Nuclei were detected with 1.0 µg/mL Hoeschst 33258 (HO33258; Sigma, St. Louis,
MO). Coverslips were mounted on VectraShield media (Vector Lab, Burlingame, CA) and sealed with clear nail polish. Mounted slips were visualized and photographed using a Leica DMIRE2 microscope (Leica, Bannockburn, IL) and OpenLab software (Improvision Inc., Lexington, MA).

**Immunoblots**

To collect cell extracts, myoblasts were stimulated with HG and rinsed with ice-cold PBS-V. Cells were then incubated with lysis buffer at pH 7.4 (28) containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl (Fisher, Pittsburgh, PA), 50 mM NaF (Fisher, Pittsburgh, PA), 10 mM Na$_3$VO$_4$ (Sigma, St. Louis, MO), 5 mM EDTA (Fisher, Pittsburgh, PA), 1.0 mM Na$_4$P$_2$O$_7$ (Fisher, Pittsburgh, PA), 1.0% Triton X-100 (Sigma, St. Louis, MO), 10% glycerol (Fisher, Pittsburgh, PA), 0.1% SDS (Fisher, Pittsburgh, PA), 1.0% Deoxycholic acid (Sigma, St. Louis, MO), 1.0 mM phenylmethysulfonyl fluoride (Sigma, St. Louis, MO), 10 µg/mL aprotinin (Sigma, St. Louis, MO), and 10 µg/mL leupeptin (Sigma, St. Louis, MO) on ice for one hour, scraped, and collected. To pellet cell debris, extracts were centrifuged for 1000 x g at 4°C. Total protein was quantified using a Bradford protein assay (Biorad, Hercules, CA) and 1 mg of lysate was incubated overnight with 1 µg/ml of appropriate primary antibody for immunoprecipitation. To collect antibody/protein complexes, extracts were incubated for two hours at 4°C with prepared Pansorbin (Merck Biosciences, Nottingham, UK). Immunoprecipitates were separated on an 8% SDS stacking gel and transferred to a Westran PVDF membrane (Schleicher &
Schuell BioScience, Keene, N.H.). Bound protein was detected using 1:1000 dilutions of appropriate primary antibodies (HR-758, M-22, and C-20) overnight at 4°C. We utilized 4G10 primary antibody (Upstate Biotech, Waltham, MA) to identify tyrosine phosphorylation of immunoprecipitated proteins and HR-758 (Santa Cruz, Santa Cruz, CA) to detect total JAK2 protein. Detected proteins were visualized using 1:5000 dilutions of anti-mouse or anti-rabbit secondary antibodies (Amersham, Piscataway, NJ) with ECLplus (Amersham, Piscataway, NJ). Complexes were then detected using a charged-coupled device (CCD) camera FluroChem™ 8800 (Alpha Innotech, San Leandro, CA) using FluroChem™ IS-800 software (Alpha Innotech, San Leandro, CA).

**Proliferation Assay**

Proliferating cells were identified via BrdU incorporation. During stimulation, 10 μM BrdU and dCTP was added to the media. Cells were incubated with the appropriate media for 12 hours. Stimulated cells were then fixed and detected as described above. To denature the DNA to facilitate detection of the BrdU, 4M HCl was added to the cells as an additional step. Proliferated cells were identified using an anti-BrdU antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) and total nuclei were visualized using HO33258.

**Differentiation Assay**
Cells were differentiated for 72 hours in either high or low glucose DMEM. At the end of the time course, cells were washed with PBS, and 0.5 M glycyglycine buffer, pH 6.75, was added to the plates. Cells were frozen at -80°C, at completion of the time course, for 24 hours. Cells were then removed from the plates and sonicated for 15 seconds at a 50% cycling setting and centrifuged at 20,000 X $g$ for one minute. Supernatant was removed and creatine kinase (CK) activity was quantified as described by the manufacturer (Sigma, St. Louis, MO). Total protein was analyzed with the Bradford protein assay. Creatine kinase units were reported corrected for total protein in nanograms.

