An Expanding Role for Nuclear Factor κB in Muscle Stem Cells: Implications for the Treatment of Duchenne Muscular Dystrophy

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Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

Of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2013

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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AN EXPANDING ROLE FOR NUCLEAR FACTOR-KB IN MUSCLE STEM CELLS: IMPLICATIONS FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is a fatal disease characterized by progressive skeletal muscle degeneration. Inhibition of the transcription factor nuclear factor-κ B (NF-κB), and more specifically the p65 subunit, significantly improves the phenotype of *mdx* mice, a murine DMD model. However, the ubiquity of NF-κB stands as an obstacle to clinical translation. In this dissertation, we explore the roles of NF-κB/p65 in the regenerative capacity of muscle-derived stem cells (MDSCs) with the goal of identifying alternative approaches to DMD treatment. We found that both cell proliferation and myogenic potential were increased in MDSCs lacking one allele of p65 ($p65^{+/-}$). In wild type MDSCs, *in vitro* pharmacologic inhibition of the upstream activating kinase, IKKβ, increased myotube formation in a dose-dependent manner. When transplanted into *mdx* hind limb muscle, $p65^{+/-}$ MDSCs resulted in significantly larger engraftments. Furthermore, engraftments in cardiotoxin (CTX) injured muscle were associated with reduced local host necrosis and inflammation. Not only were $p65^{+/-}$ MDSCs found to be more resistant to oxidative stress, but we found that *p65* depletion improved the anti-inflammatory capacity of MDSCs *in vitro* and *in vivo* via upregulation of hepatocyte growth factor (HGF). Moreover, accelerated regeneration in *p65* haploinsufficient *mdx* mice ($mdx;p65^{+/-}$) coincided with HGF upregulation. Intraperitoneal injection of a musculotropic adeno-associated virus carrying shRNA targeting HGF reversed the phenotypic improvements of $mdx;p65^{+/-}$ mice, increasing both muscle inflammation and necrosis. These data implicate NF-

κB/p65 in muscle stem cell proliferation, differentiation, survival, and growth factor gene expression, further underlining the danger of broadly targeting such an important pathway. Finally, this research has also identified HGF as a downstream effector of NF-κB/p65 inhibition in *mdx* mice. Thus, delivery of HGF or activation of its receptor, MET, may represent a new approach to reduce chronic inflammation and preserve muscle fiber integrity in DMD.

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ACKNOWLEDGEMENTS

There are a great many people I would like to acknowledge for helping me complete this work. First, I would not have reached this point without my great mentors. Thank you to my primary advisor and mentor, Dr. Johnny Huard, for supporting me in the pursuit of my research and career goals, for teaching me how to be an independent thinker, for providing me with the resources to complete my work, and for believing in me. I want to make a special thank you also to Dr. Bing Wang for his guidance in matters of both science and life. I am very grateful for the time he took to help me design better experiments, and especially for all the time he spent in this last year making it possible for me to finish up one of the last, yet most important, experiments.

I am very thankful to my committee for their support, direction, and constructive criticism that challenged me to become a better scientist. Dr. Wendy Mars has been a great committee chair, and I am grateful for the guidance she has provided to me both as a committee member, program director, and mentor. Thank you to Dr. Jon Piganelli, for his enthusiasm and support, Dr. Paul Robbins for keeping me on track, Dr. Laura Niedernhofer for encouraging me to look at things from many different angles, and Dr. Stephen Strom, who before his move, challenged me to become a more thorough scientific researcher. And lastly, Dr. Donna Stolz, for her enthusiasm, insight, and support despite being such a late addition to my committee.

Here at the University of Pittsburgh, I am proud to have struggled alongside my fellow classmates. During graduate school I have developed lasting relationships with my fellow

students as both colleagues and friends. I am enormously grateful to the other graduate students from the Huard lab: Laurie and Lauren early on, Sarah, William, and most recently, Jihee. Jihee has listened to my trials and tribulations with abundant patience, and I have really appreciated it. Thank you to William for some really great scientific discussions and helping me to think in different directions than I would have otherwise. And most of all, Sarah, who was essentially my "student role model" throughout most of my time in the lab. She was always there for me, offering both emotional and scientific support. In many times of struggle, it was her advice that got me through.

Aside from the other students, I am tremendously grateful to all my lab mates. When I first joined the lab, Aiping was the one who taught me so many new techniques and helped me pick up a project and get work started. Throughout my stay, she was an invaluable asset and colleague. I am also grateful to Ying; a lot of what I have accomplished would not have been possible without her hard work and support. She never once hesitated to go out of her way to lend a helping hand. Also, Xuequin, who joined the lab around the same time as me, and I thank her for fruitful discussions and personal support, and Minakshi, who always believed in me, encouraged me to push through any obstacles I encountered, and who just made the lab a brighter place. I am also very thankful to the awesome and hardworking students I've had during my time here: Kayla and Timothy, they were a tremendous help to me. There are so many others in the lab who have helped get to where I am today that its hard to name them all, but I lastly want to thank Michelle, Jessica, Nick, and Seth, for keeping things in order and always coming to my aide when needed.

I also cannot fail to thank the professors who shaped my early scientific endeavors at Wheeling Jesuit University: Dr. Mary Railing, Dr. Ken Rastall, Dr. Rob Shurina; and at WVU,

Dr. Peter Gannett. A special thanks to Dr. Shurina, for teaching me how to think critically (both in and out of the box), and his continued willingness to be my mentor and offer candid advice.

Perhaps most importantly, were it not for some of the amazing (non-scientist) people in my life I do not think I would have reached this point. I have been blessed to have friends that I know are there with me through the good and bad. But most of all, I am tremendously thankful to my family, especially my parents, grandparents, and sister, who have been a source of tremendous strength and support, and even my infant nephew, Luca, just for reminding me about what is really important. My parents have especially played a tremendous role in my education; from the earliest years I can remember, they encouraged my curiosity and taught me to always make my own decisions and not rely on the opinions of others. Throughout my life, I cannot even recall one time where my parents have discouraged me from pursuing my dreams. Even when I doubted myself the most, their encouragement kept me going.

1.0 INTRODUCTION

1.1 Duchenne Muscular Dystrophy

Muscular dystrophy encompasses about 40 different genetic disorders characterized by ongoing skeletal muscle degeneration, functional decline, and wasting (Bogdanovich, Perkins et al. 2004). The most severe form, Duchenne muscular dystrophy (DMD) results from frameshift mutations in the dystrophin gene, whose protein product is a critical component of the membrane-associated dystrophin-glycoprotein complex (Hoffman, Brown et al. 1987). Located on the X chromosome, it is particularly prone to deletions and point mutations due to its large size (2.4Mb, ~1% of the X-chromosome). Dystrophin itself is a 427 kD protein with four basic structural domains: (a) an N-terminal domain, through which it binds actin, (b) a central rod domain (c) a C-terminal cysteine rich domain, (d) and a distal C-terminus. Together with dystroglycan, sarcoglycan, and syntrophin/dystrobrevin, dystrophin serves as the structural connection between the actin cytoskeleton and the extracellular matrix, allowing for the transmission of external forces. Dystrophin deficiency results in the loss of membrane integrity and subsequently leads to degeneration (Pichavant, Aartsma-Rus et al. 2011). In the early years of life, only a mild pathology is observed, likely due to sufficient regeneration mediated by muscle stem cells (Webster and Blau 1990). Eventually however, the stem cell pool becomes exhausted, resulting in the replacement of functional skeletal muscle with fibrosis and adipose

tissue. Excessive degeneration of striated muscle results in kyphosis and scoliosis of the spine, and by early adolescence loss of ambulation occurs in approximately 95 percent of patients, confining them to a wheelchair. By the age of 20, about 90 percent of patients die due to respiratory or cardiovascular failure (Tiidus 2008).

There is no FDA approved treatment for chronic muscle wasting diseases such as DMD. Rather, current clinical therapies focus on disease management to improve patient quality of life. Off label prescription of corticosteroids, such as prednisone and deflazacort, is the current gold standard for DMD (McNeil, Davis et al. 2010). The mechanism of action is not fully defined, however several possibilities have been proposed, including the suppression of inflammation and altered muscle anabolic metabolism (Rifai, Welle et al. 1995). Corticosteroid use has had a tremendous impact on patient quality of life, delaying wheelchair use by up to 5 years. At the same time, use of corticosteroids for more than 2 years, the length of time necessary for clinical benefit, results in numerous side effects, including growth retardation, eye cataract development, weight gain, and skin fragility (Biggar, Harris et al. 2006).

The most common animal model of DMD is the "X-linked muscular dystrophy," or *mdx,* mouse. Due to a naturally occurring mutation in exon 23 of the dystrophin gene, *mdx* mice do not express dystrophin. Skeletal muscle degeneration begins to occur at approximately three weeks of age, resulting in necrosis and mononuclear cell infiltration. This degenerative phase is quickly followed up by robust skeletal muscle regeneration around 5-6 weeks. Although degeneration/regeneration cycles continue throughout the *mdx* lifespan, which is only slightly reduced compared to wild type mice, the severity of the dystrophic phenotype becomes mild around 12 weeks of age for incompletely understood reasons. Eventually, around 18 months, the regenerative potential of *mdx* limb skeletal muscle declines, resulting in fibrosis and atrophy

(Pastoret and Sebille 1995). Although the slow development of dystrophic pathology in the *mdx* mouse may be disadvantageous in that it does not accurately reproduce the natural history of DMD, the dystrophic phenotype demonstrated during 3-12 weeks of age indeed mimics early DMD pathophysiology (Figure 1). Interestingly, the severity of the dystrophic phenotype appears to vary by muscle group. The diaphragm, for example, does demonstrate a progressive decline in regeneration accompanied with extensive endomysial fibrosis and adipose deposition (Stedman, Sweeney et al. 1991; Muller, Vayssiere et al. 2001). *Mdx* mice begin to exhibit

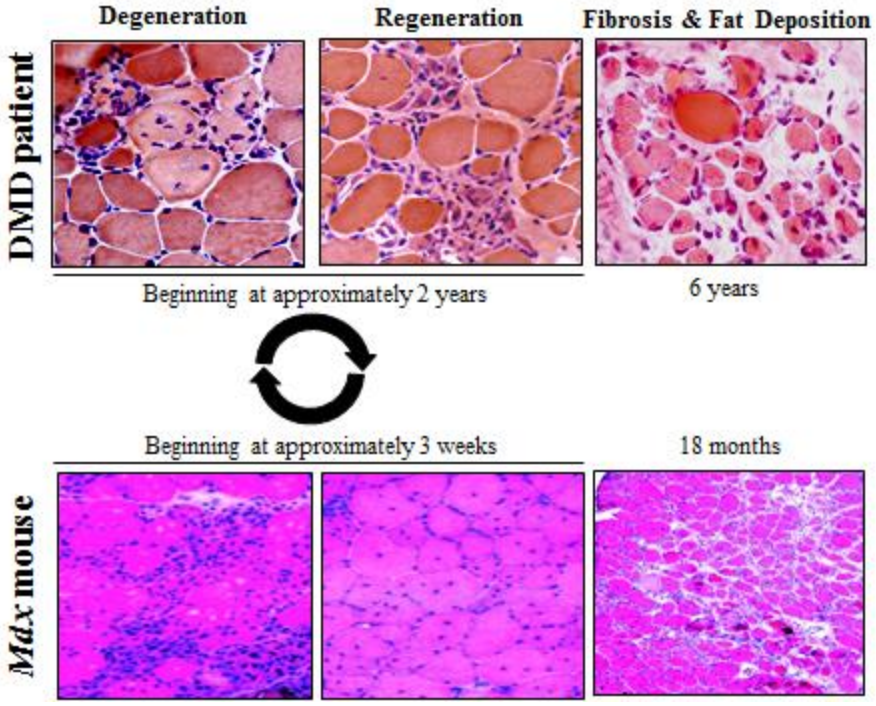

Figure 1. Comparison of DMD and *mdx* limb histopathology.

Following the onset of initial degeneration, both DMD patients and *mdx* mice undergo repeated degeneration/regeneration cycles. Eventually this results in progressive decline of regeneration and increased fibrotic and fatty tissue deposition. (DMD and *mdx* images adapted from http://neuromuscular.wustl.edu/pathol/dmdpath.htm and (Deconinck, Rafael et al. 1997)).

respiratory decline at approximately 3 months of age. By 14 weeks of age, the specific force generated by *mdx* diaphragm declines by 50 percent compared to wild type and exhibits increased susceptibility to fatigue. In human patients, respiratory failure is one of the major causes of death. Although not fully elucidated, evidence suggests that diaphragm dysfunction may contribute to the reduced lifespan of *mdx* mice (Chamberlain, Metzger et al. 2007; Huang, Cheng et al. 2011; Partridge 2013).

The *mdx* mouse is the common DMD mouse model, but in order to more closely represent the clinical progression of DMD, additional mouse models have been developed. During fetal development, the dystrophin-related protein utrophin localizes to the sarcolemma. Post-natally, utrophin is replaced by dystrophin except at myotendinous and neuromuscular junctions. In adult *mdx* mice, however, utrophin is again expressed at the muscle membrane, suggest it may play a compensatory role in mice(Matsumura, Ervasti et al. 1992). Thus, Deconinck and colleagues (1997) developed the utrophin/dystrophin double-knock out mouse (*mdx;utrophin*$^{-/-}$; *dKO*). Compared to *mdx* mice, the *dKO* mouse demonstrates degeneration of the diaphragm by 6 days of age, and by 6 weeks shows overt pathologies including decreased mobility, respiratory decline, kyphosis, and muscle weakness (Deconinck, Rafael et al. 1997). *dKO* mice have been particularly useful for studying DMD-related cardiomyopathy, which occurs in humans but not *mdx* mice (Chun, O'Brien et al. 2012). Additionally, mice heterozygous for the utrophin gene (*mdx;utrn*$^{+/-}$*)* have also been used for research. They have a longer lifespan than *dKO* mice, but still exhibit signicantly more inflammation and fibrosis than *mdx* mice(Zhou, Rafael-Fortney et al. 2008).

1.2 Skeletal Muscle Regeneration

1.2.1 Degeneration and Inflammation

The skeletal muscle repair process may be divided into four phases: degeneration, inflammation, regeneration, and remodeling/fibrosis. Although the early events of muscle injury vary, typically the injury is initiated by damage to cytoskeletal elements, leading to loss of membrane integrity. Once the membrane has been compromised, calcium (Ca^{2+}) homeostasis is disrupted, leading to its intracellular accumulation. Subsequently, Ca^{2+} influx results in protease activation (ie calpain) and necrosis of the myofiber (Tiidus 2008). This is rapidly followed by the migration of mononuclear cells, first neutrophils and then macrophages, which invade the tissue to clear necrotic debris and activate muscle stem cells. Neutrophils accumulate rapidly, often within an hour of injury. Although the exact identity of the chemoattractant molecules responsible for neutrophil accumulation during the inflammatory stage of regeneration is unknown, both muscle and endothelial cells are capable of producing inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor α (TNFα) (Nagaraju 2001). Acting as vasodilators, these factors stimulate edema and monocyte accumulation.

Macrophages have the primary roles of phagocytosis of cellular debris and the release of stimulatory cytokines (Chazaud, Sonnet et al. 2003). This role appears to be critical, as impaired macrophage migration leads to impaired regeneration (Bryer, Fantuzzi et al. 2008). Evidence suggests that two distinct macrophage phenotypes are involved with skeletal muscle repair. Macrophages with an inflammatory, or "M1" phenotype (CD68+Ly6C+CD163-CD206-), enter muscle to promote inflammation, clear debris, and activate muscle stem cells (Arnold, Henry et

al. 2007) . These cells then take on an anti-inflammatory (CD68-Ly6C-CD163+CD206+), or "M2", phenotype. *In vitro*, M1 macrophages stimulate myoblast proliferation, whereas M2 macrophages stimulate myoblast differentiation (Chazaud, Sonnet et al. 2003; Sonnet, Lafuste et al. 2006).

The role of inflammation in muscle repair is not always straightforward. In the case of chronic diseases, such as DMD, inflammation can impede, rather than promote, tissue repair. For example, knockout of inducible nitric oxide synthase (iNOS) reduces myofiber necrosis in *mdx* mice. Furthermore, it has been reported that pathological muscle necrosis can be improved up to 80% following macrophage depletion (Villalta, Nguyen et al. 2009; Villalta, Deng et al. 2011). There is also a significant improvement in dystrophic pathology in perforin and dystrophin double knockout mice, suggesting cytotoxic T-lymphocytes may also be involved (Spencer, Walsh et al. 1997). The role of inflammation in DMD will be discussed further in Section 1.4.2.

1.2.2 Satellite Cells and Regeneration

Skeletal muscle has a robust capacity for repair. Post-natal muscle regeneration is mediated by satellite cells (SC), a population of "professional" muscle stem cells. Other cell populations with myogenic potential have been isolated from skeletal muscle, such as side population cells, pericytes, and myogenic endothelial cells (Asakura, Seale et al. 2002; Zheng, Cao et al. 2007; Crisan, Yap et al. 2008); however, the importance of these for muscle homeostasis has yet to be fully elucidated. Normally quiescent in a niche located within the basal lamina but outside the muscle membrane, or sarcolemma, activated SC break quiescence and enter the cell cycle following injury. SC can be identified in muscle cross-sections by their

expression of paired box transcription factor 7 (Pax7), which is critical for the maintenance of the SC population (Araujo, Bonuccelli et al. 2013). In the mouse, there is no single membrane marker to identify SCs, so a combination of two or more can be used for isolation by fluorescence activated cell sorting (FACS), including CD34, integrin- α7, and m-cadherin, among others (Sacco, Doyonnas et al. 2008). In humans, there is not a reliable set of markers to isolate SCs, although in practice, CD56 is used to isolate muscle progenitor cells (Peault, Rudnicki et al. 2007).

As shown in Figure 2, proliferating SC form at least two distinct populations (Conboy and Rando 2002). So-called "reserve" SC continue to express Pax7 and eventually return to quiescence, while the remaining SC lose expression of Pax7 and begin to express members of the basic helix-loop-helix (bHLH) transcription factor family, including MyoD, which regulate muscle lineage progression (Zammit, Golding et al. 2004). These $Pax7^{-}MyoD^{+}$ muscle progenitor cells, or myoblasts, eventually fuse and form new muscle fibers, a process termed myogenesis. This stage can be identified histologically by central nucleation of muscle fibers in tissue sections. As the fibers mature, the nuclei move to the periphery and the regeneration process is completed (Peault, Rudnicki et al. 2007).

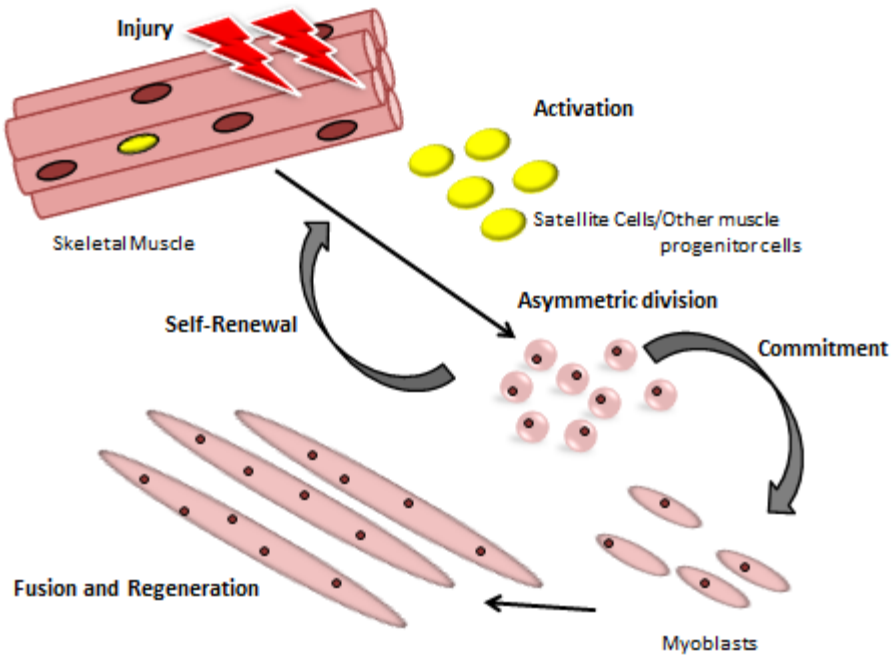

Figure 2. Skeletal muscle is regenerated through the activation of muscle stem cells

Disruption of skeletal muscle homeostasis by injury results in satellite cell activation. During population expansion, muscle stem cells undergo asymmetric division to produce myoblasts, committed muscle precursors, while a portion retains "stemness" in order to repopulate the niche. Following a proliferative phase, myoblasts eventually fuse to produce new muscle fibers.