**Statistical Analysis**

C2C12 cells in all experiments were randomly assigned to treatments and the data described is representative of a minimum of two separate replicates. Results were analyzed by one-way ANOVA and LS means contrasts and significance was determined as $p \leq 0.05$.

**RESULTS**

*Low glucose media inhibits proliferation and terminal differentiation of C2C12 cells*
We first sought to determine the affect of low glucose (LG) media on normal proliferation and terminal differentiation of C2C12 cells. Cells were plated in LG media and then either refreshed with new low or high glucose (HG) media. We found that incubation with LG media greatly decreased the ability of myoblasts to proliferate ($P \leq 0.0001$; Figure 1A). These results indicate that C2C12 myoblasts require HG media for normal cell proliferation.

We next examined the requirement for high glucose media in terminal differentiation of C2C12 cells. Previous studies indicated that restricted glucose does not inhibit myogenesis (29) and can even increase the efficacy of differentiation of primary satellite cells (30). Cells were plated in LG media at 80-90% confluency and then changed to a restricted serum HG or LG media for 72 hours. We found that CK activity in cells differentiated in LG media was greatly reduced as compared to cells differentiated in HG media ($p \leq 0.00001$; Figure 1B). These results demonstrate that media containing HG is required for both normal proliferation and terminal differentiation of C2C12 myoblasts.

**Low glucose media inhibits migration of C2C12 myoblasts**

Migration of skeletal muscle myoblasts is critical for normal fusion into multinucleated myotubes (31). Since culture of C2C12 cells in LG media inhibited myogenesis, we examined the ability of myoblasts to close a wound in the cell monolayer. Cells were plated in LG and then wounded the following day. Cells were changed to either HG media or refreshed in LG media immediately before
wounding. Cells were photographed throughout the time course and the percentage
of wound closure was determined (Figure 2B). Our results indicate that incubation
of C2C12 myoblasts in low glucose media inhibits cell migration (p ≤ 0.00001; Figure
2A).

**Treatment of C2C12 myoblasts with high glucose activates JAK2**

Since prior reports have indicated that JAK2 is activated by high glucose (HG) in
other tissue types (22,24), we sought to determine if treatment with HG increases
JAK2 phosphorylation in skeletal muscle myoblasts. Cells were plated in LG and
then stimulated with HG for five or fifteen minutes. We found that JAK2 is rapidly
upregulated by HG treatment. When we used immunofluorescence to analyze cells
stimulated with HG we found that JAK2 phosphorylation is maximal five minutes
following stimulation. In addition, activated JAK2 appears to concentrate in the
perinuclear/nuclear region of the cell (Figure 3A). When assayed by western blot
analysis we found that the greatest increase of tyrosine phosphorylated JAK2 (pTyr)
at fifteen minutes of stimulation (Figure 3B). These results indicate that JAK2 is
activated by treatment with high glucose in C2C12 myoblasts.

**High glucose effects nuclear translocation, but not phosphorylation, of STAT1
and STAT3**
Traditionally, JAK2 exerts its influence via activation of the STAT transcription factors. High glucose stimulation activates STAT proteins in a number of cell types (22, 25). To determine if the STAT proteins were stimulated by treatment with HG, C2C12 myoblasts were stimulated for five and fifteen minutes. We first examined the location of STAT proteins in the cell in response to HG treatment. We found that STAT1 appeared to partially translocate to the nucleus in response to HG stimulation (Figure 4A). This relocation peaked at five minutes and essentially returns to control levels following fifteen minutes of stimulation. Interestingly, we found that STAT3 was predominantly located in the nucleus of cells in LG media, although protein was still detectable in the cytoplasm. When stimulated with HG it appeared that the vast majority of STAT3 protein was located in the nucleus, with an even greater depletion of STAT3 in the cytoplasm than in LG-treated cells. This effect was observed at both five and fifteen minutes of stimulation (Figure 4B). Interestingly, at five minutes of stimulation, we noted several small, punctuate, concentrations of STAT3 located in the cytoplasm of stimulated cells. STAT5 did not show any response to HG stimulation (data not shown). Finally, we examined the tyrosine phosphorylation of STAT1 and STAT3 in response to incubation in HG media. We did not observe an increase in tyrosine phosphorylation in response to high glucose stimulation (Figure 5). These results suggest that STAT1 and STAT3 translocate to the nucleus in response to high glucose, although the precise role of these proteins is still unclear.