1.2.3 Muscle-derived Stem Cells

Although the main source of progenitors for muscle regeneration is from the SC pool, there are several different populations of progenitor cells with myogenic potential in skeletal muscle. These include, but are not limited to, side population cells, mesoangioblasts, muscle-derived stem cells, PW1+ interstitial cells, and myogenic endothelial (myoendothelial) cells (Naldini, Weidner et al. 1991; Peault, Rudnicki et al. 2007). The developmental origin of these myogenic populations remains incompletely defined, and thus their relationship to the satellite cell compartment is unknown.

Pre-plating, a method used to isolate myogenic populations from skeletal muscle, separates muscle progenitors based on their adhesion properties to certain substrates, particularly collagen 1 and gelatin (Rando and Blau 1994). The most slowly-adhering fraction of cells (SACs), obtained by successive re-plating of non-adherent cells, was found to contain a population of stem and progenitor cells. SACs have been identified in mouse, rat, turkey, and human skeletal muscle, and have been given many different names, including muscle-derived stem cells (MDSC), primary muscle progenitor cells, and skeletal muscle $CD34^{+}/CD45^{-}$ cells (Gharaibeh, Lu et al. 2008). For our purposes, we will refer to murine SACs as MDSCs. Unlike satellite cells, MDSCs bear endothelial ($CD31^{+}CD144^{+}$), myogenic ($CD34^{+}$) and mesenchymal progenitor ($Sca\text{-}1^{+}$) cell surface markers. Compared to myoblasts, MDSCs demonstrate a remarkable intramuscular engraftment capacity in skeletal and cardiac muscle. (Jankowski, Haluszczak et al. 2001; Qu-Petersen, Deasy et al. 2002). In addition to skeletal muscle, donor murine MDSCs have been found to aid in the repair of recipient bone and cartilage defects, and improve heart function in murine models of acute myocardial infarction (Rose, Peng et al. 2003; Deasy, Li et al. 2004; Payne, Oshima et al. 2007).

Numerous investigations suggest that the increased regenerative potential of MDSCs relative to myoblasts is due, at least in part, to (1) a higher resistance to oxidative stress and by (2) the release of trophic factors, such as vascular endothelial growth factor (VEGF). Stress resistance is thought to be mediated by elevated levels of the endogenous antioxidants glutathione, aldehyde dehydrogenase, and superoxide dismutase (Urish, Vella et al. 2009; Drowley, Okada et al. 2010; Vella, Thompson et al. 2011). The identification of stress resistance as an important stem cell characteristic has also demonstrated by more recent studies in which muscle stem and progenitor cells were isolated based on their ability to survive long-term typsin

incubation induced stress(Shigemoto, Kuroda et al. 2013). Soluble factors, such as VEGF, have also been found to promote tissue repair (Payne, Oshima et al. 2007; Beckman, Chen et al. 2013). In this respect, MDSCs show similarities to mesenchymal stem cells, for which the therapeutic efficacy of stem cell-derived soluble factors is well supported (Nauta and Fibbe 2007; Caplan and Correa 2011).

It has been hypothesized that MDSCs may represent an early progenitor of the SC lineage due to their capacity for long term self-renewal *in vitro* (a stem cell characteristic) combined with an extensive myogenic differentiation potential (Deasy, Gharaibeh et al. 2005). Using a serial muscle injury model, a sub-population of SCs has been described to have the capacity of self-renewal; but these cells cannot undergo an extended period of culture *in vitro*, indicating their commitment to the muscle lineage (Peault, Rudnicki et al. 2007; Sacco, Doyonnas et al. 2008). Finally, in contrast to SCs, SACs, and prospectively MDSCs, can be isolated from Pax7 deficient mice, suggesting a post-natal origin that is "upstream" of the myogenic lineage (Seale, Sabourin et al. 2000; Lu, Cummins et al. 2008)

Slowly-adhering cells isolated from human skeletal muscle have also been found to display skeletal and cardiac muscle regeneration properties (Chirieleison, Feduska et al. 2011; Okada, Payne et al. 2011). Furthermore, clinical trials of human SAC-based therapies are currently underway for the treatment of urinary incontinence and ischemic heart failure (Carr, Steele et al. 2008; Health 2013). Additionally, Rouger and colleagues recently reported that systemic delivery of allogeneic wild type canine SACs to dystrophic dogs significantly improves disease phenotype. Donor cells were found to not only contribute to muscle regeneration, but they replenished the satellite cell niche, resulting in long-term dystrophin expression (Rouger, Larcher et al. 2011). Thus, MDSCs are particularly promising for use in cell therapies.

1.3 Nuclear Factor κB Transcription Factors

1.3.1 Classical NF-κB activation

NF-κB is a ubiquitously expressed transcription factor with key roles in immunity, development, cancer, and more recently, cell differentiation (Hacker and Karin 2006). In the past several years, NF-κB has been receiving substantial attention for its prominent role in skeletal muscle disorders, and in particular, muscular dystrophy. Rather than referring to a single protein, the name "NF-κB" refers to a group of proteins that share the presence of an N-terminal Rel homology domain (RHD). The NF-κB family includes 5 members: p65/RelA, c-Rel, RelB, p50 (processed from the precursor p105), and p52 (processed from the precursor p100). NF-κB proteins dimerize via their RHD and bind to specific sites in DNA, referred to as κB sites (Karin and Ben-Neriah 2000). Transcriptional activity of NF-κB is typically transient, and as such, is tightly regulated. Under normal conditions, NF-κB is sequestered in the cytoplasm by association with inhibitory κB (IκB) proteins, which mask the NF-κB nuclear localization sequence (NLS). IκBs form a group of proteins characterized by the presence of multiple ankyrin repeats, which mediate binding to NF-κB dimers (Li and Nabel 1997). The most well studied activation pathway, referred to as the classical or canonical pathway, is stimulated by inflammatory molecules such as TNFα and IL-1 and results in the nuclear translocation of a p65/p50 heterodimer. As shown in Figure 3, classical NF-κB depends on the activity of the trimeric IκB kinase (IKK) complex, which phosphorylates two sites on IκB proteins, targeting them for polyubiquitination and degradation via the 26s proteasome. The IKK complex includes 2 catalytic subunits, IKK-α and -β, and one regulatory subunit, the NF-κB essential modulator, abbreviated NEMO (also known as IKKγ), which is absolutely required for classical activation.

Although both IKKα and -β contain an N-terminal catalytic domain, the classical pathway is highly dependent on the activity of IKKβ (Solt, Madge et al. 2009).

There are numerous upstream activators of NF-κB, but two important protein families often associated with its activation are TNF receptor-associated factors (TRAFs) and receptor interacting proteins (RIPs). These are especially important for activation in response to TNFα, TLR ligands, and IL-1 (Karin and Lin 2002). Following receptor binding, intracellular adaptor proteins such as TNF receptor type 1-associated death domain protein (TRADD) and myeloid differentiation primary response gene 88 (MyD88) recruit TRAFs and RIPs which then interact with NEMO as a scaffold for IKK complex activation. In this way, numerous pathways converge on NF-κB (Hacker and Karin 2006).

1.3.2 Non-classical NF-κB activation

In contrast to the classical pathway, a second activation pathway referred to as the alternative or non-canonical pathway, results in the nuclear translocation of p52/RelB dimers and is independent of IKKβ and NEMO (Senftleben, Cao et al. 2001). As shown in Figure 3, in response to a small set of stimuli, including lymphotoxin-β (LTβ), CD40 ligand (CD40L), and B-cell activating factor (BAFF), TRAF2/5 is recruited to docking sites on the intracellular portion of the receptor, leading to the activation of NF-κB inducing kinase (NIK) (Hauer, Puschner et al. 2005). In turn, NIK activates an IKKα dimer which then positively regulates the processing of p100 to p52. Often p100 is bound in the cytoplasm to RelB, such that once processed, a p52/RelB dimer can translocate to the nucleus and induce gene transcription. At first, alternative activation was thought to be mostly restricted to B cell survival and differentiation, but its role

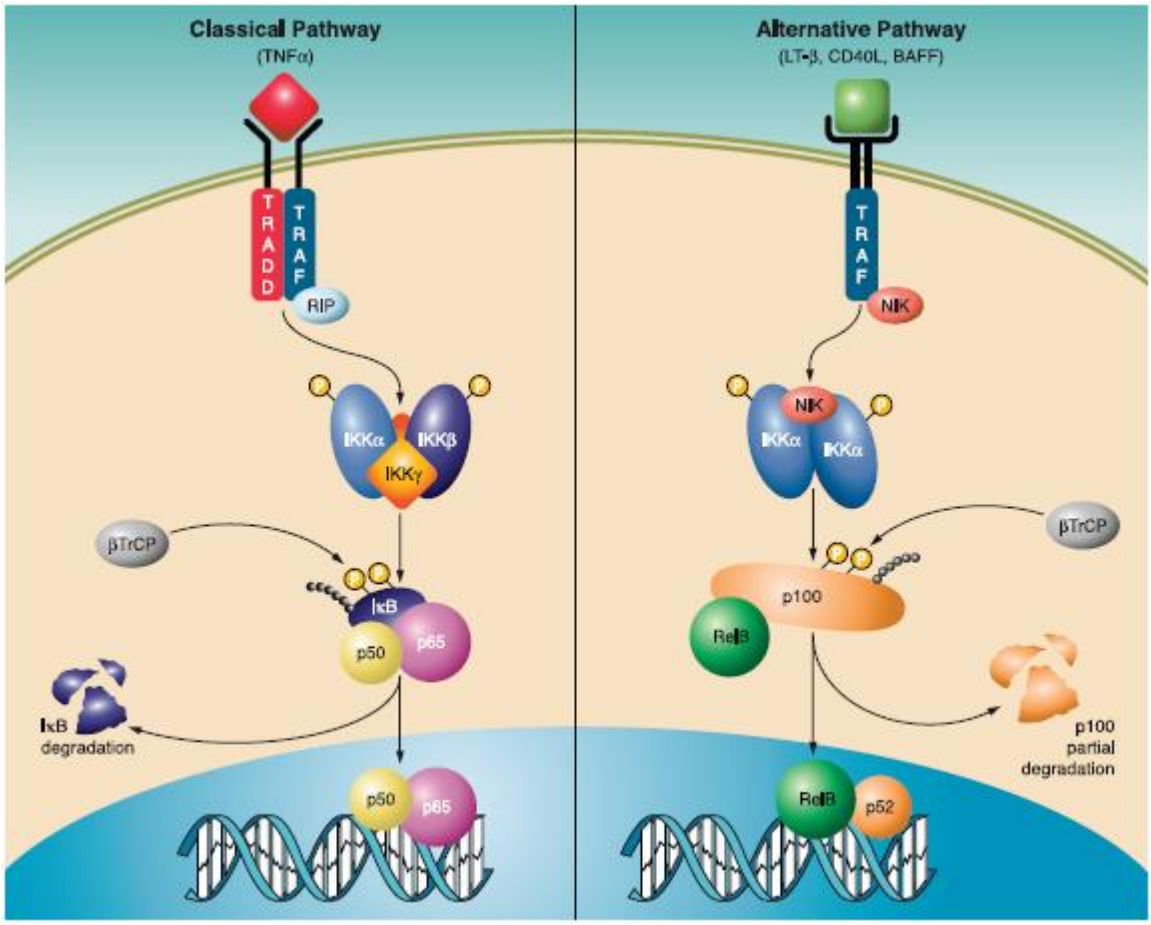

Figure 3. Activation Pathway of NF-κB Transcription Factors

Activation of NF-κB may proceed via the classical pathway or an alternative pathway. The classical requires IKKβ and IKKγ (NEMO) and results in p50/p65 DNA binding. In contrast, the alternative pathway requires IKKα and results in p52/RelB DNA binding. Image from (Bakkar and Guttridge 2010)

has expanded to include thymic organogenesis, secondary lymphoid tissue development, and skeletal muscle energy homeostasis (Dejardin 2006). For example, during myoblast differentiation, IKKα activity and p52/RelB transcriptional activity increases, resulting in increased mitochondrial biogenesis. Interestingly, this may be one mechanism accounting for the shift from glycolytic metabolism in myoblasts to oxidative phosphorylation in mature myotubes. (Bakkar, Wang et al. 2008). The role for non-canonical signaling is still expanding, and future

studies may identify novels role for this pathway in skeletal muscle development, regeneration disease, or aging.

1.3.3 Atypical NF-κB activation

Antioxidants, such as N-acetyl-cysteine, have been reported to suppress NF-κB activation, suggesting this transcription factor is redox sensitive. Indeed, NF-κB can be activated by oxidative stress. Reactive oxygen species (ROS) do not appear to induce NF-κB activity by either the classical or non-classical pathway, but by a heterogeneous collection of mechanisms collectively termed "atypical" (Gloire and Piette 2009). Antioxidant genes, including manganese superoxide dismutase and glutathione-s-transferase, are among NF-κB target genes; thus ROS-induced NF-κB activation has generally been considered to be pro-survival. (Pantano, Reynaert et al. 2006).

The mechanism of this type of signaling remains poorly understood, but is thought to occur by both indirect (ie. effect on redox-sensitive kinases) and direct mechanisms. For example, oxidation of "reactive cysteines" on either p50 or IKKβ can induce their activation. It is important to note however, that oxidation of amino acid residues on NF-κB and related proteins is not always activating. For example, oxidation of a methionine residue in IκBα has been reported to block NF-κB activation in response to TNFα. Similarly, certain anti-inflammatory molecules, such as the prostaglandin, PGA, were found to inactivate IKKβ by modifying cysteine residues (Rossi, Kapahi et al. 2000). Given the complexity of this system, and conflicting reports, it is likely that the role of oxidative stress in NF-κB activity is context and cell-type dependent.

1.4 NF-κB In Muscle Disease

1.4.1 NF-κB suppresses myogenesis

Summarized in Figure 4, classical NF-κB has been implicated in muscle lineage progression and may disrupt myogenesis by at least three mechanisms: induction and stabilization of cyclin D1, induction of the gene repressor YinYang1 (YY1), and destabilization of MyoD mRNA (Guttridge, Mayo et al. 2000; Wang, Hertlein et al. 2007; Dahlman, Wang et al. 2009).

Before myoblasts can undergo fusion and differentiation, cyclin D1 expression is down regulated, arresting cells in G-phase of the cell cycle. During proliferation, cyclin D1 functions to activate cyclin dependent kinases (CDK) 4 and 6, promoting the activity of the transcription factor E2F and allowing entry into S-phase. Several NF-κB binding sites have been found within the cyclin D1 promoter (Asakura, Seale et al. 2002). Although preferential binding is reported to be by a nonclassical p52/B-cell lymphoma 3-encoded protein (Bcl3) heterodimer, there appears to be some redundancy, as cyclin D1 transcription was not found to change in *p52*, *Bcl3*, *p50*, *c-Rel*, or *p65* null (-/-) mouse embryonic fibroblasts (MEFs). Although cyclin D1 mRNA levels were unaltered, $p65^{-/-}$ MEFs were found to have lower levels of cyclin D1 protein and CDK 4 activity. Dahlman and colleagues (2009) went on to determine that p65 is able to interact directly with cyclin D1 and promote its stability, evidenced by a significantly reduced cyclin D1 half-life in $p65^{-/-}$ cells (Guttridge, Albanese et al. 1999; Dahlman, Wang et al. 2009). More importantly, $p65^{-/-}$ primary myoblasts were found to more rapidly withdraw from the cell cycle compared to $p65^{+/+}$ myoblasts when placed under low serum conditions. Thus, the interaction between p65

and cyclin D1 likely promotes proliferation and prevents or delays cell cycle exit (Dahlman, Wang et al. 2009).

A negative role for NF-κB in myogenesis may continue once myoblasts have exited the cell cycle. The classical p65/p50 dimer induces the expression of YY1, a transcriptional repressor of a number of myogenic genes required for terminal differentiation, including muscle creatine kinase and myosin heavy chain IIb. At myofibrillary gene promoters, YY1 recruits members of the polycomb repressor complex to deacetylate histones and silence gene expression (Wang, Hertlein et al. 2007). In addition to myofibrillary gene suppression, YY1 has also been found to repress the promyogenic microRNA (miR), miR-29, itself a repressor of YY1 (Wang, Garzon et al. 2008). Furthermore, NF-κB, specifically p65, regulates myogenesis on the post-transcriptional level by binding to a destabilization element within the MyoD transcript (Guttridge, Mayo et al. 2000; Wang, Garzon et al. 2008). For these reasons, the halting of classical NF-κB activity is critical for myogenic differentiation. Declining NF-κB activity in turn lowers YY1 and increases MyoD at the same time. MyoD can then replace YY1 at myogenic gene promoters and recruit other activators, including CREB-binding protein (CBP)/p300 (Asakura, Seale et al. 2002; Wang, Garzon et al. 2008).

The physiologic role of NF-κB in regulating muscle progenitor activation, proliferation, and finally differentiation, is strongly supported by *in vivo* studies using transgenic mice. Conditional knockout of skeletal muscle IKKβ was found to result in elevated numbers of $CD34^{+}/Sca\text{-}1^{-}$ muscle progenitor cells, prospectively SCs (Acharyya, Villalta et al. 2007). A similar finding was reported by another group, who found that muscle-specific deletion of IKKβ increased the number of nuclei per regenerating fibers at 10 days post-injury, suggesting an increased contribution from the SC compartment (Mourkioti, Kratsios et al. 2006). As

differentiation occurs, classical NF-κB signaling is replaced by activation of the alternative pathway which plays a role regulating mitochondrial content and energy homeostasis.

Activation of classical NF-κB in post-mitotic myocytes may play a role in promoting skeletal muscle degeneration. For example, exposure of differentiated myotubes *in vitro* to the combination of TNFα and interferon gamma results in the loss of MyoD and the contractile protein MyHC in an NF-κB dependent manner, presumably through destabilization of MyoD transcript (Guttridge, Mayo et al. 2000). Although not conclusively demonstrated in *mdx* mice *in vivo*, the regulation of skeletal muscle homeostasis by NF-κB has been confirmed using transgenic mice expressing a constitutively active form of IKKβ in skeletal muscle. These mice displayed severe muscle atrophy accompanied by functional deficits (Cai, Frantz et al. 2004). Interestingly, an increase in the levels of inflammatory cytokines was not found. However, these mice did have increased expression of the muscle-specific E3 ubiquitin ligase muscle ring finger 1 (MuRF-1). This may be relevant during the later stages of muscular dystrophy, which are characterized by the development of extensive fibrosis and muscle wasting. It is important to note, however, that the development of fibrosis in the respiratory musculature of the *mdx* mouse has been reported to occur in an NF-κB *independent* manner. This conclusion was based on findings using the proteasome inhibitor Pyrollidine dithiocarbamate (PDTC) to inhibit NF-κB (Graham, Singh et al. 2010). While PDTC does decrease NF-κB activity by stabilizing IκB levels, it is not specific to the NF-κB pathway. Future studies using transgenic models or more specific small molecule inhibitors will be required to better understand what part, if any, NF-κB plays in the development of fibrosis.

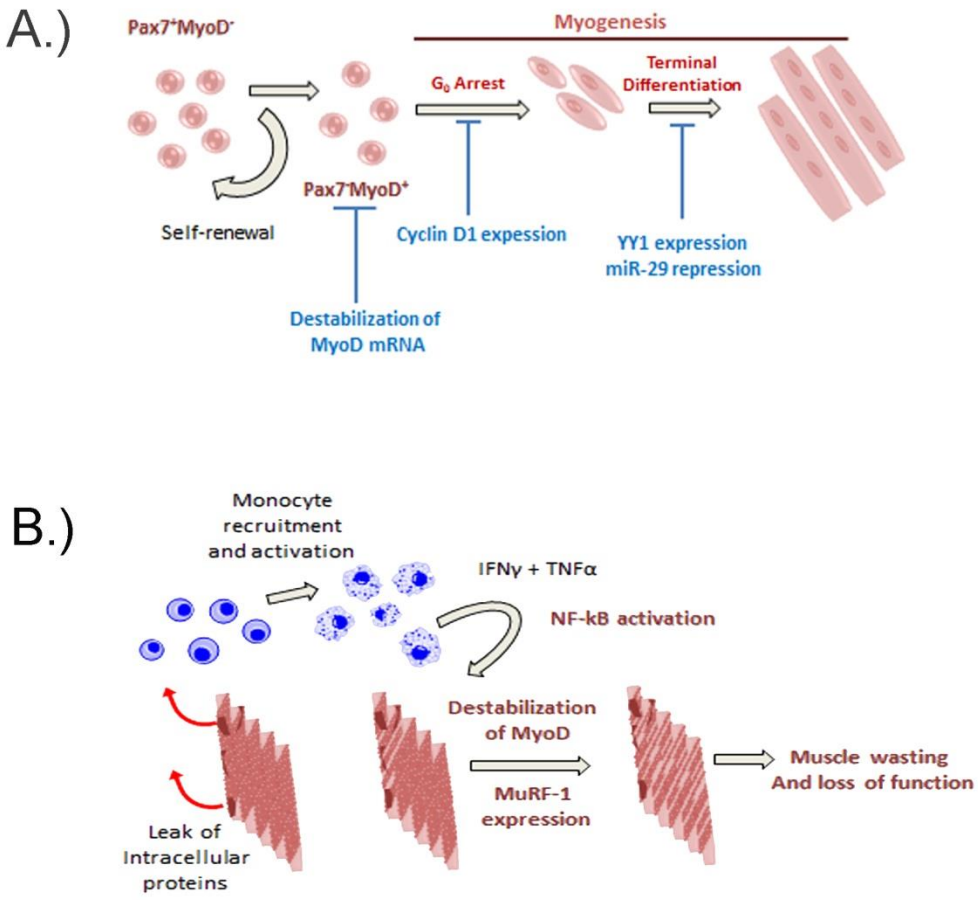

Figure 4. NF-κB negatively regulates post-natal myogenesis.

(A) A model of NF-κB mediated repression of post-natal myogenesis. Based on the evidence gathered from *in vitro* and *in vivo* studies, NF-κB may suppress myogenesis at multiple steps along lineage progression. Following the activation of Pax7+ muscle progenitor cells, NF-κB/p65 may delay differentiation by promoting cell cycle progression through p65-mediated stabilization of cyclin D1 and destabilization of the myogenic transcription factor MyoD. Later on, in myoblasts, NF-κB can induce transcription of YinYang1, a repressor of multiple myogenic genes including the terminal differentiation genes muscle creatine kinase and myosin heavy chain (MyHC). (B) Necrotic muscle recruits inflammatory immune cells that release tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), amongst other cytokines. In addition to the destabilization of MyoD transcripts, these factors promote muscle degeneration by upregulating MuRF1, a muscle specific E3 ubiquitin ligase, in an NF-κB dependent manner.

Although classical NF-κB plays a suppressive role during myogenic differentiation, it remains a critical transcription factor for tissue homeostasis, and a number of NF-κB target genes are important for cell survival. Target genes include the anti-apoptotic proteins XIAP and c-IAP family members, which inhibit caspase activity to block the extrinsic apoptotic pathway, as well as Bcl-2 family members, such as Bcl-xL, Bcl-2 and NR13, which exert an anti-apoptotic effect through disruption of the intrinsic (mitochondrial) apoptotic pathway(Karin and Lin 2002). Indeed, deletion of either *p65* or *IKKβ* results in embryonic lethality. A pro-survival role for NF-κB has also been identified in myoblasts, where it has been reported to induce antioxidant genes in response to oxidative stress (Catani, Savini et al. 2004). Therefore, in our discussion of the negative role of NF-κB in myogenesis, we must keep in mind that this transcription factor still serves an important role in skeletal muscle homeostasis.

1.4.2 NF-κB is dysregulated in DMD

NF-κB has been found to be persistently activated in both DMD patients and the *mdx* mouse (Monici, Aguennouz et al. 2003; Acharyya, Villalta et al. 2007). Of note, aberrant activity has been identified not only in immune cells, but in the muscle fibers themselves (Acharyya, Villalta et al. 2007). Initially this was believed to be in response to the elevated cytokine levels found in dystrophic muscle. However, the observation that *mdx* myotubes derived from primary myoblasts *in vitro* also show increased NF-κB activity, suggests that this explanation is oversimplified (Altamirano, Lopez et al. 2012). This finding has been disputed by others (Acharyya, Villalta et al. 2007), and the exact cause of upregulation remains unclear. Several different mechanisms have been proposed, including induction by mechanical stretch, in

response to elevated intracellular calcium concentrations, as a consequence of oxidative stress, and finally, changes in the expression levels of the NF-κB subunits themselves.

A number of NF-κB target genes are survival factors, such as B-cell lymphoma 2 (Bcl-2) and cellular inhibitor of apoptosis protein-1 (cIAP1). It is possible that NF-κB is activated in dystrophin deficient skeletal muscle fibers as a survival mechanism in response to mechanical stress (Dogra, Changotra et al. 2006). This is based on the observation that IKK is a downstream target of Akt, the activity of which is significantly higher in the diaphragm of *mdx* mice compared to normal mice. *Ex vivo*, passive mechanical stretch of *mdx* diaphragm is sufficient to induce increased Akt activity. Stretch of WT diaphragm also leads to an increase in Akt activity, but to a much lesser degree than in the *mdx* diaphragm. Furthermore, IKK activation following stretch could be inhibited using small molecule inhibition of phosphoinositide 3-kinase (PI3-K) (Dogra, Changotra et al. 2006). However, more studies will be required to identify the physiological importance of this finding.

Rather than PI3-K/Akt, another group attributes the initiation of NF-κB signalling as a response to excess intracellular calcium ($[Ca^{2+}]$) found in dystrophin deficient muscle (Altamirano, Lopez et al. 2012). The influx of extracellular calcium to damaged muscle fibers is a well described process. However, resting calcium levels are elevated in *mdx* myotubes *in vitro*, and culture in low calcium or calcium free solution did not lead to a complete normalization of resting [Ca2+]. Thus, dysregulation of calcium levels may also be due to leak from the sarcoplasmic reticulum, potentially by increased activity of the inositol triphosphate receptor (IP3R) (Altamirano, Lopez et al. 2012). The physiological significance of the dystrophic abnormality in calcium homeostasis is demonstrated by the improvement of *mdx* histopathology following the overexpression of sarcoplasmic reticulum Ca(2+) ATPase 1 (SERCA1) via adeno-

associated virus gene therapy (Goonasekera, Lam et al. 2011). In contrast to the previous hypothesis, in which PI3K/Akt/NF-κB activity plays a pro-survival role, this model of activation focuses on the participation of NF-κB in promoting calcium-driven necrosis by the induction of iNOS, which could potentially induce oxidative stress (Altamirano, Lopez et al. 2012). Thus, increased levels of nitric oxide radicals produced by iNOS activity could further activate redox sensitive NF-κB. Such a process could lead to a positive feedback loop maintaining the chronic NF-κB activation found in dystrophic muscle.

Indeed, oxidative stress may play a key role in both the pathogenesis of DMD and NF-κB activation, as markers of oxidative stress have been found in *mdx* mice and DMD patients. NF-κB induced by ROS leads to the upregulation of NF-κB target genes, many of which are inflammatory mediators. Indeed, treating *mdx* mice with the vitamin E analogue, IRFI-042, reduced serum levels of muscle creatine kinase, the presence of which is an indirect measurement of muscle fiber damage. Not only was the DNA binding activity of NF-κB reduced, but also the levels of TNFα (Messina, Altavilla et al. 2006). Similarly, others have reported TNFα reduction and decreased membrane permeability following treatment with the antioxidant N-acetylcysteine (Whitehead, Pham et al. 2008; de Senzi Moraes Pinto, Ferretti et al. 2013).

Finally, Singh and colleagues (2009) propose that increased NF-κB activity is not entirely due to increased stimulation, but to increases in the expression of NF-κB subunits (Singh, Millman et al. 2009). They made the observation that in addition to an increased fraction of phosphorylated IκBα, total levels of IκBα are increased. However, IκBα is among the first target genes induced by NF-κB, so this observation may be secondary to the elevated activity. Total levels of IKKβ were also found to be increased. Despite the increase in total protein, the fraction

of phosphorylated IKKβ was also significantly increased. The investigators point out that, with the exception of p65, NF-κB subunits are NF-κB target genes (Singh, Millman et al. 2009). Therefore, even in this model, some external stimulus would still be required to account for the initial activity of NF-κB, which could then be self-perpetuated.

It is likely that a combination of these mechanisms, in addition to other ones not yet identified, is responsible for the excessive NF-κB activity found in DMD. Common to all of these findings, however, was increased IKK activity, implicating the classical NF-κB pathway. Although there is some evidence for a positive role of NF-κB in preserving dystrophin deficient muscle fibers, the preponderance of evidence points to NF-κB as exacerbating the pathologies of DMD.

1.4.3 Blockade of NF-κB attenuates dystrophic pathology in murine DMD models

Genetic studies by Acharyya and colleagues (2007) have demonstrated that deletion of one allele of *p65*, but not p*50*, increases the number of regenerating fibers and decreases the extent of inflammation in *mdx* muscle. Using conditional knockouts, they were able to delete IKKβ in myeloid cells or muscle fibers of *mdx* mice, respectively. Using this approach, they found inflammation to be reduced by deletion in myeloid cells, and regeneration to be enhanced by knock-out in muscle fibers (Acharyya, Villalta et al. 2007). Additionally, using adeno-associated viral vectors (AAV), the local delivery of a dominant negative IKKβ mutant increased muscle regeneration in aged (11months+) *mdx* mice and decreased necrosis in both young and aged mice (Tang, Reay et al. 2010). More recently, we have reported that silencing of p65 via AAV mediated delivery of p65 short hairpin RNA (shRNA) reduced the severity of the *mdx*

phenotype (Yang, Tang et al. 2012). Silencing of p65 in *mdx* muscle was accompanied by a reduction in inflammatory cell infiltrate and increased muscle membrane integrity. These investigations implicate classical NF-κB in muscular dystrophy and establish that the beneficial effect of NF-κB blockade is derived from targeting NF-κB in both immune and muscle cells.

To date, both specific and non-specific NF-κB inhibitors have shown success in reducing the severity of the phenotype of *mdx* muscle (Grounds and Torrisi 2004; Carlson, Samadi et al. 2005; Hodgetts, Radley et al. 2006; Acharyya, Villalta et al. 2007; Pan, Chen et al. 2008; Tang, Reay et al. 2010; Reay, Yang et al. 2011; Yang, Tang et al. 2012; Araujo, Bonuccelli et al. 2013; de Senzi Moraes Pinto, Ferretti et al. 2013). Nonspecific targeting with PDTC has been found to preserve skeletal muscle fibers and reduce damage to the respiratory musculature (Carlson, Samadi et al. 2005). Similarly, curcumin administration was reported to reduce serum muscle creatine kinase and lower levels of TNFα (Pan, Chen et al. 2008). Indeed, despite some disagreement as to the exact cause of activation, numerous studies have repeatedly demonstrated that inhibition of NF-κB has a therapeutic effect on the *mdx* phenotype.

Rather than non-specific NF-κB inhibitors such as PDTC, several groups have undertaken a more specific pharmacologic approach targeting NEMO, required for the function of the IKK complex (Whitehead, Pham et al. 2008). Decoy peptides corresponding to the NEMO binding domain (NBD) of IKKα and -β have been designed to prevent activation of the classical pathway. Success with this approach has varied between different groups, likely due to different methods and durations of administration. For example, it was reported that NBD-peptide decreased necrosis in the diaphragm of treated mice after 4 weeks and was accompanied by a modest increase in the specific force of the muscle. Yet there was no change in serum muscle creatine kinase, indicating that protection from necrosis in the majority of the

musculature was unlikely (Whitehead, Pham et al. 2008). Another group also reported that the damage (and subsequent necrosis) to the diaphragm was reduced up to 79 percent following 4 weeks of treatment (Peterson, Kline et al. 2011). NBD-peptide therapy has also shown promise treating DMD associated cardiomyopathy, which is one of the main causes of patient death (Schram, Fournier et al. 2013). Unlike DMD patients, *mdx* mice do not exhibit any cardiac abnormalities until later in life. The utrophin-dystrophin double knockout (*dKO*) mouse has a much more severe phenotype, with a lifespan of approximately 8-10 weeks (Deconinck, Rafael et al. 1997; Wang, Li et al. 2009; Delfin, Zang et al. 2012; Isaac, Wright et al. 2013). Therefore, many investigations into treatments for cardiac symptoms have used the *dKO* mouse. Linear muscle preparations demonstrated *ex vivo* that treatment with NBD increased the active force development of *dKO* cardiac muscle. However, histological examination found no change in fibrosis development, suggesting that the improvement in contractile function occurs in a manner independent of tissue remodeling (Delfin, Xu et al. 2011). Despite the variability between reports, overall a positive effect has been achieved using NBD-peptide in muscular dystrophy.

1.5 Hepatocyte Growth Factor (HGF)

1.5.1 HGF participates in muscle regeneration

Skeletal muscle regeneration is a highly coordinated process, regulated by both transcription factors, such as members of the bHLH family, and paracrine factors, "relaying" messages between different cell types. For example, myeloid cell-derived factors, such as TNFα, stimulate myoblast proliferation during the early inflammatory stage of muscle repair (Chazaud,

Sonnet et al. 2003; Arnold, Henry et al. 2007). Of particular interest for the studies contained in this dissertation, hepatocyte growth factor (HGF) is one such critical factor. Through antibody neutralization experiments, HGF has been identified as the key factor for the ability of crushed muscle extract to stimulate satellite cell proliferation (Tatsumi, Anderson et al. 1998).

HGF, also known as scatter factor, is an 84 kD heterodimeric protein, formed by an α-(69 kD) and β-subunit (34 kD) joined via a disulfide bond (Naldini, Weidner et al. 1991). The receptor of HGF, MET, is also a heterodimer, formed by an extracellular α-subunit joined to a transmembrane β-subunit. Several downstream cascades are activated by MET, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K). Thus, the roles of HGF are quite diverse, including mitogenic, morphogenic, angiogenic, anti-inflammatory, and anti-apoptotic properties.

HGF is stored in the extracellular matrix of many different tissues as an inactive, single chain pro-form, and therefore its biological activity is regulated by extracellular proteases. Urokinase-type plasminogen activator (uPA), HGF activator (HGF-A), plasma kallikrein, coagulation factors XII and XI, matriptase, and hepsin have all been reported to cleave the single-chain HGF to produce the active heterodimer (Mars, Zarnegar et al. 1993; Nakamura, Sakai et al. 2011). The HGF stored in in skeletal muscle has been reported to be in both the pro- and active form (Sheehan, Tatsumi et al. 2000; Tatsumi and Allen 2004). Indeed, mice deficient in uPA have defective muscle regeneration, mediated, at least in part, by reduced myoblast proliferation and differentiation (Sisson, Nguyen et al. 2009). Conversely, deletion of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of uPA, results in accelerated muscle regeneration (Koh, Bryer et al. 2005).

The current evidence suggests that the levels of active HGF in skeletal muscle following injury appear to be controlled by macrophage-derived uPA, which is critical for leukocyte migration into injured muscle (Novak, Bryer et al. 2011). However, myoblasts also express uPA in response to basic fibroblast growth factor (FGF-2) (Miralles, Ron et al. 1998). These two distinct sources of protease may create distinct pools of active HGF that have unique functions in a given context. Based on the work contained in this dissertation, the role of HGF in skeletal muscle regeneration may not only be in the initial phase of repair, where it promotes leukocyte migration and myoblast proliferation, but also in the final phase of repair, as we shall discuss below.

1.5.2 HGF/MET and Wnt/GSK-3/β-catenin pathway cross-talk during inflammation

The anti-inflammatory properties of HGF/MET signaling are of particular interest for this dissertation. Indeed, HGF has been implicated in the resolution of inflammation in many tissues, such as the liver, kidney, and skeletal muscle (Trusolino, Bertotti et al. 2010). This effect appears to be mediated by the both the suppression of inflammatory cytokine expression in inflammatory cells and the induction of anti-inflammatory genes, such as *IL-10*. Downstream of MET/PI3K/Akt activation in macrophages, the phosphorylation of the serine/threonine protein kinase glycogen synthase kinase-3β (GSK3β) plays an important role in mediating HGF's anti-inflammatory effect (Gong, Rifai et al. 2008). Ubiquitously expressed, GSK3β is constitutively active under basal conditions, but is inactivated by phosphorylation at Ser9. Originally identified and named for its role in glucose metabolism, GSK3β has been found to have a much larger role in cell signaling than previously thought, with over 50 known target substrates (Martin, Rehani et al. 2005). Gong and colleagues (2008) have demonstrated that a subset of NF-κB target genes,

including *IL-6* and *MCP-1*, requires phosphorylation of p65 at Ser-468 by GSK3β for efficient transcription. Inactivated GSK3β reduces the associations of p65 with the co-activators CBP/p300 (Martin, Rehani et al. 2005; Gong, Rifai et al. 2008; Coudriet, He et al. 2010).. Furthermore, Martin and colleagues have reported that the decreased DNA binding of p65 allows for the association of CREB and p300, inducing anti-inflammatory gene expression, namely IL-10 (Martin, Rehani et al. 2005).

GSK3β is most often associated with the Wnt/β-catenin pathway, where it acts as a negative regulator of β-catenin. Some groups have considered whether increased β-catenin may also play a role in the anti-inflammatory function of HGF. For example, Dai and colleagues have reported that in kidney tubular epithelial cells, the anti-inflammatory action of GSK3β inactivation downstream of TGFβ depends on β-catenin activity (Dai, Wen et al. 2011). In hepatocytes, β-catenin has been reported to associate with the cytosolic portion of MET, allowing nuclear translocation of β-catenin independent of Wnt/Frizzled activation. In this situation, β-catenin was responsible for sequestering p65, thus reducing its transactivation potential (Monga, Mars et al. 2002). The events occurring after GSK3β phosphorylation are likely to be ligand- or cell-type dependent, but these models are not mutually exclusive. Thus, HGF/Met may lead to the inactivation of GSK3β and attenuate inflammatory gene expression by decreasing NF-κB/p65 DNA binding.

2.0 NF-κB/P65 NEGATIVELY IMPACTS THE MYOGENIC POTENTIAL OF MDSCS

2.1 Abstract

Inhibition of the IκB kinase (IKK)/nuclear factor κB (NF-κB) pathway enhances muscle regeneration in injured and diseased skeletal muscle, but it is unclear exactly how this pathway contributes to the regeneration process. In this study, we examined the role of NF-κB in regulating the proliferation and differentiation of muscle-derived stem cells (MDSCs). MDSCs isolated from the skeletal muscles of $p65^{+/-}$ mice (haploinsufficient for the p65 subunit of NF-κB) had enhanced proliferation and myogenic differentiation compared to MDSCs isolated from wild-type (wt) littermates. In addition, selective pharmacological inhibition of IKKβ, an upstream activator of NF-κB, enhanced wt MDSC differentiation into myotubes *in vitro*. The $p65^{+/-}$ MDSCs also displayed a higher muscle regeneration index than wt MDSCs following implantation into adult mice with muscular dystrophy. Additionally, using a muscle injury model, we observed that $p65^{+/-}$ MDSC engraftments were associated with reduced inflammation and necrosis. These results suggest that inhibition of the IKK/NF-κB pathway represents an effective approach to improve the myogenic regenerative potential of MDSCs and possibly other adult stem cell populations. Moreover, our results suggest that the improved muscle regeneration

observed following inhibition of IKK/NF-κB, is mediated, at least in part, through enhanced stem cell proliferation and myogenic potential.

2.2 Introduction

Chapter 2 is adapted from a published article in *Molecular Therapy*:

Lu A*[1], Proto JD*[1], Guo L[1], Tang Y[1], Lavasani M[1], Tilstra JS[2], Niedernhofer LJ[2], Wang B[1], Guttridge DC[3], Robbins PD[2], Huard J[1]. (2011) NF-κB negatively impacts the myogenic potential of muscle-derived stem cells. *Mol Ther* 20(3):661-8.

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NF-κB is a ubiquitously expressed nuclear transcription factor that is evolutionarily conserved. In mammals, the NF-κB family consists of five subunits: p65 (RelA), c-Rel, RelB, p50 and p52 (Verma, Stevenson et al. 1995). Transcriptionally active NF-κB exists as a dimer, with the most common form being a p50/p65 heterodimer. Under non-stress conditions, the heterodimer is maintained in an inactive state in the cytoplasm via its interaction with inhibitor of κB (IκB) proteins. Classic NF-κB activation is mediated by IκB kinase (IKK), a large, 700-

900 kDa complex consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit termed IKKγ, or NF-κB essential modulator (NEMO). In response to a variety of stimuli, including pro-inflammatory cytokines, bacterial products, viruses, growth factors, and oxidative stress, the complex is activated. Activated IKKβ phosphorylates IκB, leading to its polyubiquitination and subsequent degradation by the 26S proteasome. IκB degradation allows NF-κB to translocate to the nucleus where it binds to its cognate DNA site, as well as co-activators such as CBP/p300, to induce gene expression. Dysregulation of this pathway can result in chronic activation of IKK or NF-κB, and is seen in several pathophysiological states including cancer, rheumatoid arthritis, sepsis, muscular dystrophy, heart disease, inflammatory bowel disease, bone resorption, and both type I and II diabetes.