DISCUSSION
In this study we investigated the role of glucose levels in normal C2C12 proliferation, migration, and terminal differentiation. We also examined if the JAK/STAT pathway is activated in response to high glucose (HG). We first identified that low glucose (LG) levels are required for normal proliferation of C2C12 myoblasts. This result was not surprising as increased glucose concentration stimulates proliferation in a number of cell types, including VSMCs (24). Diabetes, a disease identified by impairment of glucose homeostasis, has been identified to cause atherosclerosis (32). Atherosclerosis is caused by both aberrant proliferation and migration of vascular smooth muscle cells (33). Accordingly, HG stimulates migration of VSMCs (34). We also found that HG was sufficient to stimulate migration in C2C12 myoblasts and that culture of these cells in LG media severely attenuated their ability to close a wound in the monolayer. Thus, our results demonstrate that C2C12 myoblast proliferation and migration are stimulated by HG.

We also show that culture of cells in LG, restricted serum media greatly inhibited the ability of these cells to terminally differentiate. This result is surprising because earlier work identified either no impact of glucose restriction on skeletal muscle myogenesis (29) or found that LG, restricted serum media was ideal for differentiation of ovine satellite cells (30). Differentiated cells display increased expression of the GLUT4 glucose transporter (9,11,12) and this gene is controlled by MEF2 in C2C12 myotubes (35). It is of interest that increased GLUT4 expression greatly improved the ability of insulin-resistant db/db mice to moderate glucose homeostasis (36). We saw a substantial impact of culture in LG on the ability of
C2C12 cells to differentiate, despite the inclusion of insulin in our differentiation media. The inclusion of insulin is of note, as stimulation with insulin is capable of increasing GLUT4 in C2C12 myotubes (37). In addition, incubation with insulin is sufficient to induce myogenesis in C2C12 myoblasts (38,39). However, in our system, it appears that LG negatively impacts the ability of cells to undergo appropriate myogenesis, even in the presence of insulin. Our results conclude that HG media is necessary for normal proliferation, migration, and terminal differentiation of C2C12 myoblasts.

Finally, we demonstrate that JAK2 is activated in response to HG in skeletal muscle myoblasts. JAK2 has been demonstrated to be activated in both mesangial cells and VSMCs in response to HG (21-24). In addition, HG stimulates association of JAK2 with the angiotensin II (Ang-II) AT_1 receptor (23). These results have suggested that HG increases the activation of JAK2 by Ang-II and this action may mediate cell growth of mesangial cells (21). In addition, JAK2 appears to regulate synthesis of TGF-β and fibronectin when stimulated by HG (22). We found that JAK2 is activated in response to stimulation with HG in skeletal muscle myoblasts. In addition, we observed a localization of activated JAK2 protein in the nuclear/perinuclear region of the myoblast in response to stimulation with HG. This response is intriguing, because we have previously observed a similar pattern of activated JAK2 when cells are cyclically stretched (40). These results suggest that JAK2 activation appears to have a similar cellular localization in response to different stimuli. Our previous research has also indicated a role for JAK2 in normal proliferation and migration of C2C12 myoblasts (41). These results are of particular
interest as all of our previous experiments were conducted in HG DMEM, the recommended base media for culture of C2C12 cells. These results demonstrate that JAK2 is activated in response to HG and suggest a larger role for this kinase in regulation of the cellular response to glucose in skeletal muscle.

Finally, we observed an intriguing response of STAT proteins to high glucose. Previous work has indicated that STAT proteins are activated in response to HG (21-23,25). Our results indicated that STAT1 and STAT3 appear to increase nuclear localization in response to HG stimulation. However, we did not see a HG-stimulated increase in STAT phosphorylation for either of these proteins. We observed no response of STAT5 in either assay (data not shown) despite an earlier study identifying an activation of STAT5 in response to glucose in the skeletal muscle of fasted mice (25) and in mesangial cells treated with HG (21,22). Previously, STAT1 and STAT3 were identified as activated by HG in mesangial cells (21-23). It is possible that we did not stimulate the cells for a sufficient period of time, although these previous studies observed tyrosine phosphorylation of these proteins as early as five minutes following stimulation with HG (21,23). Thus, these results suggest a role for the STAT proteins in response to HG stimulation, but more research in skeletal muscle myoblasts is needed to clarify this potential function.

Taken together, the results of this study demonstrate the necessity for adequate glucose in the media of cultured skeletal muscle cells. They also demonstrate that JAK2, like in other cell types, is activated by HG in C2C12 myoblasts. These results also illustrate the care that must be taken when analyzing signaling proteins in cell culture, as choice of media can play a large role in the
stimulation of proteins being analyzed. This research demonstrates that JAK2, and potentially the JAK/STAT pathway, is involved in the response of cultured skeletal muscle cells to high glucose. However, future work is needed to elucidate the precise mechanism by which this important kinase functions in this system.

REFERENCES


**FIGURE LEGENDS**

Figure 1. Low glucose media inhibits proliferation and terminal differentiation of C2C12 cells. (A) Cells were plated in low glucose media and then incubated with in high glucose (HG; 25 mM) or low glucose (LG; 5.5 mM) DMEM growth media that contained BrdU (10 μM) and dCTP (10 μM) for the final 12 hours of culture. The percentage of proliferated cells was determined by dividing the number of FITC-positive nuclei by the total number of nuclei identified by HO33258. ****Significantly
different \( (p \leq 0.00001) \). (B) Cells were plated in low glucose media and then incubated with in high glucose (HG; 25 mM) or low glucose (LG; 5.5 mM) DMEM restricted serum differentiation media for 72 hours. Creatine kinase (CK) units were determined as described by the manufacturer's protocol (Sigma) and standardized to total protein in nanograms. \( **** \)Significantly different \( (p \leq 0.00001) \).

Figure 2. Low glucose media inhibits migration of C2C12 myoblasts. (A) Cells were plated in low glucose media and then incubated with in high glucose (HG; 25 mM) or low glucose (LG; 5.5 mM) DMEM growth media for eight hours after wounding. After eight hours of treatment the percentage of the wound covered by migrating cells was determined from photographs. Cells incubated in low glucose had impaired migration \( (p \leq 0.00001) \) as compared to cells cultured in high glucose media. (B) Cells were photographed at the start of the time course (T0) and eight hours after wounding (T8).

Figure 3. Treatment of C2C12 myoblasts with high glucose activates JAK2. (A) Cells were plated in low glucose media and then stimulated with in high glucose (HG; 25 mM) DMEM growth media for five and fifteen minutes. Following incubation in HG media, cells were fixed and detected as described in materials and methods. Phospho-JAK2 \( (p\text{JAK2}) \) was detected using p JAK2\(^{1007/1008} \) antibody. (B) JAK2 was immunoprecipitated from cell extracts of cells stimulated with HG for five and fifteen minutes. Immunoprecipitates were detected using phospho-tyrosine (pTyr)
4G10 antibody and primary HR-758 JAK2 antibody as described in materials and methods.

Figure 4. High glucose affects nuclear translocation of STAT1 and STAT3 (A and B). Myoblasts were plated in low glucose media and then stimulated with 25 mM DMEM growth media for five and fifteen minutes. Following stimulation, cells were fixed and total STAT1 (A) and STAT3 (B) were detected as described in materials and methods and nuclei were detected using HO33258 (HO).