The NF-κB pathway, long recognized as an important component of innate and adaptive immunity, has also more recently emerged as a key player in the regulation of skeletal muscle homeostasis (Langen, Schols et al. 2001). Furthermore, activation of NF-κB in skeletal muscle has been linked to cachexia, muscular dystrophies, and inflammatory myopathies (Baghdiguian, Martin et al. 1999; Kumar, Lnu et al. 2003; Monici, Aguennouz et al. 2003; Hunter and Kandarian 2004; Acharyya, Villalta et al. 2007). Conversely, knock-out of p65, but not other subunits of NF-κB, enhances myogenic activity in MyoD-expressing mouse embryonic fibroblasts (MEFs) (Bakkar, Wang et al. 2008). Genetic depletion of p65 enhances muscle regeneration in both *mdx* and wild-type murine skeletal muscle (Acharyya, Villalta et al. 2007). What remains unclear, however, is how reduction of NF-κB activity positively impacts muscle.

Given that the repair of damaged tissues is mediated by adult stem cell populations, we hypothesized that NF-κB activity negatively regulates muscle stem cell function. In this study, we specifically focus on the role of p65 in regulating muscle-derived stem cell (MDSC) growth

and differentiation. This population of adult stem cells is capable of restoring muscle function (Payne, Oshima et al. 2007; Ambrosio, Ferrari et al. 2010). As complete knockout of p65 ($p65^{-/-}$) results in embryonic lethality, we isolated MDSCs from the skeletal muscles (SKM) of $p65^{+/-}$ mice and wt littermates (Beg, Sha et al. 1995). We observed that, *in vitro*, p65 haploinsuffiency was associated with increased cell proliferation and myogenic differentiation. Pharmacologic inhibition of IKK/NF-κB also enhanced myogenic differentiation. We also demonstrated that $p65^{+/-}$ MDSCs have a higher capacity for muscle regeneration after implantation into dystrophic, *mdx* mouse SKM. Furthermore, we show that muscle inflammation and necrosis post-injury is decreased following $p65^{+/-}$ MDSC implantation into cardiotoxin injured SKM. These results suggest that reducing the activity of the IKK/NF-κB pathway is an effective approach to improve the myogenic potential of MDSCs and possibly other adult stem cell populations. Our results provide a novel mechanistic insight as to why the inhibition of this pathway promotes SKM healing.

2.3 Materials and Methods

Animals: The C57BL/6J mice heterozygous for p65/RelA were originally described by Amer Beg (Beg, Sha et al. 1995). The mdx/SCID (C57BL/10ScSn DMD^{mdx}/J/CB17-$Prkdc^{scid}$/J) and C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal protocols used for these experiments were approved by the Children's Hospital of Pittsburgh's Institutional Animal Care and Use Committee.

Isolation of MDSCs from $p65^{+/-}$ mice. The mice were sacrificed at 5 months of age and muscle stem cell isolation was performed as previously described via a modified preplate

technique (Gharaibeh, Lu et al. 2008). Briefly, the SKM tissue was minced and processed through a series of enzymatic dissociations: 0.2% of collagenase type XI (Sigma-Aldrich, St. Louis, MO) for 1 hr, 2.4 units/ml of dispase (Invitrogen, Carlsbad, CA) for 45 min, and 0.1% of trypsin-EDTA (Invitrogen) for 30 min at 37^{o}C. After enzymatic dissociation, the muscle cells were centrifuged and re-suspended in proliferation medium (DMEM supplemented with 10% FBS, 10% HS, 0.5% chicken embryo extract, and 1% Penicillin-streptomycin), and the resulting cell suspension from both *p65*$^{+/-}$ and wt muscle were plated in collagen type I coated flasks. Different populations of muscle-derived cells were isolated based on their adhesion characteristics. After 7 days, late preplate populations (slowly adhering cells) were obtained and cultured in proliferation medium. The slowly adhering fraction of muscle cells has been previously shown to contain MDSCs (Gharaibeh, Lu et al. 2008). For all experiments, congenic *p65*$^{+/-}$ and *p65*$^{+/+}$ MDSCs of the same passage number were compared.

p65 staining and ArrayScan Assay. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), rinsed 2 times with PBS, and the cells' membrane permeabilized for 10 min with 0.1% Triton X-100 in PBS. A 10% goat serum blocking solution was used for 1 hr and the cells were incubated with a 1:200 dilution of rabbit polyclonal anti-p65 (Abcam, Cambridge, MA) for 1 hr at RT. After washing 3 times, the cells were incubated for 30 min with Cy3-conjugated anti-rabbit IgG (1:500, Sigma-Aldrich). The nuclei were revealed by 4', 6-Diamidino-2-phenylindole (DAPI) staining. Nuclear localization of the NF-κB subunit p65 was measured via ArrayScan^{Tm}. This technique allows for the rapid, automated quantification of p65 and DAPI colocalization, as identified by immunocytochemistry in cells grown on a 96-well plate. Recordings were taken from multiple fields of view per well, generating data representative of each well.

Western Blot assay. The cell populations isolated from *p65*$^{+/-}$ and wt mice were cultured in proliferation medium and stimulated with TNFα (10 ng/ml) for 30 min prior to harvesting. Cells were then lysed in Laemmli Sample Buffer (Bio-Rad, Hercules, CA), boiled for 5 min, and centrifuged at 4000 rpm for 5 min. Each sample was loaded on a 10% SDS-polyacrylamide gel, which was run for 2 hrs and then transferred for 1.5 hrs at 100 volts while stirring on ice. The membrane was blocked with 5% bovine serum albumin (Sigma) in PBS for 1 hr and then incubated with rabbit anti-phospho-NF-κB/p65 monoclonal antibody (1:1000, Cell signaling, Danvers, MA) overnight at 4°C. After washing 3 times with Tris-buffered saline tween-20 (TBST), the membrane was incubated with goat anti-rabbit IgG (H+L) (1:5000, Pierce, Rockford, IL) for 50 min at room temperature (RT). Blots were developed by ECL solution (Pierce).

RT-PCR analysis. Total RNA was extracted from cells using Nucleo Spin RNA II column (Clontech, Mountain View, CA). Following isolation, cDNA was synthesized with SuperScript™ II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR was performed with Taq polymerase (Invitrogen) as per the manufacturer's instructions and PCR products were separated by electrophoresis with 1% agarose gels. The primers used for PCR are listed in Table 1. Each set of oligonucleotides was designed to span two different exons to avoid background amplification of genomic DNA. The data were quantified by densitometry using Adobe Photoshop 7.0.

Gene	Forward primer	Reverse primer	Location
Sca-1	CCTACTGTGTGCAGAAAGAGC	CAGGAAGTCTTCACGTTGACC	89–331
CD34	GCAGCTTTGAGATGACATCACC	CTCAGCCTCCTCCTTTTCACA	498–715
MyoD1	ACAGTGGCGACTCAGATGCATC	GCTGCAGTCGATCTCTCAAAGC	708–1105
Desmin	AACCTGATAGACGACCTGCAG	GCTTGGACATGTCCATCTCCA	615–873
CD31	AGAGCTACGTCATTCCTCAG	GACCAAGTGTGTCACTTGAAC	474–988
CD144	CACCAACAAAAACCTGGAACA	CCACCACGATCTTGTATTCAG	425–729
β-Actin	TCAGAAGGACTCCTATGTGG	TCTTTGATGTCACGCACGAT	234–722

Table 1. Primer Sequences used in Chapter 2.

Pax7 and MyoD staining. *P65*$^{+/-}$ and wt cells were fixed and permeabilized with 2% paraformaldehyde plus 1% Triton X-100 for 30 min at 4°C and rinsed 2 times with PBS. Cells were blocked with 5% HS and then incubated with a 1:100 dilution of mouse monoclonal anti-Pax7 (DSHB, Iowa City, Iowa) and anti-MyoD (Santa Cruz biotechnology, Santa Cruz, CA) over night at 4°C. After washing 3 times, the cells were incubated for one hour with biotinylated anti-mouse IgG (1:300, Vector Lab, Burlingame, CA), which acted as a secondary antibody. Streptavidin 594 conjugate (1:500, Invitrogen) was added in the last step. The nuclei were revealed by DAPI staining. Negative control staining was performed by an identical procedure, with the exception that the primary antibody was omitted.

In vitro Assessment of Cell Proliferation. In order to compare the proliferative potential of *p65*$^{+/-}$ MDSCs to wt MDSCs, we used a previously described live cell imaging system [LCI] (Kairos Instruments LLC) (Deasy, Jankowski et al. 2003). Brightfield images were taken at a 100x magnification at ten min intervals over a 72 hour period in three fields of view per well, with three wells per population. The images were combined to generate a movie using ImageJ software (NIH). Proliferation was assessed by counting the number of cells per field of view, n, over twelve hours. All six populations were also plated in 96-well plates in quadruplicate (500 cells/well) and cultured under normal conditions for 72 hours. At this time, 20 uL of CellTiter96 AQueous One Reagent (Promega, Madison, WI) was added to each well and incubated in 5% CO_2 at 37 °C. Following another 3 hour incubation, absorbance at 490 nm was read with a 96-well plate reader.

Myogenic differentiation assay and fast Myosin Heavy Chain (MyHC-f) staining. After 15 passages, cells were plated on 12 well plates (30,000 cells per well) with DMEM

supplemented with 2% FBS to stimulate myotube formation. Three days later, immunocytochemical staining for fast skeletal Myosin Heavy Chain (MyHC-f) was performed. After rinsing 3 times with PBS, cells were fixed for 2 min in cold methanol, blocked with 10% horse serum for one hr and then incubated with a mouse anti-MyHC-f (1:250, Sigma, clone MY-32) for 2 hr at RT. The primary antibody was detected with a secondary anti-mouse IgG antibody conjugated with Cy3 (1:300, Sigma) for 15 min. The nuclei were revealed by DAPI staining. The percentage of differentiated myotubes was quantified as the number of nuclei in MyHC-f positive myotubes relative to the total number of nuclei.

Selective Inhibition of IKKβ. To determine the effects of IKK/NF-κB inhibition on wt MDSCs during myogenic differentiation, we utilized IKK-2 Inhibitor IV (IKKi), or 2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (Calbiochem, San Diego, CA), a reversible competitive inhibitor of IKKβ ATP binding. Cells were plated at 10^5 cells per well in 6 well plates and exposed to IKK inhibitor in differentiation medium. Cells were treated with either 1, 3, or 5 uM IKKi. Lysates were collected at 0 min, 14, 24, 48, and 72 hrs following treatment. NF-κB activity and myogenic differentiation was assessed by western blot for phosphorylated NF-κB/p65 (1:1000, Cell signaling, Danvers, MA) and MyHC (1:500, Sigma, clone MY-32), as detailed above.

Cell implantation and Dystrophin staining. MDSCs from *p65*$^{+/-}$ and wt muscle were grown in proliferation medium until the cell number was sufficient for injection. A total of $3x10^5$ viable cells was suspended in 20 μl of Hank's balanced salts solution (HBSS) and injected into the gastrocnemius muscles of 8-12 wk-old mdx/SCID mice using a Hamilton syringe. The same number of cells was injected into the gastrocnemius muscles of 8 wk-old wt C57BL/6J mice that had been injured 1 day earlier by a 30 μL intramuscular injection of 2μM cardiotoxin (CTX;

Sigma) in PBS. The cell suspension was mixed with green fluorescent-labeled beads prior to injection to detect the injection sites. Six or fourteen days after implantation, the mice were sacrificed and the gastrocnemius muscles were harvested and flash frozen in liquid nitrogen-cooled 2-methylbutane. Serial cryosections 10 μm in thickness were obtained for immunohistochemical analyses. Cryosections were fixed with 5% formalin and blocked with 5% donkey serum in PBS for 1 h, then incubated with rabbit anti-dystrophin (1:300, Abcam) for 2 hr at RT. The sections were exposed to secondary 594-conjugated anti-rabbit IgG (1:500, Invitrogen) in PBS for 30 min. The nuclei were revealed by DAPI staining. Immunostaining was visualized and images were taken by fluorescence microscopy (Nikon Eclipse E800). Northern Eclipse software was used for quantitative analysis of the regenerated dystrophin-positive myofibers. A series of pictures were taken, and Adobe Photoshop 7.0 was used to construct a composite picture of the dystrophin-positive myofibers, which were then manually counted.

Retroviral transduction of MDSCs . MDSCs were plated at an initial confluence of 30-40% and retrovirally transduced (in the presence of Polybrene[8 μg/ml]) to express the beta-galactosidase gene (LacZ), as previously described(Li and Huard 2002).

LacZ staining The cryosections were fixed in 1% glutaraldehyde and incubated 3 hours with 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal) substrate at room temperature (RT). Sections were counterstained with Eosin.

CD14 staining. Cryosections were fixed with 5% formalin and blocked with 5% donkey serum in PBS for 1 h, then incubated with rat anti-CD14 (1:200, Biolegend, San Diego, CA) overnight at 4ºC. This was followed by a 1 hr incubation with biotinylated anti-rat IgG (1:300, Vector). Streptavidin Cy3 conjugate (1:500, Sigma) was added in the last step followed by

several rinses in PBS. Following CD14 staining, five random pictures per section were taken and the number of CD14 positive cells was counted manually.

Mouse IgG staining and quantification of necrosis. Muscle sections were fixed with 5% formalin and blocked with 10% horse serum in PBS for 1 hr, then incubated with biotinylated anti mouse IgG (1:300; Vector) for 1 hr at RT. This was followed by a 15 min incubation with streptavidin Cy3 conjugate (1:500, Sigma). The nuclei were revealed by DAPI staining. The IgG positive area was measured and quantified as the percentage of mouse IgG expressing area per total area using Northern Eclipse software.

Statistical analysis. All results are given as the mean ± standard deviation. Means from $p65^{+/-}$ and wt or treated and untreated were compared using Students' *t*-test. Differences were considered statistically significant when the p-value was <0.05.

2.4 Results

Isolation and phenotypic characterization of MDSCs from $p65^{+/-}$ and wt mice.

To examine the effect of NF-κB activity on MDSC function, we purified populations of muscle stem cells from the SKM of mice heterozygous for the p65 subunit of NF-κB ($p65^{+/-}$) and wt littermates. Using a modified preplate technique (Gharaibeh, Lu et al. 2008), we isolated independent populations of MDSCs from three mice of each genotype. To confirm that p65 haploinsufficiency reduced basal levels of NF-κB activity, nuclear p65 was measured via ArrayScan^{Tm}. Nuclear, or active, p65 was found to be 30% lower in $p65^{+/-}$ compared to wt MDSCs (**Fig. 5a**). Upon activation, NF-κB subunits undergo post-translational modifications, such as phosphorylation, to enhance their activity. Immunoblot analysis revealed that the level

of phosphorylated p65 (P-p65) was also reduced; however, stimulation with TNFα led to an increased level of P-p65 in both wt and *p65*$^{+/-}$ MDSCs (**Fig. 5b**), demonstrating that basal, but

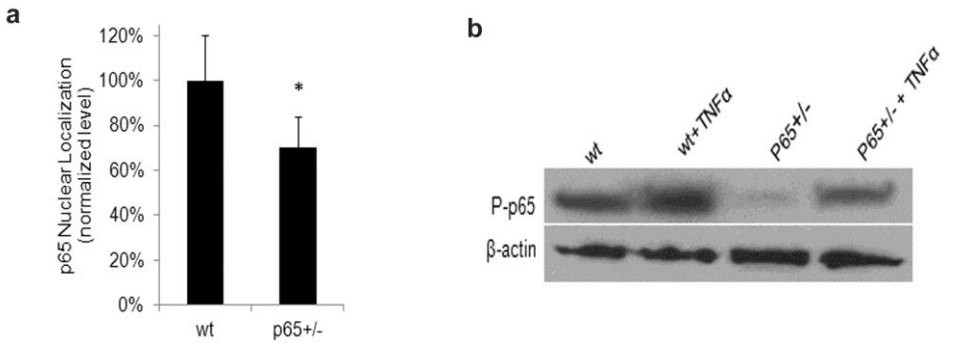

Figure 5. Basal levels of phosphorylated p65 Levels are reduced in *p65*$^{+/-}$ MDSCs

Muscle-derived stem cells (MDSCs) obtained from the skeletal muscles (SKM) of *p65*$^{+/-}$ mice have a lower level of activated p65 compared to wild-type (wt) MDSCs. (a) ArrayScan analysis of nuclear p65 in MDSCs isolated from *p65*$^{+/-}$ and wt mice. Error bars indicate "mean + SD." (b) Immunoblotting for phosphorylated p65 in whole cell lysates of MDSCs before and after tumor necrosis factor-α (TNFα) stimulation for 30 minutes.

not induced, NF-κB activity is affected by knocking-out one allele of *p65*. To confirm the MDSC phenotype of *p65*$^{+/-}$ and wt cells, each population was analysed for the expression of stem (CD34, Sca-1), myogenic (MyoD and desmin), and endothelial (CD144, CD31) cell markers by RT-PCR. For each of the markers, there was variability in expression between cell populations of a single genotype, but upon quantification, no significant differences were found between the different genotypes, with the exception of CD144, which was elevated in *p65*$^{+/-}$ MDSCs. (**Figs. 6a,c, $p<0.05$**). Such variability in marker expression has been previously reported and interpreted as evidence that these cell populations contain a mix of stem and committed progenitor cells (Jankowski, Haluszczak et al. 2001; Sacco, Doyonnas et al. 2008). We next examined the expression Pax7 and MyoD protein by immunostaining, and also found no significant difference between *p65*$^{+/-}$ and wt cells (**Figs. 6b,d**). These results suggest that genetic reduction of p65 does not dramatically alter the marker phenotype of MDSCs.

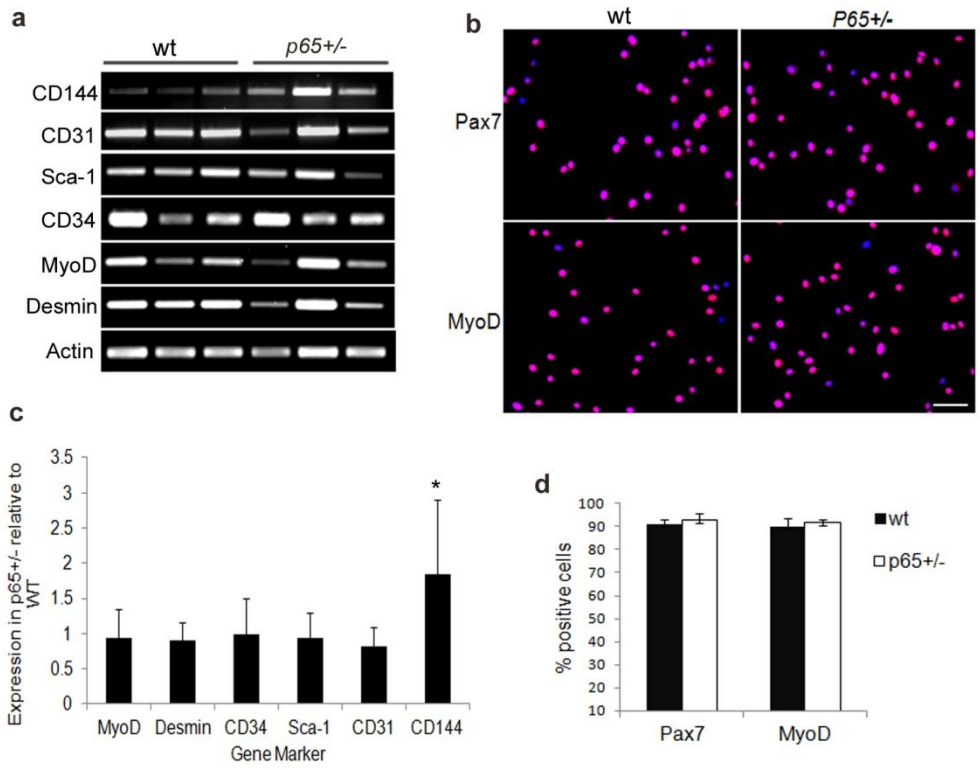

Figure 6. Phenotypic Characterization of wt and *p65*$^{+/-}$ MDSCs.

(a) RNA was isolated from three independent cell populations of each genotype. RT-PCR was performed to characterize the MDSC populations for the expression of stem (CD34 and Sca-1), endothelial (CD31 and CD144) and myogenic (MyoD and desmin) cell markers. (b) Immunostaining for the muscle stem cell markers Pax7 and MyoD was also performed (bar = 25 μm). (c) Quantification of RT-PCR results. Error bars indicate "mean + SD" (n = 3 independent experiments). (d) Quantification of Immunostaining of Pax7 and MyoD.

p65$^{+/-}$ *MDSCs proliferate faster than* wt *MDSCs.*

NF-κB is known to regulate cell division, so we investigated whether p65 reduction would alter MDSC proliferation. *p65*$^{+/-}$ and wt MDSCs were plated in collagen-coated flasks and expanded in growth medium for 10 to 12 passages. Cells were then transferred to 24 well plates and proliferation was measured using a previously described Live Cell Imaging (LCI) system (Deasy, Jankowski et al. 2003). We observed that *p65*$^{+/-}$ MDSCs proliferated significantly faster than wt cells (**Fig. 7a**). Equal numbers of cells were also plated on a 96 well plate and grown for

three days at which point the differences in cell number were determined using an MTS assay. This assay demonstrated a similar significant increase in cell proliferation in *p65*$^{+/-}$ MDSCs (**Fig. 7b**) suggesting that NF-κB, and in particular p65, limits the proliferation of MDSCs.

***p65*$^{+/-}$ *MDSCs have enhanced myogenic differentiation compared to wt cells*.**

We next measured the ability of the *p65*$^{+/-}$ and wt MDSCs to undergo myogenic differentiation *in vitro*. Equal numbers of cells were plated in a 24 well plate and switched to differentiation medium once the cells adhered. After 3 days the majority (80%) of the *p65*$^{+/-}$ cells had differentiated into myotubes, as determined by immunodetection of myosin heavy chain (**Fig. 8a**). The differentiation potential of the *p65*$^{+/-}$ MDSCs was significantly greater than the wt MDSCs (60%; $p<0.01$; **Fig. 8b)**. These results demonstrate that NF-κB, and in particular p65, represses MDSC differentiation *in vitro*.

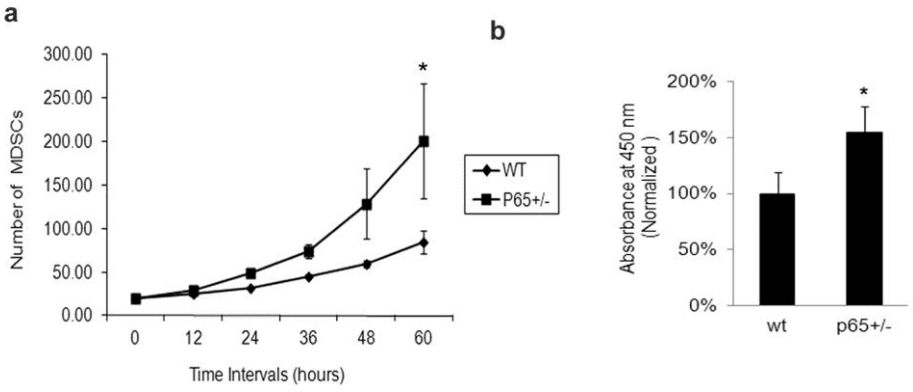

Figure 7. Proliferation of p65+/- MDSCs

Muscle-derived stem cells (MDSCs) isolated from p65+/– mouse skeletal muscles (SKM) have a higher rate of proliferation than wild-type (wt) cells. (a) Cell proliferation rate was measured by live Cell Imaging and (b) by an MTS assay ($P < 0.05$).

Pharmacologic inhibition of IKK β increases myogenic differentiation in vitro.

To confirm this finding , we tested whether a pharmacologic inhibitor of NF-κB could enhance MDSC myogenic potential *in vitro*. Wt MDSCs were exposed to differentiation medium containing various doses of IKK-2 inhibitor IV (IKKi), a specific, reversible inhibitor of IKKβ. Cell lysates were collected at 0, 1, 14, 24, 48, and 72 hrs following treatment. Accordingly, MyHC levels dramatically increased, beginning at 14 hrs (**Figs. 9a,c**). As expected, we observed a robust time-dependent decrease in p-p65 that was dose-dependent (greater at 3 μM than 1 μM; **Fig. 9b)**. We next examined NF-κB activity in wt and $p65^{+/-}$ MDSCs at various time points during myogenic differentiation by immunodetection of P-p65 and MyHC. In wt cells, beginning at 48 hrs post-transition to differentiation medium, the levels of p-p65 were detectably reduced (**Fig. 9d**). This occurred more rapidly (by 24 hrs) in $p65^{+/-}$ cells. Similarly, accumulation of MyHC was greater at earlier time points (14 hrs) in $p65^{+/-}$ cells than wt. This timeframe for MyHC accumulation is similar to that observed in wt cells treated with IKKi (**Fig. 9a**). In order

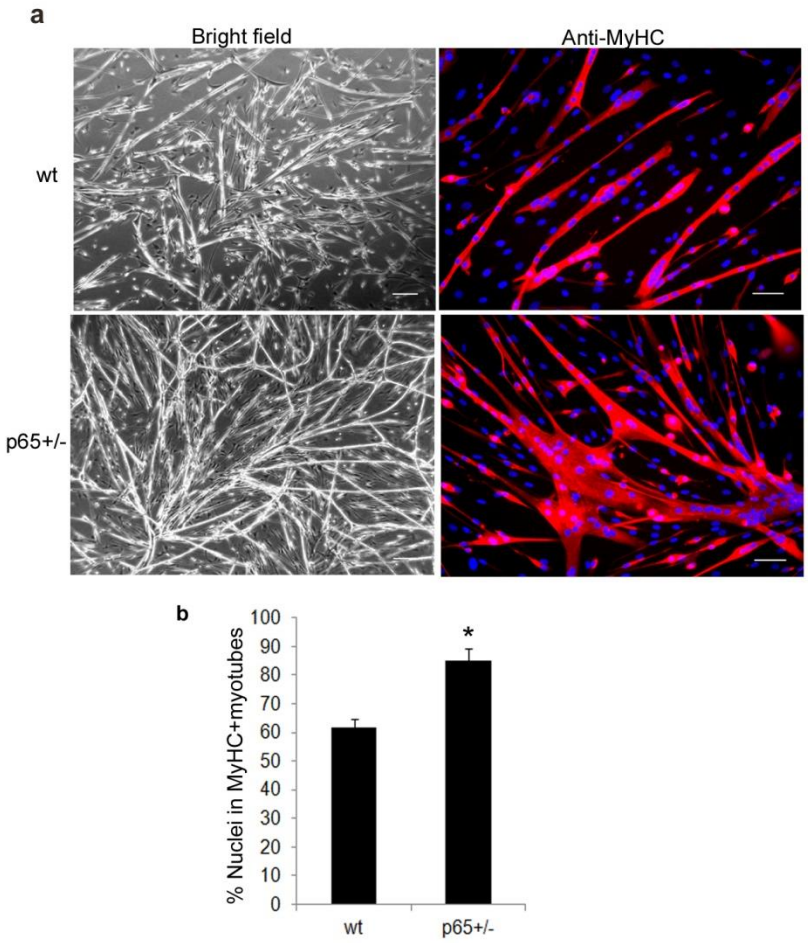

Figure 8. Myogenic differentiation is enhanced in *p65*$^{+/-}$ MDSCs.

(**a**) MDSCs were cultured in myogenic differentiation medium for 3 days, during which cell fusion into multinucleated myotubes was monitored using bright field microscopy and then confirmed by immunostaining for MyHC. (**b**) Quantitation of MyHC positive myotubes. The percentage of differentiated myotubes was quantified as the number of nuclei in MyHC positive myotubes relative to the total number of nuclei. A total of three populations of *p65*$^{+/-}$ and wild-type (wt) MDSCs were tested ($P < 0.05$). In panel "**a**" all bars = 200 μm on bright field and all bars = 50 μm on MyHC immunostaining.

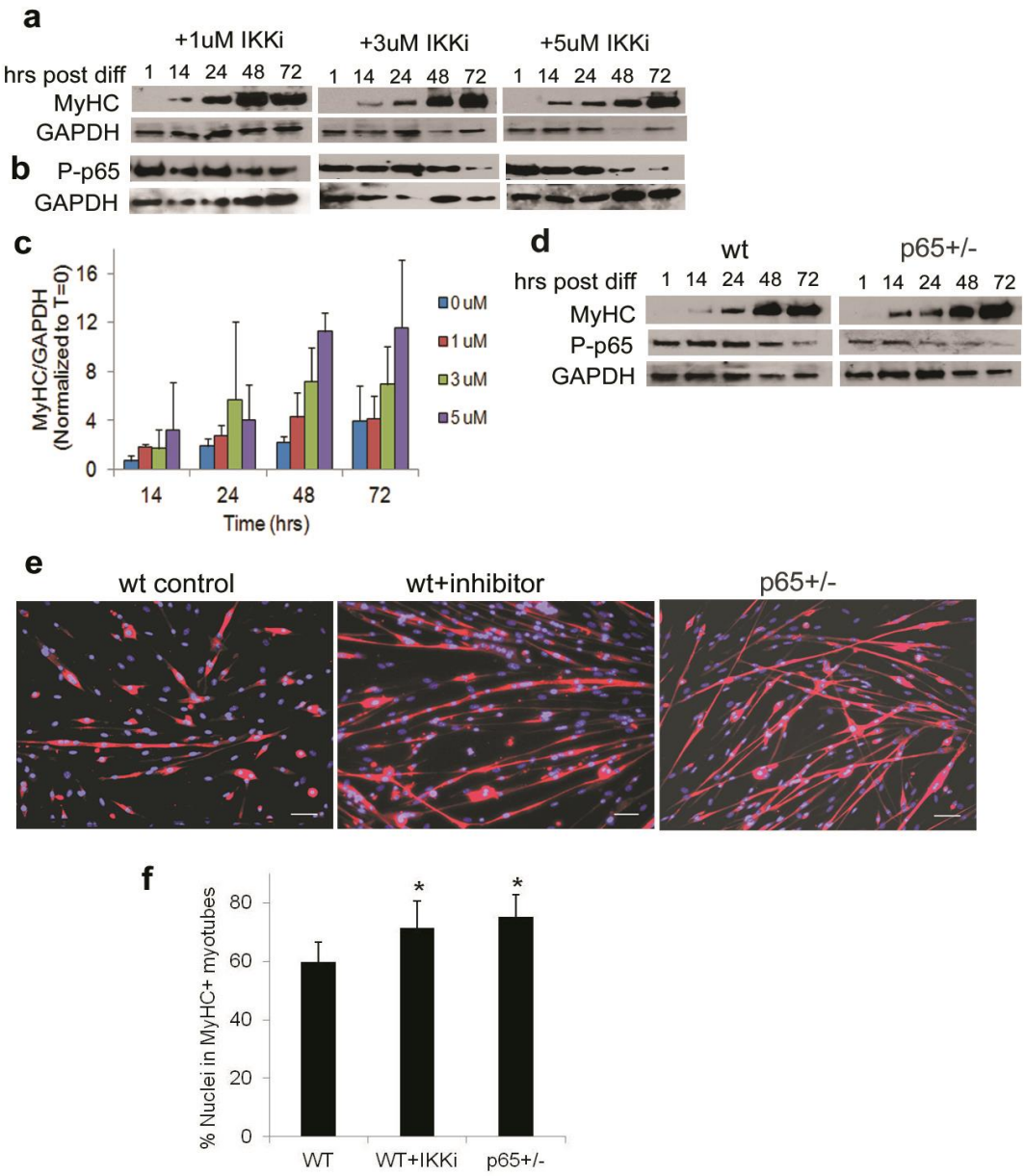

Figure 9. Pharmacologic inhibition of IKKβ enhances myogenic differentiation of WT MDSCs in a dose dependent manner

(**a**) Western blot for myosin heavy chain (MyHC) over 72 hours of wt MDSCs treated with 1, 3, or 5 uM IKKi during differentiation. (**b**) Western blot for P-p65 over 72 hours of wt MDSCs treated with 1, 3, or 5 uM IKK inhibitor (IKKi) during differentiation. (**c**) Quantification of **b**, MyHC levels during differentiation (n = 3 independent experiments). (**d**) In parallel, wt and $p65^{+/-}$ MDSCs were cultured in differentiation medium and lysates were collected at the various time points indicated. Lysates were used for western blot for MyHC and P-p65 levels. (**e**) wt MDSCs, $p65^{+/-}$ MDSCs, and wt MDSCs treated with IKKi (5 uM), were grown under fusion conditions for 72 hours and immunostained for MyHC expression (**f**) Quantification of MyHC staining ($P < 0.05$). In panel "**e**" all bars = 100 μm.

to verify that increased MyHC expression was concomitant with increased myotube formation, we treated wt MDSCs with 5 μM IKKi. After three days, differentiation was assessed by immunofluorescence detection of MyHC. As shown in **Fig. 9e,** compared to non-treated controls, the inhibitor caused a significant increase in myotube formation. The level of myogenic differentiation was comparable to that of *p65*$^{+/-}$ MDSCs ($p<0.01$; **Fig. 9f**). These results provide strong support that the MDSC myogenic potential can be improved using NF-κB inhibition *ex vivo*.

p65*$^{+/-}$ *MDSCs have a greater capacity for muscle regeneration in vivo compared to wt MDSCs.

To determine if genetic depletion of p65 increases the engraftment and muscle regenerative capacity of MDSCs *in vivo*, we examined the ability of *p65*$^{+/-}$ and wt MDSCs to regenerate muscle fibers following intramuscular implantation into an immunocompromised model of DMD. For these experiments, $3x10^5$ *p65*$^{+/-}$ and wt MDSCs were injected into the gastrocnemius muscles of 8 wk-old dystrophin-deficient SCID (*mdx*/SCID) mice. Fourteen days post-implantation, significantly more dystrophin-positive myofibers were detected in the muscle injected with *p65*$^{+/-}$ MDSCs than in muscle injected with wt MDSCs ($p<0.01$; **Figs. 10a,b**). These results confirm our *in vitro* observations and may provide a novel mechanism as to why IKK inhibitors have been reported to improve muscle regeneration (Tang, Reay et al. 2010).

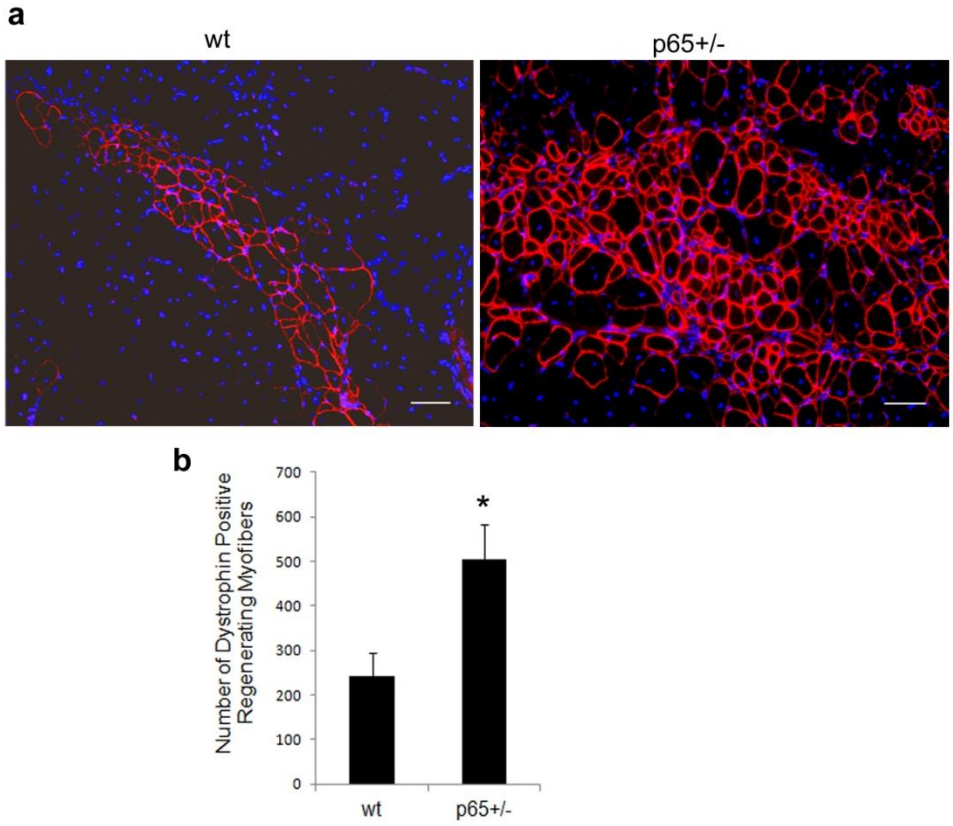

Figure 10. *p65*$^{+/-}$ MDSCs generate larger engraftments that WT MDSCs when transplanted into *mdx* mice.

(**a**) Gastrocnemius cryosections from 8-week-old mdx/SCID mice in which *p65*$^{+/-}$ and wild-type (wt) MDSCs were implanted. Engraftment was determined by immunostaining for dystrophin (red). Three populations of *p65*+/– and wt MDSCs were transplanted into 12 mice in two independent experiments. (**b**) Quantitation of regenerated dystrophin-positive myofibers ($P < 0.05$). In panel "**a**" all bars = 50 μm. Error bars indicate "mean + SD" ($n = 12$).

Transplantation of p65$^{+/-}$ *MDSCs post-injury reduces SKM inflammation and necrosis*.

The results above suggest that lowering basal levels of NF-κB activity increased the ability of MDSCs to engraft and differentiate following intramuscular injection (**Fig 10**). However, while it is possible this is mediated through enhanced proliferation and differentiation, the exact mechanism as to why more dystrophin-positive myofibers were found within the *p65*$^{+/-}$ MDSC engraftment sites remains unclear. Surrounding the engraftments of the wt MDSCs, we observed numerous cells positive for the macrophage marker CD14 as detected by

immunofluorescent staining (data not shown), whereas the *p65*$^{+/-}$ MDSC engraftment sites were surrounded by fewer numbers of CD14+ cells (data not shown). As the mdx/SCID is an

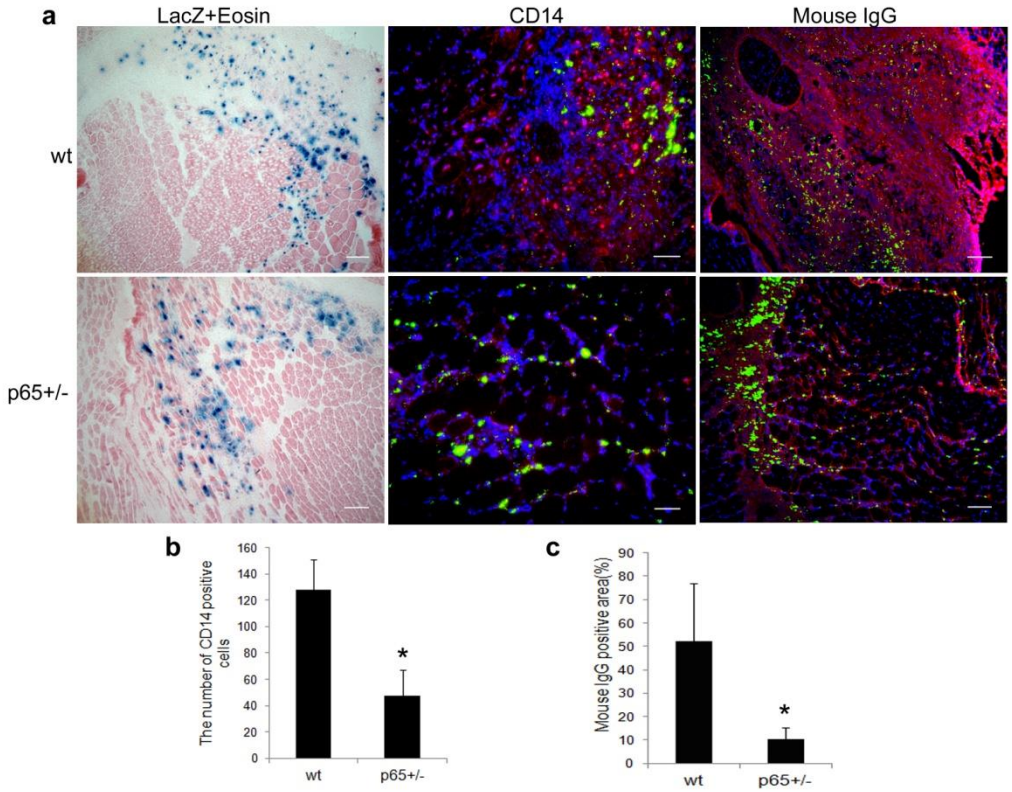

Figure 11. Local inflammation and necrosis is reduced surrounding *p65*$^{+/-}$ MDSC engraftments

p65$^{+/-}$ MDSCs attenuate muscle inflammation and necrosis. (**a**) Gastrocnemius cryosections from 8-week-old C57BL/6J mice, which were injected with *p65*$^{+/-}$ or wt MDSCs 24 hours post-CTX injury. LacZ and eosin staining identified the injection area and immunostaining for CD14 (red) and mouse immunoglobulin G (IgG) (red) identified macrophages and necrotic tissue, respectively. In immunological stains, fluorescent green beads in C57BL/6J muscle sections confirmed the location of injection sites. (**b**) Quantitation of CD14+ cells (the data represent six muscles per group). (**c**) Necrotic area in the gastrocnemius muscles was identified by IgG staining and quantified based on the total positive area per image (the data represent six muscles per group). In panel "**a**" all bars = 100 μm on LacZ+eosin and mouse IgG staining. All bars = 50 μm on CD14 staining.

immunocompromised mouse model with a high level of background inflammation, we decided to further investigate this phenomenon using the well-established cardiotoxin (CTX) muscle injury model in immunocompetent wild type mice. In order to confirm that transplanted *p65*$^{+/-}$ MDSCs are able to reduce inflammation in host skeletal muscle, we injected *p65*$^{+/-}$ and wt

MDSCs into the gastrocnemius muscles of 8 wk-old C57BL/6J mice 24 hours post-CTX injury. Six days post-transplantation, the wt MDSC engraftment area demonstrated a greater number of inflammatory cells surrounding the wt donor MDSCs than the *p65*$^{+/-}$ MDSCs. Furthermore, numerous centrally located nuclei, characteristic of regenerating muscle fibers, were found within the *p65*$^{+/-}$ MDSC injection sites. Consistent with observations made in *mdx*/SCID mice, the *p65*$^{+/-}$ MDSC engraftment area was associated with significantly fewer CD14 positive cells than the wt MDSC engraftment area ($p<0.01$**; Figs.11a, b**). There was also a significant (42%) reduction in tissue necrosis, as determined by quantification of mouse IgG staining **(**$p<0.01$; **Figs.11a, c**). These results indicate that the improved engraftment and differentiation of *p65*$^{+/-}$ MDSCs is potentially due to their ability to attenuate the inflammation and necrosis that typically occurs after muscle injury.