Figure 5. High glucose does not affect phosphorylation of STAT proteins. Myoblasts were plated in low glucose media and then stimulated with in high glucose (HG; 25 mM) DMEM growth media for five and fifteen minutes. STAT1 and STAT3 were immunoprecipitated from cell extracts and immunoprecipitates were detected using phospho-tyrosine (pTyr) 4G10 antibody and primary STAT antibodies as described in materials and methods.
Figure Three

A

Control 5 minutes 15 minutes

B

High Glucose Stimulation

anti-pTyr

anti-JAK2
Figure Four

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Figure Five

Co 5m 15m  Co 5m 15m
pTyr
STAT
STAT1  STAT3
APPENDIX B. TNFA STIMULATION OF C2C12 MYOBLASTS RESULTS IN UNUSUAL CELLULAR LOCALIZATION OF JAK2

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ABSTRACT

In this experiment we sought to determine if the tyrosine kinase, JAK2, is activated in response to tumor necrosis factor α (TNFα) stimulation in C2C12 myoblasts. TNFα is an inflammatory cytokine primarily associated with muscle wasting in skeletal muscle, although recent work has identified roles for TNFα in myoblast proliferation and myogenesis. In response to short periods of TNFα stimulation in myoblasts, activated JAK2 accumulates at the cell membrane in a bright, punctuate pattern, similar to localization of focal adhesions. These results suggest that JAK2 is responsive to TNFα in C2C12 myoblasts.

INTRODUCTION

Tumor necrosis factor α (TNFα) is a potent inflammatory cytokine primarily associated with muscle wasting in skeletal muscle (1,2). In skeletal myoblasts, TNFα induces apoptosis and inhibits differentiation at high levels (20 ng/mL) (3).
However, TNFα stimulates differentiation in myoblasts when applied at levels comparative to those observed in normal serum (1-6 ng/mL) and TNFα expression increases in response to restricted serum in C2C12 myoblasts (4). Low levels of TNFα are also sufficient to stimulate proliferation of skeletal muscle satellite cells. However, the role of TNFα in myogenesis is still controversial, as a recent study observed that moderate levels (10 ng/mL) of TNFα inhibits myogenesis through inhibition of MyoD (5).

Janus kinase 2 (JAK2) is a protein tyrosine kinase that is traditionally associated with cytokine receptors lacking intrinsic kinase ability. Activation of JAK2 by cytokine binding initiates a phosphorylation cascade resulting in dimerization and nuclear translocation of the signal transducer and activator of transcription (STAT) proteins (6). JAK2 has previously been described as associated with the type 1 TNF receptor (TNFR1) and is activated in response to TNFα signaling in adipocytes (7). JAK2 is also implicated as being involved in nitric oxide synthase (NOS) induction by TNFα (8). However, to date, no studies have examined a role for JAK2 in TNFα signaling in skeletal muscle. In this report, we describe an unusual localization of activated JAK2 in response to TNFα signaling in C2C12 myoblasts.

MATERIALS AND METHODS

C2C12 skeletal muscle myoblasts (American Type Culture Collection, Manassas, VA) were cultured in a humidified atmosphere of 5% CO₂ at 37°C. Cells were grown in DMEM (Atlanta Biologicals, Lawrenceville, GA) containing 10% fetal bovine serum
(FBS; Gibco-BRL Life Technology, Carlsbad, California), 1.0 mg/mL penicillin/streptomycin (Gibco-BRL Life Technology, Carlsbad, California) and 0.3 mg/ml L-glutamine (Gibco-BRL Life Technology, Carlsbad, California). Myoblasts were plated at 1250 cells/cm$^2$ onto glass coverslips coated with bovine fibronectin (Sigma, St. Louis, MO) in 12-well plates.