2.5 Discussion

NF-κB signaling has been implicated in the regulation of muscle degeneration and regeneration. The five mammalian NF-κB transcription factors are all expressed in skeletal muscle to modulate a variety of processes, including apoptosis, inflammation, and myoblast differentiation. Although there have been conflicting results reported as to whether NF-κB acts as a repressor or promoter of myogenesis (Lehtinen, Rahkila et al. 1996; Guttridge, Albanese et al. 1999; Kaliman and Barannik 1999; Canicio, Ruiz-Lozano et al. 2001; Langen, Schols et al. 2001; Munz, Hildt et al. 2002; Baeza-Raja and Munoz-Canoves 2004), recent results suggest that classical NF-κB signaling functions as a negative regulator of myogenesis (Bakkar, Wang et al. 2008). In addition, NF-κB activation is associated with the degeneration and/or lack of

regeneration of dystrophic muscle in *mdx* mice (Acharyya, Villalta et al. 2007). Thus, in this study, we examined the effect of NF-κB reduction on the proliferation and differentiation of MDSCs isolated from wt mice and mice heterozygous for p65. Although $p65^{+/-}$ MDSCs had a more than a 30% reduction in p65/NF-κB levels compared to the wt MDSCs, the two genotypes expressed similar stem (CD34, Sca-1), myogenic (Desmin, MyoD) and endothelial (CD144, CD31) cell markers. This result suggests that the reduction in NF-κB did not affect overall expression of MDSCs markers, albeit there is some variability in stem cell marker expression between populations of the same genotype.

We observed that MDSCs with reduced p65 levels had improved proliferation compared to wt control cells, suggesting that p65/NF-κB activity negatively controls MDSC population expansion. More importantly, we also observed that both the rate and extent of myogenic differentiation was accelerated in MDSCs with reduced p65 and in wt MDSCs treated with an IKKβ inhibitor. Together, these data suggest that NF-κB inhibits muscle stem cell differentiation. Our results are in agreement with previous studies showing that NF-κB's negative regulation of myogenesis is dependent on p65 transcriptional activity (Bakkar, Wang et al. 2008). It has been suggested previously that the negative effect of NF-κB on differentiation is mediated through the transcriptional activation of cyclin D1 and YinYang1 (YY1) (Guttridge, Mayo et al. 2000; Wang, Hertlein et al. 2007). Interestingly, we have observed a reduction in the level of cyclin D1 in $p65^{+/-}$ MDSCs compared to wt cells, but found no difference in the level of YY1 expression (data not shown).

Recent genetic evidence supports the role of IKK/NF-κB in driving the pathogenesis of muscular dystrophy, identifying this signaling pathway as a potential therapeutic target for the treatment of DMD (Acharyya, Villalta et al. 2007). The activity of NF-κB in dystrophic muscle

is associated with not only immune cells, but also regenerative muscle fibers. Thus we investigated whether the *p65*$^{+/-}$ MDSCs have a higher muscle regeneration potential than wt MDSCs after their intramuscular injection into dystrophic *mdx*/SCID skeletal muscles. Our results demonstrated that *p65*$^{+/-}$ MDSCs are more efficient at regenerating dystrophin positive myofibers compared to wt MDSCs, which is consistent with the enhanced ability of the *p65*$^{+/-}$ MDSCs to differentiate in culture.

We also assessed inflammation around the engrafted site by immunofluorescent staining for CD14, a macrophage marker. While we found very few CD14 positive cells within the injection sites of the *p65*$^{+/-}$ cells, many CD14+ cells were detected within the wt MDSC engraftment areas. As decreased macrophage invasion in the *p65*$^{+/-}$ cell engraftment area correlated with a reduction in necrosis, it is possible that a reduction in p65 enhances the local anti-inflammatory properties of MDSCs via regulation of paracrine factors. Several cytokines under control of NF-κB, such as tumor necrosis factor alpha (TNFα) and IL-6, are potent inhibitors of myogenic differentiation (Langen, Schols et al. 2001). Thus, taken together, these results suggest that inhibition of NF-κB/p65 may enhance myogenesis by reducing inflammation and necrosis.

Other groups have demonstrated the importance of non-NF-κB proteins in muscle development and pathology. During regeneration following injury, numerous paracrine factors such as myostatin, hepatocyte growth factor (HGF), and basic fibroblast growth factor play critical roles coordinating repair (Karalaki, Fili et al. 2009). For example, myostatin acts independently of the classical TNFα and NF-κB pathway to inhibit MyoD expression and signal cachexia by reversing the IGF-1/PI3K/Akt hypertrophy pathway to increase the levels of active FoxO1, allowing for increased expression of atrophy-related genes (McFarlane, Plummer et al. 2006). **In summary**, here we described a negative role for the p65/NF-κB signaling pathway in

MDSC growth and differentiation *in vitro*, as well as muscle regeneration *in vivo*. Similarly, pharmacological inhibition of IKKβ identifies the IKK/NF-κB signaling pathway as a potential therapeutic target to improve the myogenic potential of MDSCs and muscle regeneration after injury and diseases.

3.0 HGF IS CRITICAL FOR THE BENEFICIAL EFFECT OF NF-ΚB BLOCKADE ON DYSTROPHIC MUSCLE

3.1 ABSTRACT

The ubiquitous transcription factor NF-κB/p65 has been implicated in the fatal disease Duchenne muscular dystrophy (DMD). The severity of this disease can be attenuated by NF-κB inhibition in the *mdx* mouse, a murine DMD model; but, this approach remains problematic for treating human patients. We found that deleting one allele of *p65* ($p65^{+/-}$) improved the anti-inflammatory capacity of muscle-derived stem cells *in vitro* and *in vivo* via upregulation of hepatocyte growth factor (HGF). HGF upregulation also coincided with the reduced inflammation of *p65* haploinsufficient *mdx* mice ($mdx;p65^{+/-}$). Moreover, shRNA-mediated silencing of HGF in $mdx;p65^{+/-}$ skeletal muscle ablated the beneficial effect of *p65* deficiency, worsening inflammation and necrosis. In this investigation, we identify HGF as a downstream effector of NF-κB/p65 blockade. Our findings identify a key role for HGF in modulating the resolution of inflammation during skeletal muscle repair and warrant further investigation into its potential for the treatment of DMD.

3.2 INTRODUCTION

Central to the inflammatory response, the transcription factor NF-κB has been found to be highly activated in the skeletal muscles of patients suffering from the neuromuscular disease Duchenne muscular dystrophy (DMD) (Spencer, Walsh et al. 1997; Acharyya, Villalta et al. 2007). In this fatal disease, absence of the cytoskeletal protein dystrophin results in skeletal muscle membrane instability, ongoing degeneration, and chronic inflammation. During infancy, at the earliest stages of disease, skeletal muscle is able to regenerate. However, in early adolescence effective tissue regeneration rapidly declines, partially due to excessive inflammation; patients usually die in their twenties (Spencer, Walsh et al. 1997; Acharyya, Villalta et al. 2007; Villalta, Nguyen et al. 2009; Villalta, Deng et al. 2011). It is well established from animal models of DMD, such as the dystrophin-deficient *mdx* mouse, that NF-κB activity exacerbates the dystrophic phenotype. Genetic or pharmacologic strategies blocking p65, the classical NF- κB DNA binding subunit, or its upstream activator, IκB kinase β (IKKβ), have been found to accelerate regeneration and reduce inflammation in *mdx* mice (Acharyya, Villalta et al. 2007). This type of approach is problematic for human DMD patients, however, given the broad and pleotropic role of NF-κB (Delfin, Xu et al. 2011; Peterson, Kline et al. 2011; Reay, Yang et al. 2011).

NF- κB suppression is thought to improve dystrophic muscle by promoting the differentiation of muscle progenitor cells. Indeed, p65 has been found to negatively regulate myogenesis through multiple mechanisms, including the induction of myofibrillary gene repressors and the stabilization of cyclinD1, which promotes cell cycle progression and blocks progenitor cell differentiation (Guttridge, Albanese et al. 1999; Wang, Hertlein et al. 2007; Dahlman, Wang et al. 2009). We have previously reported that NF-κB also negatively influences

the myogenic potential of muscle-derived stem cells (MDSCs) (Lu, Proto et al. 2012), a highly myogenic cell population with stem cell-like characteristics, including multi-lineage differentiation and self-renewal (Qu-Petersen, Deasy et al. 2002; Deasy, Gharaibeh et al. 2005; Gharaibeh, Lu et al. 2008). When compared to committed muscle precursor cells, or myoblasts, MDSCs demonstrate a higher intramuscular engraftment capability in both skeletal and cardiac muscle (Jankowski, Deasy et al. 2002; Payne, Oshima et al. 2005). Although the exact reason for the improved regenerative potential of MDSCs remains unclear, current evidence from animal studies suggests critical roles for resistance to stress (Urish, Vella et al. 2009; Drowley, Okada et al. 2010; Vella, Thompson et al. 2011) and the release of soluble factors such as vascular endothelial growth factor (Payne, Oshima et al. 2007; Ota, Uehara et al. 2011; Cassino, Drowley et al. 2012; Beckman, Chen et al. 2013). Many NF-κB target genes are involved in both survival and growth factor expression, and our earlier work supports a role for p65 in muscle cells that extends beyond differentiation. For example, following injection into injured WT hind limb muscle, we unexpectedly observed that necrosis and CD14+ inflammatory cell infiltration surrounding $p65^{+/-}$ MDSC engraftments was reduced compared WT cell engraftments (Lu, Proto et al. 2012). Thus, enhanced myogenesis may not account for all of the benefit of NF-κB blockade in *mdx* skeletal muscle.

In this investigation, we found that deleting one allele of *p65* improves both MDSC survival under oxidative stress and the MDSC anti-inflammatory capacity *in vitro* and *in vivo*. Our data demonstrate that *p65* deficiency results in the up-regulation of HGF, a protein known to have immunomodulatory properties in several tissues (Galimi, Cottone et al. 2001; Gong, Rifai et al. 2008; Coudriet, He et al. 2010). Secreted in a pro-form and sequestered in the extracellular matrix, HGF is thought to be one of the primary activators of satellite cells in injured skeletal

muscle (Tatsumi, Anderson et al. 1998; Miller, Thaloor et al. 2000; Sheehan, Tatsumi et al. 2000). Although critical during the early stages of muscle repair as both a stimulator of myoblast proliferation and potentially as a chemoattractant for inflammatory cells, an active role for HGF in the final stages of muscle repair has yet to be identified (Galimi, Cottone et al. 2001; Sisson, Nguyen et al. 2009). We investigated whether HGF has a specific role in mediating the attenuated dystrophic phenotype of *mdx*;*p65*$^{+/-}$ mice (Acharyya, Villalta et al. 2007). We found that the regeneration of the hind limb and diaphragm muscles of *mdx*;*p65*$^{+/-}$ mice at 4 weeks of age coincided with significant upregulation of HGF. Finally, shRNA mediated knockdown of HGF, delivered using a musculotropic adeno-associated virus, significantly increased inflammation and necrosis in the *mdx*;*p65*$^{+/-}$ diaphragm, thus reversing the beneficial effect of *p65* haploinsufficiency. Our findings provide new mechanistic insight into how modulation of NF-κB/p65 attenuates muscular dystrophy, and demonstrate that HGF plays a crucial role in the resolution of skeletal muscle inflammation. Thus, in the future, treatment of muscular dystrophy and other inflammatory myopathies might be achieved through modulation of HGF levels.

3.3 MATERIALS AND METHODS

Animals C57Bl/6 (wild type; WT) mice and C57BL/10ScSn-*Dmd*^{mdx}/J (*mdx*) mice were purchased from the Jackson laboratory (Bar Harbor, ME). *P65*$^{+/-}$ mice, originally characterized by Beg and colleagues (Beg, Sha et al. 1995), were bred with *mdx* mice to produce *mdx*:*p65*$^{+/-}$ and *mdx*:*p65*$^{+/+}$ mice. P65 heterozygotes were backcrossed into an *mdx* background for a minimum of 10 generations. Genotyping was carried out by PCR analysis of tail samples. Mice ranged in age from 5 days to 12 weeks. Specific ages for each experiment are described below.

All animal protocols used for these experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Cell Culture Primary WT and *p65*$^{+/-}$ MDSCs were obtained from 5 month old WT and *p65*$^{+/-}$ mice using the modified preplate method, as previously described (Gharaibeh, Lu et al. 2008). Cells were cultured in proliferation medium (PM) containing 10% fetal bovine serum, 10% horse serum, 1% Penicillin-Streptomycin, and 0.5% chick embryo extract in DMEM. RAW264.7 cells, a murine macrophage-like cell line (ATCC, Manassas, VA) were maintained and expanded in 10% fetal bovine serum and 1% Penicillin-Streptomycin in DMEM.

Retroviral vector construction and transduction of MDSCs To label cells prior to *in vivo* and co-culture experiments, MDSCs were retrovirally transduced to express nuclear-localized red fluorescent protein (RFPn). The retroviral vector was constructed with a combined CMV and long terminal repeat (LTR) promoter driving RFP followed by a nuclear localization sequence (NLS) derived from an SV40 large T-antigen. Briefly, cells were plated at 40% confluence and transduced at an MOI of 5 in culture medium supplemented with polybrene (8ug/mL, Sigma-Aldrich, Milwaukee, WI, USA). After transduction, cells were passaged approximately four times in order to ensure stable gene expression. Finally, transduced WT and *p65*$^{+/-}$ cells were selected by flow cytometry (FACSAria II, Bedford, MA, USA).

Measurement of Cell Proliferation. In triplicate, cells were plated in a 24 well collagen type I coated plate. Using a previously described live cell imaging system (LCI), 10x brightfield images were taken in ten minute intervals over a 72 hour period (Deasy, Jankowski et al. 2003). Our custom built LCI includes a Biobox incubator that sits atop a Nikon Eclipse TE 2000 U microscope stage, which is attached to a CCD camera (Kairos Instruments LLC, Pittsburgh, PA). Three locations to be imaged were randomly chosen per well, giving 9 fields of view per

population, per experiment. LCI was used to measure proliferation over 60 hours by counting the number of cells per field of view at 12 hour intervals using ImageJ software (NIH). For co-culture experiments, RFPn expressing WT or *p65*$^{+/-}$ MDSCs were plated with murine RAW264.7 (ATCC) cells at a ratio of 1:10 and incubated overnight in PM. The following day, cells were activated by exposure to 100 ng/mL LPS (Sigma-Aldrich) in PM. Using LCI, we tracked the activity of RFP-expressing MDSCs over a 60 hour period by capturing 10x brightfield and fluorescent images at ten minute intervals. Population doubling time (PDT) was calculated using a previously validated model (Deasy, Qu-Peterson et al. 2002). For each field of view per population, the mean PDT was defined as the average of PDT measurements calculated at 48 and 60 hrs.

***In vitro* Measurement of Cell Survival under Oxidative Stress.** Cells were exposed to oxidative stress induced by treatment with 250 μM hydrogen peroxide. In order to visualize cell death, propidium iodide (PI), a DNA-binding dye, was added to culture medium according to the manufacturer's protocols (BD Bioscience, San Jose, CA, USA). To block NF-κB activation, WT MDSCs were pretreated with the reversible ATP-competitive inhibitor of IKKβ, IKK-2 Inhibitor IV (EMD Millipore, 401481, Billirca, MA, USA) at 5uM for 24 hours prior to experiments. Using the LCI system described above, 10x brightfield and fluorescence images were taken in 10 minute intervals over 24 hours (Deasy, Jankowski et al. 2003). Identifying the number of PI+ cells per field of view out of the total cell number determined the percentage of cell death over time.

Cardiotoxin Muscle Injury model and Stem Cell Implantation WT (8-12 weeks old) mice were injured by the injection of 30 uL of cardiotoxin (4uM, CTX, Sigma, St. Louis, MO), as previously described (Charge and Rudnicki 2004). Twenty four hours later, $300x10^5$ RFPn

positive WT or *p65*$^{+/-}$ MDSCs were injected into the injured gastrocnemius muscle. At 24 hours, 72 hours, and 7 days post-injection, the animals were sacrificed and the hind limbs were harvested and frozen in 2-methylbutane pre-cooled in liquid nitrogen. The specimens were stored at –80°C and 10µm thick cryosections were obtained at -25°C. To examine muscle regeneration between genotypes, the gastrocnemius of 4-6 week old *p65*$^{+/-}$ and WT mice were injured with CTX as above and sacrificed at 1, 3, or 5 days post-injury, and tissues were harvested and snap frozen as described above.

Immunofluorescence and Histology Cryosections were fixed with 5% formalin for 5 minutes and blocked with 10% donkey serum for 2 hours. Slides were then incubated with one or more primary antibodies, including rabbit anti-RFP (1:200, Abcam, Cambridge, MA, USA), rabbit anti-mouse Ki67 (1:200, Abcam), rabbit anti-phospho(S9)-GSK3β (1:50, Abcam) or rat anti-CD68 (1:200, Abcam) in 10% donkey or goat serum. Next, sections were incubated with secondary antibodies including 594-conjugated anti-rabbit or anti-rat IgG (1:500, Invitrogen, Grand Island, NY, USA) and 488-conjugated anti-rabbit or anti-rat IgG (1:500, Invitrogen) in PBS for 30 minutes. We stained sections for embryonic myosin heavy chain (eMyHC) using a mouse anti-mouse eMyHC antibody (1:50, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA) with a mouse-on-mouse (M.O.M.) staining kit (Vector Labs, Burlingame, CA, USA), according to manufacturer's directions. To identify necrotic fibers, we used a biotinylated anti-mouse IgG antibody (1:300, Vector Labs) with the M.O.M. kit diluent, according to manufacturer's directions. Histological analysis was carried out by hemotoxylin and eosin staining (H&E), as previously described (Yang, Tang et al. 2012).

Image Acquisition and Processing Immunofluorescent or brightfield images were captured using an upright fluorescent microscope (Leica Microsystems Inc., Wetzlar, Germany)

equipped with a digital Retiga camera (QImaging, Surrey, Canada). Images were acquired using Northern Eclipse (Empix Imaging Inc., Cheektowaga, NY, USA) or QCapture (QImaging) and quantified using ImageJ software (NIH) or CellProfiler (Broad Institute, Cambridge, MA, USA). To analyze RFP, CD68, Ki-67, or eMyHC staining, 200x images were captured through the entire injury and engraftment area from the region of highest engraftment along the length of the muscle. To analyze CD68 and phospho(S9)-GSK3β staining, four 600x images were captured from the injured area of each muscle. To analyze IgG or CD68 staining on ZsGreen transduced muscle, 200x fluorescent images were captured from three locations along the length of sections from the medial region of each muscle. 100x, 200x, or 400x brightfield images were captured for H&E analysis, with the regions for imaging chosen in a similar manner as described above. Area was measured using Adobe Photoshop (Adobe Systems Inc., New York, NY, USA). Final images for figures were also prepared in Adobe Photoshop. We used the fluorochromes described above and no imaging medium was used.

***In Vitro* Inflammation Assay** RAW264.7 cells were plated in a six well plate with 10^5 cells per well, and then incubated for 48 hours to achieve high density. Conditioned medium was prepared by plating 10^6 MDSCs into a T-175 flask with 15mL of medium. Following a 24 hour incubation, the medium was collected and then filtered (0.22 μm). RAW264.7 cultures were washed with PBS and then cultured in conditioned medium with or without 100ng/mL LPS for 30 minutes, 3 hours, or 24 hours, at which time cell lysates were collected. To block MET, the HGF receptor, cells were pretreated with SU11274 (EMD Millipore, 448101) in DMEM supplemented with 1% penicillin-streptomycin (P/S) for 2 hours prior to exposure to CM (serum free) supplemented with SU11274.

Western Blot Cell and tissue lysates were prepared in RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (#2 and #3, 1:100, Sigma) and quantified using the Bio Rad Protein Assay (500-0001, Bio Rad, Hercules, CA, USA). Immunoblotting was performed as previously described(Lu, Proto et al. 2012). Membranes were incubated with rabbit monoclonal antibodies (CellSignaling, Danvers, MA, USA) to phospho(S9)-GSK3β, total GSK3β, or polyclonal rabbit anti-HGF (1:300, Thermo Scientific, Waltham, MA, USA) at 4°C overnight in 5% milk or BSA in TBST. Ponceau S (Sigma) staining was used to evaluate equal loading.

Realtime RT-PCR Total RNA was isolated using TRI Reagent (Sigma) and reverse transcribed using Maxima first strand cDNA synthesis kit (Thermo Scientific) according to manufacturer's protocols. Realtime PCR was carried out using the Maxima Syber Green Assay kit (Thermo Scientific) with an iQ5 thermocycler (Bio Rad). Primers were designed using PRIMER-Blast (NCBI) and can be found in Table 2.

Accession Number	Gene Name	Forward Sequence	Reverse Sequence
NM_007393.3	β-actin	CCACACCCGCCACCAGTTCG	TACAGCCCGGGGAGCATCGT
NM_031168.1	IL-6	TCTGCAAGAGACTTCCATCCAGTTGC	AGCCTCCGACTTGTGAAGTGGT
NM_008361.3	IL-1β	AAGCCTCGTGCTGTCGGACC	GCTTGGGATCCACACTCTCCAGC
NM_013693.2	TNFα	AGCCCACGTCGTAGCAAACCAC	CGGGGCAGCCTTGTCCCTTG
NM_001025257.3	VEGF	GGCTTTACTGCTGTACCTCC	GCAGTAGCTTCGCTGGTAGA
NM_011577.1	TGFβ	CTAATGGTGGACCGCAACAAC	CACTGCTTCCCGAATGTCTGA
U43428.1	iNOS	GCTGCCTTCCTGCTGTCGCA	CCTGACCATCTCGGGTGCGG
NM_010427.4	HGF	TCATATCTTCTGGGAGCCAGATGCT	GGTCCAAATTGACAATTGTAGGTGTAGT

Table 2. Primer Sequences used in Chapter 3.

Construction of HGF shRNA, AAV vector production, and AAV administration We designed two HGF-shRNAs, each based on previously reported siRNA sequences (Paranjpe,

Bowen et al. 2007; Bell, Cai et al. 2008). We tested the efficiency of HGF knockdown *in vitro* using C2C12 mouse myoblasts (ATCC). We chose the most efficient sequence (>60% reduction by western blot) for our continued experiments. Our shRNA targeted the sense sequence 5'-acg aag tct gtg aca ttc ctc-3' (position in gene sequence: nucleotide 718-738) and antisense sequence 5'-gcg gaa tgt cac aga ctt cgt-3'. The following oligos were synthesized by Invitrogen: GATC-sense-CTCGAG-antisense-TTTTTTT-G (forward) and AATTC-AAAAAAA-sense-CTCGAG-antisense-G (reverse). We chose to use AAV-9 due to its unique tropism towards skeletal muscle that results in high gene transfer efficiency (Bostick, Ghosh et al. 2007; Katwal, Konkalmatt et al. 2013). We designed an AAV construct containing a dual cassette consisting of the human U6 promotor driving HGF-shRNA, followed by a CMV promoter driving the ZsGreen reporter gene as previously described (Yang, Tang et al. 2012). An AAV vector with scrambled shRNA was designed as a control (ct-shRNA). At five days of age, we performed intraperitoneal (i.p) injection with 100uL of virus tittered at $5x10^{12}$ v.g.per mL . Four weeks later, animals were sacrificed and the skeletal muscles were harvested. Each experimental and control group contained 4-6 mice.

Statistics Data is reported as mean +/- standard error of the mean (sem). To compare 2 groups, a Student's t-test was used to determine significance. To compare three or more groups, we used a one-way ANOVA followed by Tukey post-hoc analysis. A p-value less than 0.05 was considered significant.

3.4 RESULTS

P65 haploinsufficiency improves donor cell survival in injured muscle at 3 and 7 days post-injection

Recently, we reported that genetic loss of one *p65* allele improved MDSC engraftment following intramuscular (i.m.) injection into the injured hind limb muscles of WT mice (Lu, Proto et al. 2012). Whether changes in stress resistance accounted for this finding remained unclear. To assess the potential role of NF-κB in MDSC survival and subsequent engraftment in skeletal muscle we measured the survival of WT and $p65^{+/-}$ MDSCs following i.m. transplantation using the well-established cardiotoxin (CTX) muscle injury model (Charge and Rudnicki 2004). Prior to injection, we labeled WT and $p65^{+/-}$ MSDCs by retroviral transduction to express nuclear-localized red fluorescent protein (RFPn). At 3 days and 7 days post-injection, we found significantly more $p65^{+/-}$ donor cells present compared to WT cells (**Figure 12 A, B**; $p<0.001$). The number of WT cells declined rapidly from days 1 to 3. During the same period, there was no statistically significant decline in the number of $p65^{+/-}$ cells (**Figure 12 B**; WT d3 or d7 versus d1, $p<0.05$). It is unlikely that the higher numbers of $p65^{+/-}$ cells are due to proliferation, as co-localization of RFPn with the cell cycle marker Ki67 indicated a similar fraction of proliferating donor cells between groups from days 1 to 3 (**Figure 12 A, C**).

Genetic or pharmacologic blockade of NF-κB alters MDSC resistance to oxidative and inflammatory stress

Previous investigations from our group have identified oxidative stress resistance as an important determinant of MDSC regenerative potential and a predictor of engraftment success

(Deasy, Lu et al. 2007; Urish, Vella et al. 2009). To ascertain what impact NF-κB inhibition has on MDSC resistance to oxidative stress, we conducted *in vitro* assays using live cell imaging (LCI) (Deasy, Jankowski et al. 2003). Interestingly, as shown in **Figure 12 D**, in the 24 hours following exposure to H_2O_2-induced oxidative stress, we found the survival of $p65^{+/-}$ cells to be increased compared to WT cells (76% versus 64%, $p<0.05$). To confirm if this effect was dependent on upstream IKK complex activation, we utilized a specific, ATP-competitive small molecule IKKβ inhibitor, IKK-2 inhibitor 4 (IKKi), to treat cells before H_2O_2 exposure. Following treatment, WT cell survival increased to a level similar to that of $p65^{+/-}$ cells (vs p65+/-, $p=0.57$), implicating the classical NF-κB pathway in cell survival (**Figure 12 D)**.

In addition to oxidative stress, donor cells are also confronted by an inflammatory milieu *in vivo* (Wollert and Drexler 2010). To more closely examine how inflammation might affect the expansion of the donor cell pool, we co-cultured MDSCs with RAW cells *in vitro* (ratio of 1:10). For these experiments, we again used RFP-transduced MDSCs to allow easy discrimination between cell types. We determined MDSC population doubling time (PDT) using a previously validated model of cell population growth (Deasy, Jankowski et al. 2003). In the absence of LPS, $p65^{+/-}$ MDSCs maintained a higher proliferative state compared to WT cells (PDT of 19.3 versus 31.7 hrs, $p<0.05$), as we previously reported (**Figure 12 E**) (Lu, Proto et al. 2012). However, when LPS (100ng/mL) was added to the co-culture system, the PDT of $p65^{+/-}$ cells significantly increased (19.3 to 31 hrs, $p<0.05$), indicating a reduction in the rate of cell proliferation. In contrast, WT MDSCs did not demonstrate a significant change in PDT ($p=0.817$) following the addition of LPS to culture medium. Decreasing proliferation under inflammatory conditions and/or having a higher resistance to oxidative stress, may account for the improved survival of $p65^{+/-}$ MDSCs following i.m. transplantation.

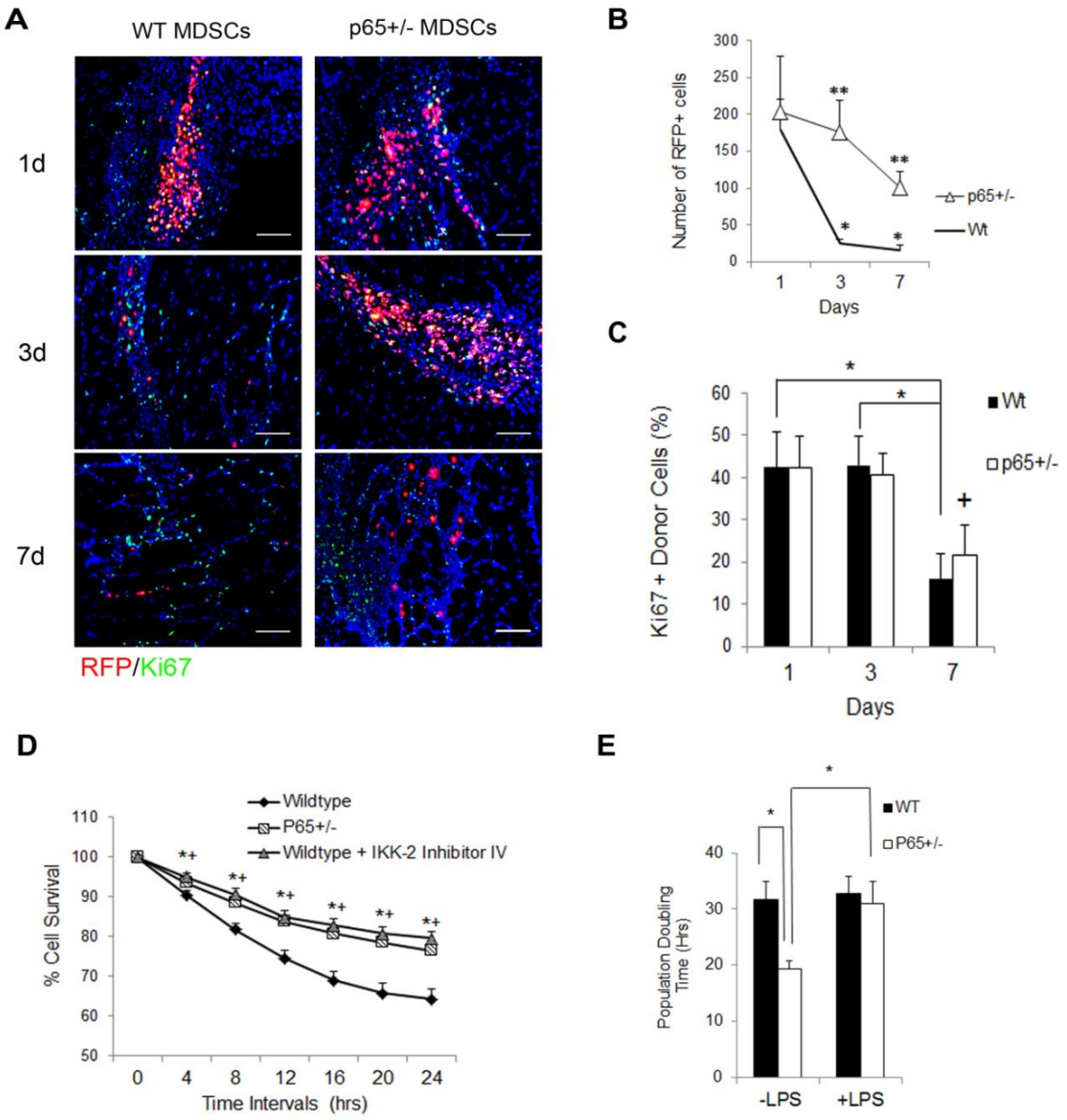

Figure 12. Survival is increased in *p65*$^{+/-}$ MDSCs *in vivo* and in *vitro*.

(A) Immunofluorescent staining of tissue sections for the proliferation marker Ki67 (green) and RFPn (donor cells, red) demonstrates reduced attrition of *p65*$^{+/-}$ MDSCs up to one week post-injection. (B) Quantification of RFPn+ cells indicated a significant decline in WT cells within the first week, while *p65*$^{+/-}$ MDSCs displayed a much slower decline in number (**versus WT, $p \leq 0.001$; *versus Day 1, $p \leq 0.05$). (C) Ki67 positivity indicated that there were no differences in proliferation at days 1 and 3 (*$p \leq 0.05$; $^{+}$versus day 1 $p \leq 0.10$). (D) LCI demonstrated that, relative to WT MDSCs, a higher number of *p65*$^{+/-}$ or IKK-2 inhibitor IV treated cells survived under H_2O_2-induced oxidative stress after 24 hrs (**p65*$^{+/-}$ versus WT, $p<0.05$; $^{+}$WT+Inhibitor versus WT, $p<0.05$). (E) When co-cultured with RAW267.4 cells (1:10) in the presence of LPS, the population doubling time of *p65*$^{+/-}$ MDSCs significantly increased, reflecting a decreased rate of proliferation. WT MDSCs demonstrated no significant changes (*$p<0.05$). For A-C: Scale bar: 100 µm. n=8-9 mice per group. Data represented in D and E as mean +/- sem for at least 3 experiments.

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$p65^{+/-}$ MDSC engraftments accelerate the resolution of inflammation and increase the number of regenerating fibers in recipient muscle relative to WT MDSC engraftments

We next identified inflammatory cell infiltration in injured muscle by immunofluorescent staining of tissue sections for the activated macrophage marker CD68 (**Figure 13 A**). One week post-transplantation we found $p65^{+/-}$ cell engraftments to be associated with reduced numbers of macrophages compared to WT MDSC engraftments (**Figure 13 B**). This is in confirmation of our previous finding that $p65^{+/-}$ engraftments were associated with reduced numbers of CD14+ monocytes at one week post-injury, relative to WT engraftments (Lu, Proto et al. 2012). Inflammatory resolution is closely associated with tissue regeneration. We examined muscle regeneration at one week post-transplantation by staining for the embryonic isoform of myosin heavy chain (eMyHC), expressed only by immature, regenerating muscle fibers. eMyHC+ fibers were easily identified in and around the donor cell engraftments (**Figure 13 C**)**.** Although not statistically significant, $p65^{+/-}$ engraftments tended to have greater numbers of associated total eMyHC+ myofibers (**Figure 14 A**, p=0.12), with the majority being host-, rather than donor-derived (**Figure 14 B,** p=0.10).

P65 haploinsufficiency increases the suppressive effect of MDSC-conditioned medium on IL-6 induction in LPS-activated RAW264.7 cells.

A number of soluble factors regulating inflammation and regeneration are under the control of NF-κB; thus, we hypothesized that MDSC-secreted factors differed between WT and $p65^{+/-}$ cells (Karin and Lin 2002; Sen and Smale 2010). To answer this question, we first compared the ability of WT- and $p65^{+/-}$ MDSC-conditioned medium (CM) to modulate the

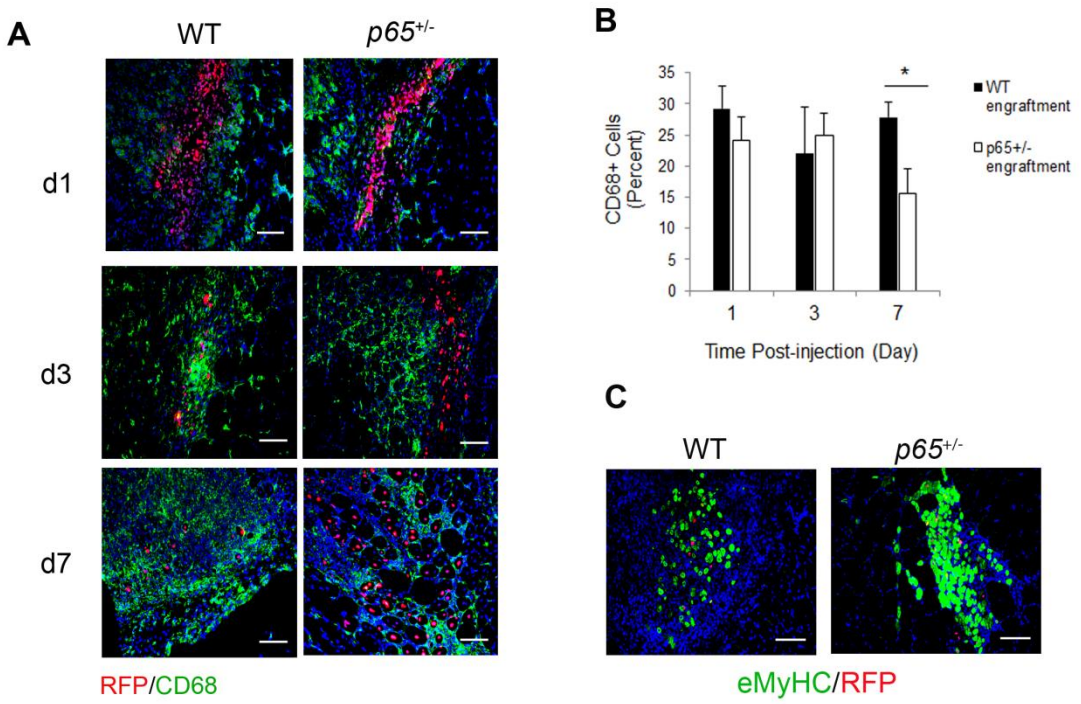

Figure 13. Donor *p65*$^{+/-}$ MDSC engraftments promote the repair of recipient muscle.

(A) Immunofluorescent staining of tissue for the macrophage marker CD68 (green) indicated that injuries were infiltrated by macrophages within 24 hrs post-injection (48 hrs post-injury), which continued to persist at 7 days (bottom). (B) Quantificantion of CD68 positivity within 20x images indicates that *p65*$^{+/-}$ MDSC engraftments have significantly less CD68+ cells present at 7 days. (C) eMyHC+ fibers (green) could be identified in or around donor cell engraftments at 7 days. n=3-4 mice per group. Scale bar: 100 µm

expression of the prototypical cytokines *Tnfα*, *IL1β*, and *IL6* in a murine macrophage cell line (RAW264.7; RAW) stimulated with endotoxin (LPS). Briefly, RAW macrophages were simultaneously exposed to LPS and conditioned medium (CM) from either WT or *p65*$^{+/-}$ MDSCs. Treatment with or without LPS in fresh medium served as controls. Exposure to WT- and *p65*$^{+/-}$-CM was associated with reductions in all three factors, but we found that it was the RAW cells stimulated in *p65*$^{+/-}$ -CM that expressed the lowest amount of *IL-6* (a 10 fold decrease in *p65*$^{+/-}$-CM versus WT-CM, $p<0.05$) (**Figure 15 A**). These results demonstrate that immunomodulatory factors are secreted by MDSCs and are at least partially under the control of the NF-κB transcription factor p65.

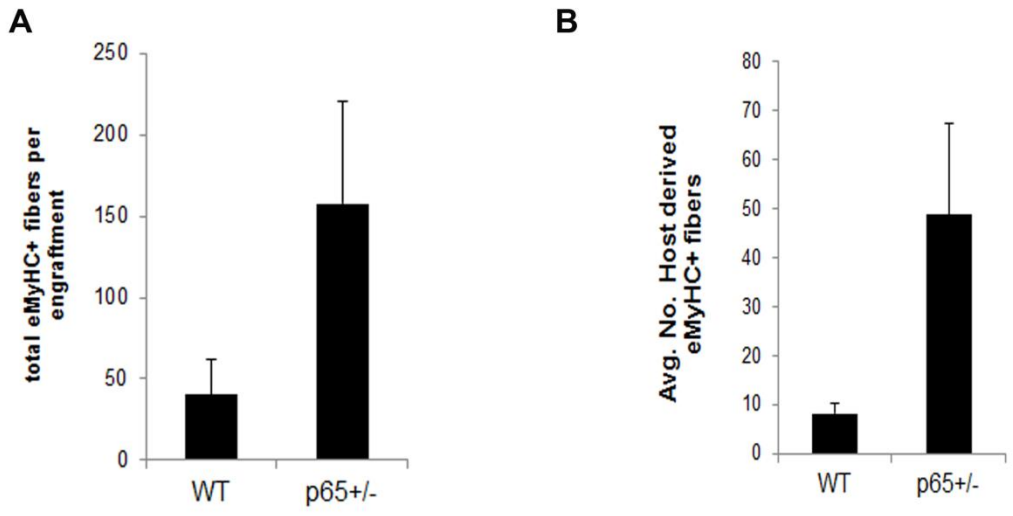

Figure 14. Muscle regeneration is increased by *p65*$^{+/-}$ MDSC engraftment

(A) On average, engraftments of *p65*$^{+/-}$ MDSCs had a higher total number of eMyHC+ fibers relative to WT engraftments (p=0.12). (B) The number of host-derived eMyHC+ fibers per field of view (200x images) was higher in *p65*$^{+/-}$ engraftments compared to WT MDSC engraftments (p=0.10). n=7-8 mice per group.