Following stimulation with 5 ng/ml TNFα (Boehringer Mannheim, Indianapolis, IN), cells were rinsed with phosphate buffered saline containing sodium orthovanadate (PBS-V; 1 mM Na$_3$VO$_4$) and fixed for 30 minutes (1% formaldehyde and 0.5% sucrose in PBS). After fixation, cells were rinsed with PBS-V and incubated with 1% IGEPAL (Sigma, St. Louis, MO) for five minutes. Cells were incubated in blocker for 20 minutes (5% goat serum, 0.4% BSA, and 0.2% Triton X-100 in PBS) before overnight incubation in PBS with the appropriate antibodies. The primary phospho-antibody used for JAK2 was pJAK2$^{1007/1008}$ (Biosource, Camarillo, California) at a 1:500 dilution. Following incubation with the primary antibody, cells were rinsed and incubated overnight with a 1:500 dilution of anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Coverslips were mounted on VectraShield media (Vector Lab, Burlingame, CA) and sealed with clear nail polish. Mounted slips were visualized and photographed using a Leica DMIRE2 microscope (Leica, Bannockburn, IL) and OpenLab software (Improvision Inc., Lexington, MA).

RESULTS AND DISCUSSION
In order to identify if TNFα activates JAK2 in C2C12 myoblasts, cells were stimulated with 5 ng/ml of TNFα for five and fifteen minutes. To our surprise, we observed a very unusual phenotype of activated JAK2 in response to TNFα. In previous work, we identified that phosphorylated JAK2 (pJAK2) accumulates in the nuclear/perinuclear domain when activated (9,10). Instead, TNFα appears to stimulate pJAK2 localization at the cell membrane (Figure 1A). This result is much more in line with a traditional function of JAK2 at the level of the cytokine receptor. It also suggests a role for JAK2 in binding to TNFRs, as JAK2 appeared in a bright, punctuate pattern along the length of the cell membrane. However, we have also observed the nuclear/perinuclear localization of JAK2 in response to the IL-6-like cytokine, LIF and FGF (unpublished results). Combined, these results indicate that the response of JAK2 to TNFα stimulation is unique and not simply indicative of a cytokine-mediated effect. A similar patterning has also been observed with stimulation with IGF-1 (unpublished results). Treatment with TNFα also causes pJAK2 to be visible in a pattern similar to localization of phosphotyrosine proteins in focal adhesions (Figure 1B). This response could be indicative of a novel signaling function. Clearly, more work is required to identify a potential requirement for JAK2 in TNFα signaling and to explore the function of this unusual patterning of JAK2.

FIGURE LEGEND

TNFα stimulation of C2C12 myoblasts results in unusual cellular localization of JAK2. (A) Following stimulation with 5 ng/ml TNFα for 5 (5min) or 15 minutes
(15min), cells were fixed and detected as described in materials and methods. Phospho-JAK2 (pJAK2) was detected using pJAK21007/1008 antibody. Stimulation with TNFα induces JAK2 localization at the cell membrane and in a linear, regular pattern in the apparent cytoplasm. (B) Enlarged photographs of cells stimulated with TNFα for 5 (5min) or 15 minutes (15min).

REFERENCES

Figure One

A

Control | 5 minutes | 15 minutes

pJAK2

B

pJAK2 | pJAK2

5 min | 15 min
APPENDIX C. PROTOCOL FOR DETECTING IMMUNOFLUORESCENCE USING BIOFLEX PLATES

1. Plate cells on bioflex membranes that are either purchased pre-coated with a suitable surface or coated prior to use.

2. Stretch cells as indicated by protocol.
   *Note: If detecting cells with immunofluorescence it is VERY important to not use the posts supplied with older Flexcell systems (3000 and earlier). To use the posts properly they must be coated with silicone which greatly interferes with visualization of fluorescence. Instead, stretch the plates without using the posts.

3. Gently aspirate (or pipet) off culture media and rinse cultures 1-2x in 0.1M PO₄ Buffer (PBS) for one minute each rinse.

4. Fix cells (see fixative recipe below) for 30-40 minutes.

5. Gently rinse cultures 2x in PBS, five minutes each rinse. It is very important all the formaldehyde is removed.

6. Incubate with 1% IGEPAL in PBS for 5 minutes at RT. Aspirate.

7. Incubate with blocker (see recipe below) for 20 minutes at RT.

8. Prepare 1° antibodies diluted in PBS.
   ***To save ab/phalloidin: Place droplets (30-50 ul) of ab dilution on parafilm lined culture dish (10 or 20 cm) spaced accordingly. Carefully remove coverslips from wells and place (cell side down) on respective droplets. To maintain humidity, line the lid of the dish with wetted Kimwipe.***

9. To remove the silastic membrane from the plate:
a. First start with a sharp, new razorblade (the sharpness of the blade is critically important).

b. Remove blocker ONLY from the well you are currently working on. Do not aspirate all the wells at once as this will cause them to dry out. Aspirate one well at a time, just before you start to remove it.

c. Cut an approximately round portion of the membrane off. Use as much of the well as possible, typically ~70-80%.

d. Use dissecting scissors to snip off the last piece of membrane still attached. Be very carefully not to overly stretch the membrane when removing it. Use a pair of forceps to support the membrane when you cut it out.

e. Dip the membrane once in PBS (in beaker), blot edge on a clean Kimwipe and then place, cell side down, on the prepared primary antibody.

10. Incubate for 6-8 hours in the primary antibody at RT or overnight at 4°C.

11. Prepare secondary antibody diluted in PBS, with or without 0.2% Triton.

   ***Follow above procedure to save antibody, and be sure to cover dish with a light blocking cover (cardboard or aluminum foil covered lid)***

12. Remove membranes from humidity dish and gently rinse by dipping in PBS (in beaker). Blot edge of membrane gently on a kimwipe.

13. Incubate for 90 minutes in the 2° antibody at RT to o/n at 4°C. Excessive incubation times in the 2° antibody may increase background fluorescence.

14. Remove the membrane from humidity dish, and gently rinse by dipping twice in PBS (in beaker). Blot edge on kimwipe to remove excess PBS.
15. After blotting, dip twice into ddH$_2$O water (in beaker), blot slip edge on a kimwipe, and then trim membrane, using dissecting scissors so it is approximately the same size as a round glass coverslip (See figure one).

16. Gently place membrane (cell side down) onto Vectashield droplet (3-4 ul) placed on a microscope slide.

17. Place an additional droplet of Vectashield on top of the membrane (non-cell side) and drop a round coverslip on top of the membrane. This will sandwich the membrane in glass to aid visualization.

18. Seal edges of slip AND membrane by rimming lightly with clear nail polish. Let dry for ~one minute.

19. Store slides in a dark place (e.g. a slide box) at 4°C until visualization.

**EQUIPMENT**

*Forceps*
*New razor blades*
*Dissecting scissors*
*Parafilm*
*20 cm culture dish*
*Clear nail polish*
*Slides*
*Cardboard or aluminum foil cover for dark steps*
*Kimwipes*
*Two small beakers (~50 mls)*
*Slide box/folder*

**SOLUTIONS**

**4% Paraformaldehyde in 0.1M PO$_4$ buffer (for use in below sol'n)**
Paraformaldehyde  4 g
0.1 M PO₄ buffer  100 mL
(or add 10.8 mls 37% solution to 89.2 mls PBS for 100 ml of solution)

I.  *Store at 4°C*

**1% Paraformaldehyde, 0.5% Sucrose in 0.1 M PO₄ (Fixative)**

- Sucrose 0.25 g
- 4% Paraformaldehyde 12.5 ml
- 0.1 M PO₄ buffer 37.5 ml

**Blocking solution-can also be used to dilute the primary antibody**

5% goat serum, 0.4% BSA in 0.2% Triton X-100 (diluted in PBS).

*Add Triton before the BSA*

Centrifuge this solution prior to use at 14,000xg for five minutes at RT.

Do not use Triton if you wish to maintain the integrity of the tissue.

**1% IGEPAL in PBS**

**0.1M PBS**

**ddH₂O**

---

**FIGURE ONE**

- Vectashield
- Cells
- Bioflex membrane
- Coverslip
- Slide
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First, I would like to thank my outstanding major professor, Jim Reecy. He made me the scientist I am by challenging me daily, inspiring me always, and humoring my stubborn need to do things "my way." Thank you for giving me the freedom to take my research any way I dreamt it. I've had a fabulous graduate experience.

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