Factors in MDSC-conditioned medium increase Ser9 phosphorylation on GSK3β

In macrophages, a subset of inflammatory genes, including IL-6, requires the activity of the serine/threonine kinase glycogen synthase kinase 3β (GSK3β) for efficient gene transcription (Steinbrecher, Wilson et al. 2005). Downstream from the activation of receptor tyrosine kinases, GSK3β is a target for phosphorylation by proteins such Akt, which serves to inactivate GSK3β (Martin, Rehani et al. 2005; Dogra, Changotra et al. 2006; Beurel, Michalek et al. 2010). Inactivation of GSK3β by phosphorylation at Ser9 acts as a regulatory switch whereby cytokine gene expression is attenuated (Beurel, Michalek et al. 2010). By western blot, we found that RAW264.7 cells activated in *p65*$^{+/-}$-CM demonstrated a striking +3.5-fold ($p \leq 0.05$) increase in

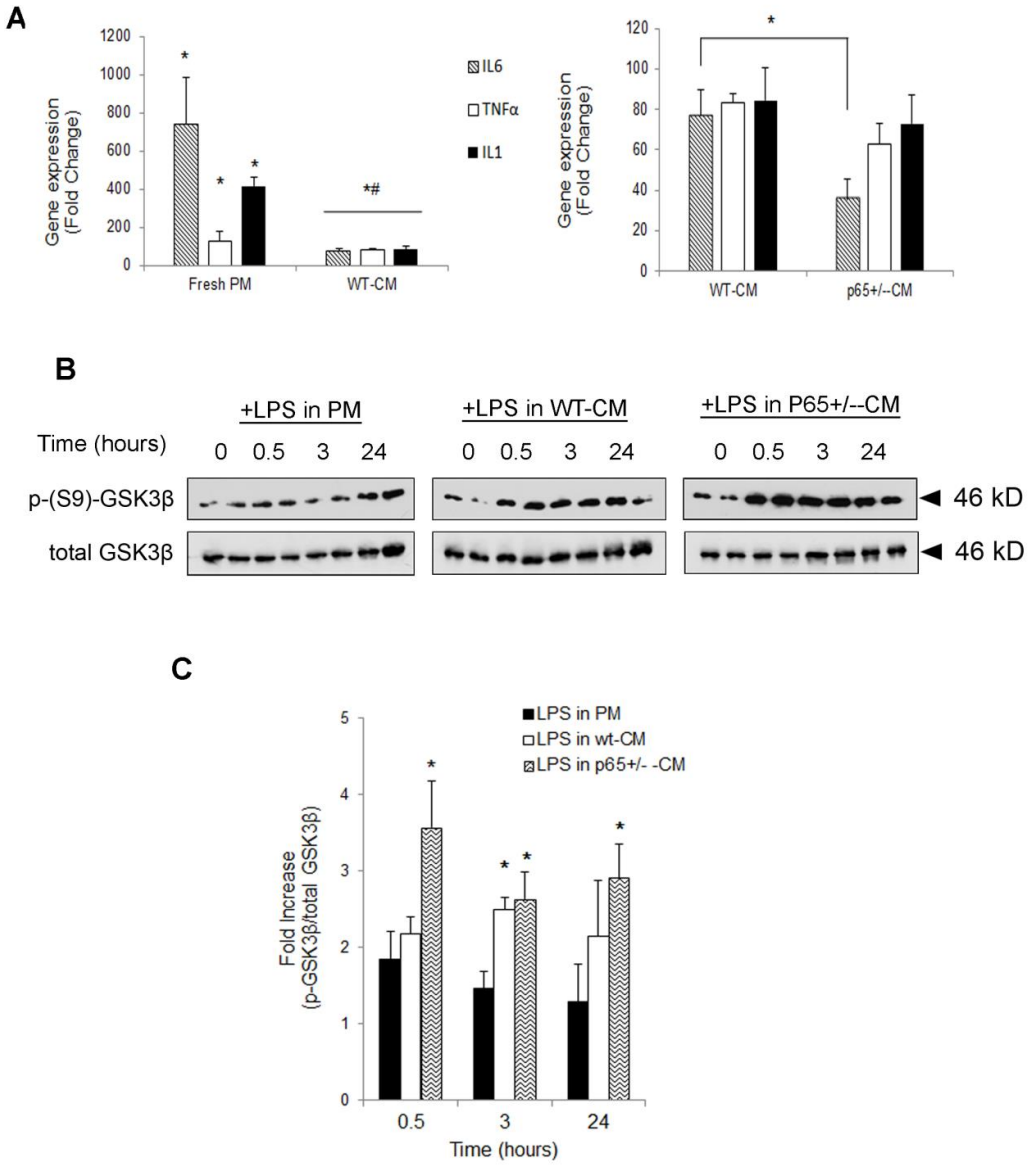

Figure 15. MDSC conditioned medium reduces cytokine expression in LPS-activated RAW264.7 cells and is associated with inactivation (Ser9 phosphorylation) of GSK3β

(A) Real time RT-PCR demonstrated that IL-1, TNFα, and IL-6 expression was attenuated in both WT- and $p65^{+/-}$-CM treated groups, with IL-6 significantly more decreased by $p65^{+/-}$-CM compared to WT-CM (*versus No LPS, p≤0.05; #versus +LPS, p≤0.05; +versus LPS+WT-CM, p≤0.05). (B) Western blot demonstrated that activation of RAW264.7 cells in PM induces an increase in pS9-GSK3β within 30 minutes, a response which was amplified in both WT- and $p65^{+/-}$-CM. (C) Densitometric analysis revealed that when activated in $p65^{+/-}$-CM, the fraction of pS9-GSK3β increased by +3.5-fold in 30 minutes, an amount significantly higher than WT-CM and PM groups (*p≤0.05). Data represented as mean +/- sem of at least 3 independent experiments.

GSK3β phosphorylation within 30 minutes and remained at +2.9-fold ($p \leq 0.05$) up to 24 hours. In contrast, when activated in WT-CM, the fraction of pS9-GSK3β did not peak until 3 hours (approximately +2.5 fold, $p \leq 0.05$) and then declined over the next 24 hours (**Figure 15 B, C**). In fresh medium, p-GSK3β increased by approximately +2-fold before decreasing over the next 24 hours (**Figure 15 B, C**). Based on these data, *p65*$^{+/-}$-CM induces RAW265.7 pS9-GSK3β more strongly than WT-CM, resulting in a greater suppression of IL-6.

Pharmacologic inhibition of the HGF receptor, MET, reverses the effect of p65*$^{+/-}$*-CM on GSK3β inactivation in RAW264.7 cells

To identify candidate anti-inflammatory factors differentially expressed by *p65*$^{+/-}$ MDSCs, we began with a number of genes known to have immunomodulatory properties in mesenchymal stem cells, including *Tgfb1*, *Vegf*, *Hgf* , *IL-4*, *IL-10*, and *Inos* (Nauta and Fibbe 2007). Of these factors, HGF to be significantly upregulated (**Figure 16 A**, $p \leq 0.05$). Although detected in whole skeletal muscle, IL-4 and IL-10 transcripts were detected in neither WT nor *p65*$^{+/-}$ MDSCs (data not shown). HGF has been reported to modulate the IL-6 production in activated macrophages, and thus act as an anti-inflammatory factor. The anti-inflammatory activity of HGF/MET is thought to occur through downstream inactivation GSK3β, a mechanism that would be in line with our findings.To assess the relative importance of HGF, we blocked the activation of its receptor, MET, using SU11274 (Sigma), a small molecule selective inhibitor. In order to eliminate potential MET activation by serum-containing medium, we carried these assays out under serum-free conditions. As shown in **Figures 16 B and C**, GSK3β phosphorylation 30 minutes post-exposure to LPS in *p65*$^{+/-}$-CM, was attenuated by MET

inhibition in a dose dependent manner. In contrast SU11274 had minimal effect in LPS and serum free medium alone, suggesting that HGF was likely mediating this response.

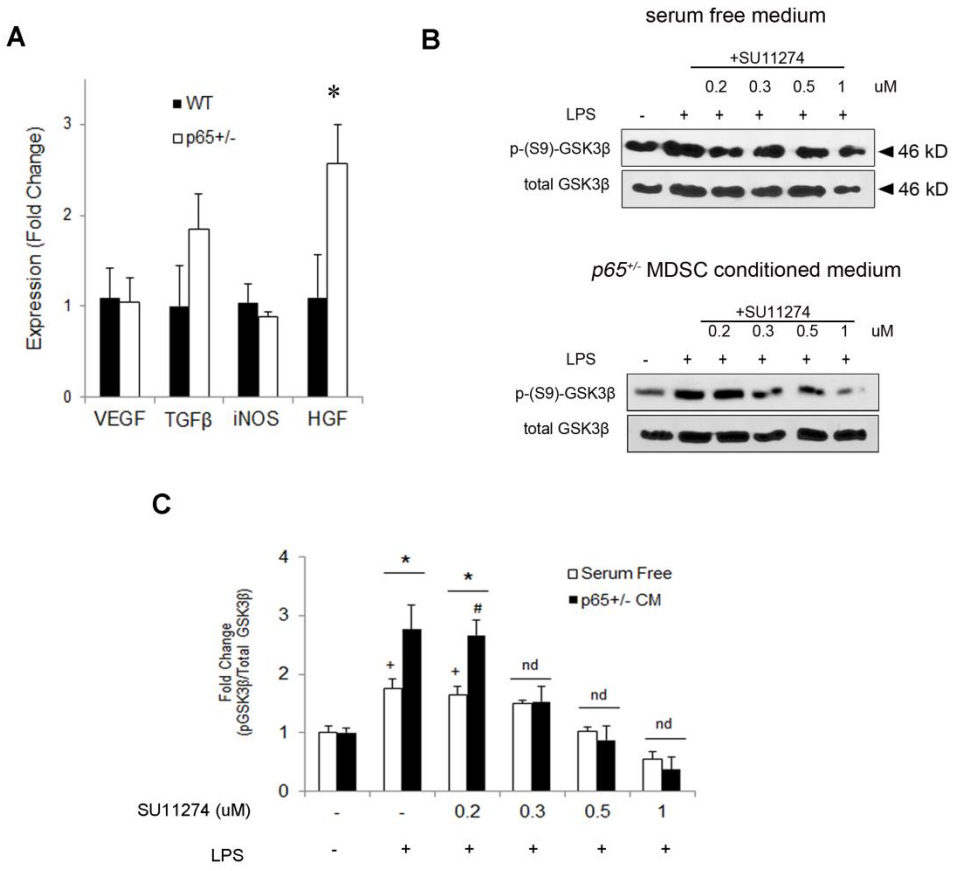

Figure 16. Decreased NF-κB activity is associated with up-regulated HGF expression in MDSCs.

(A) Real time RT-PCR analysis revealed that HGF is significantly up-regulated in *p65*$^{+/-}$ compared to WT MDSCs. (B) Inhibition of the HGF receptor, MET, blocks the induction of GSK3β phosphorylation in RAW264.7 cells exposed to LPS in *p65*$^{+/-}$ -CM after 30 minutes. (C) Inhibition of pS9-GSK3β occurred in a dose dependent manner following treatment with SU11274 ($p<0.05$; **p65*$^{+/-}$ CM vs SF; +SF vs SF Ctrl; #CM vs SF Ctrl). Data represented as mean +/- sem of at least 3 independent experiments.

Accelerated regeneration of p65$^{+/-}$ muscle is associated with elevated HGF and inactivation of GSK3β in macrophages.

To identify if an HGF/Met/GSK3β pathway is activated during muscle regeneration *in vivo*, we conducted CTX muscle injury experiments on the hind limb muscles of 4-5 week old *p65*$^{+/-}$ and WT mice. The injured muscles were harvested at 1, 3, and 5 days post-injury. We measured HGF expression over this period and found it to be significantly higher in *p65*$^{+/-}$ muscle 3 days post-injury (**Figure 17 A**). This coincided with *de novo* fiber formation, indicated by H&E staining of tissue cross sections (**Figure 17 B, middle panel, arrows**). Consistent with the earlier findings of Archaaya and colleagues (2007) regarding accelerated muscle regeneration in *p65*$^{+/-}$ mice, the mononuclear cell infiltrate was greatly reduced at 5 days post-injury (**Figure 17 B, bottom panel**) (Acharyya, Villalta et al. 2007). Slightly higher levels of HGF mRNA were detected in uninjured *p65*$^{+/-}$ muscles, but this was not statistically significant ($p=0.09$). Similarly, western blot demonstrated *p65*$^{+/-}$ muscle to have a modest, but not significant increase in total basal HGF protein (**Figure 17 C**). Finally, by western blot we also observed that Ser9-phosphorylation of GSK3β occurred in both WT and *p65*$^{+/-}$ skeletal muscle following injury, but was sustained longer (5 days) in *p65*$^{+/-}$ mice (**Figure 17 D, E**). Although informative, western blot analysis of pS9-GSK3β using whole skeletal muscle extracts did not allow us to specifically examine macrophages. Thus, we next stained tissue sections for CD68 and phospho-GSK3β and examined the number of dual positive macrophages (**Figure 17 F**). A substantially higher number of phospho-GSK3β+/CD68+ macrophages was found in *p65*$^{+/-}$ muscle at all three time points tested (**Figure 17 G**, $p \leq 0.05$). However, we noted no significant difference in the total number of infiltrated CD68+ macrophages (data not shown). These results demonstrate that the

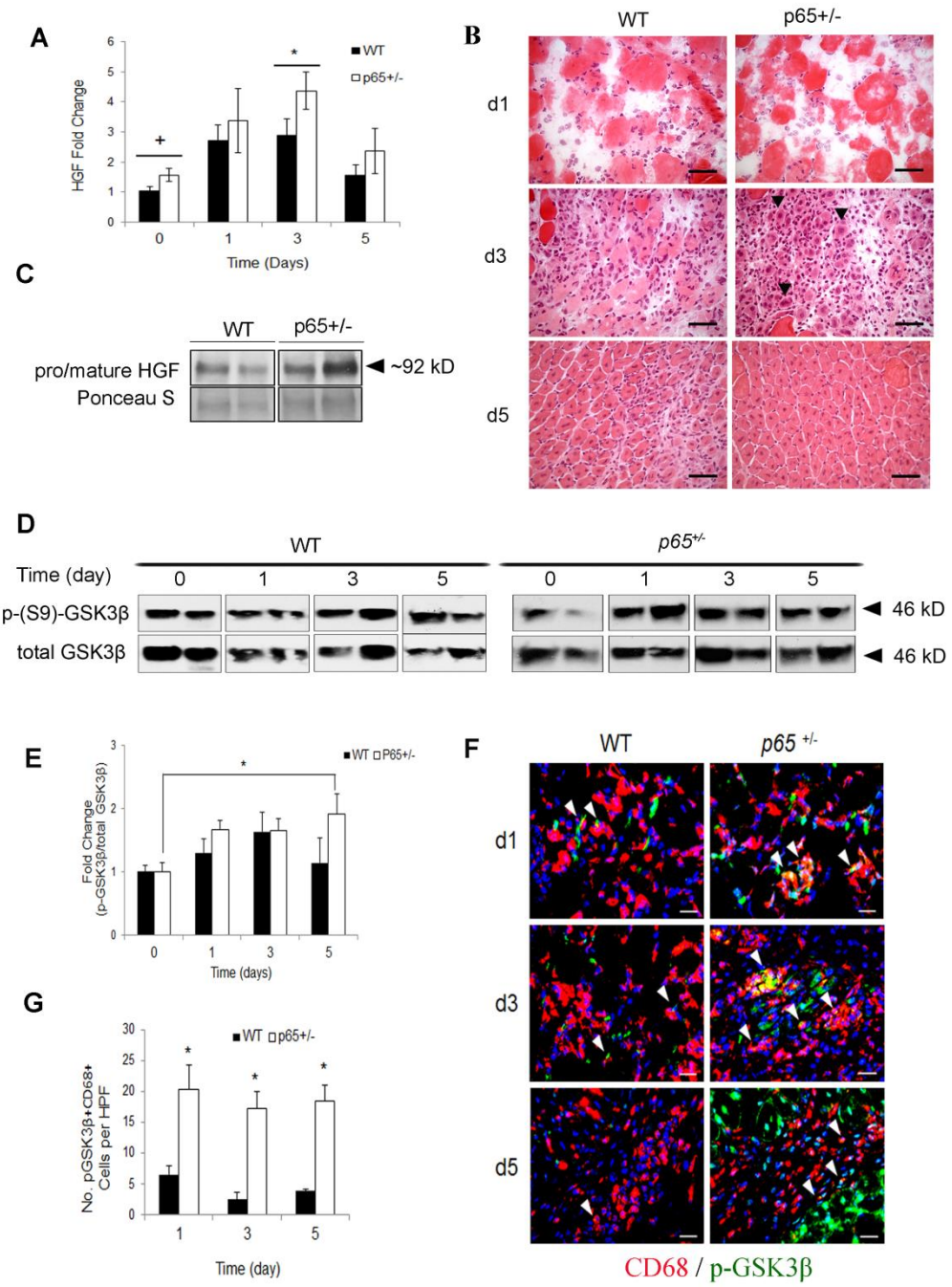

Figure 17. Accelerated regeneration correlates with HGF up-regulation.

(A) HGF expression is significantly up-regulated in *p65*$^{+/-}$ muscle at 3 days post-CTX injury (*p≤0.05 vs. 3d WT; +p<0.10 vs. d0 WT). (B) H&E staining indicate that compared to WT muscle, the gastrocnemius muscle of *p65*$^{+/-}$ mice regenerates more rapidly (arrows) following CTX injury. (C) Nonreducing SDS-PAGE and western blot analysis of muscle extracts shows a slight increase in total HGF in uninjured *p65*$^{+/-}$ muscles. (D) Phospho-GSK3β is maintained at 5 days post injury in *p65*$^{+/-}$ muscle, while in WT muscle a (E) significant change in phosphorylation could not be detected. The displayed immunoblot images from extracts of WT and *p65*$^{+/-}$ muscles are all taken from the same membranes, respectively. (F) p-GSK3β+ macrophages were identified in injured skeletal muscle by immunofluorescent co-staining for pS9-GSK3β (green) and CD68 (red, arrow). (G) Quantification as the number of p-GSK3β+ macrophages per high power field (HPF, 600x) indicated that a significantly higher number of p-GSK3β+/CD68+ macrophages were found in *p65*$^{+/-}$ skeletal muscle compared to WT skeletal muscle at 1, 3, and 5 days post injury (p≤0.05). For A-E, Scale bar: 50 μm, n=6-8 mice per group. For F and G, Scale bar: 20 μm, n=3 mice per group

accelerated regeneration of *p65*$^{+/-}$ skeletal muscle correlates with HGF up-regulation in whole muscle and GSK3β inactivation in macrophages.

HGF is upregulated in mdx;p65*$^{+/-}$ *skeletal muscle and is associated with an attenuated dystrophic phenotype

At this point, we hypothesized that the accelerated inflammatory clearance and muscle regeneration found in *p65*$^{+/-}$ mice may be partially attributed to HGF. Therefore, we next sought to determine if elevation of HGF contributes to the reported improved phenotype of *mdx;p65*$^{+/-}$ mice (Acharyya, Villalta et al. 2007). We examined HGF expression in the gastrocnemius (GAS) muscles of *mdx*;*p65*$^{+/-}$ and *mdx;p65*$^{+/+}$(*mdx*) littermates during both the degenerative (~4 weeks) and regenerative phases (~6 weeks) of the dystrophic pathology. Strikingly, HGF was up-regulated +5.7 fold in the *mdx;p65*$^{+/-}$ mice at four weeks of age compared to age-matched WT mice (**Figure 18 A,** $p<0.05$). In contrast, HGF expression in *mdx* GAS only increased +2.7 fold compared to WT mice. At this time, H&E staining of *mdx*:*p65*$^{+/-}$ GAS tissue revealed a reduced mononuclear cell infiltrate and significantly enhanced regeneration compared to *mdx* littermates, quantified as the number of centrally nucleated fibers (**Figure 18 B, C,** $p<0.05$) (Acharyya, Villalta et al. 2007). By six weeks, we detected no significant difference between HGF expression or centrally nucleated fibers in *mdx* and *mdx*;*p65*$^{+/-}$ GAS (**Figure 18 A, C).** We also examined muscle tissue at 1 week of age, before the onset of muscle degeneration, and found no significant differences between either of these two genotypes compared to WT muscle **(Figure 18 A).** This demonstrated that upregulation of HGF occurred following the onset of muscle degeneration and was associated with the regenerative stage of the *mdx* dystrophic pathology.

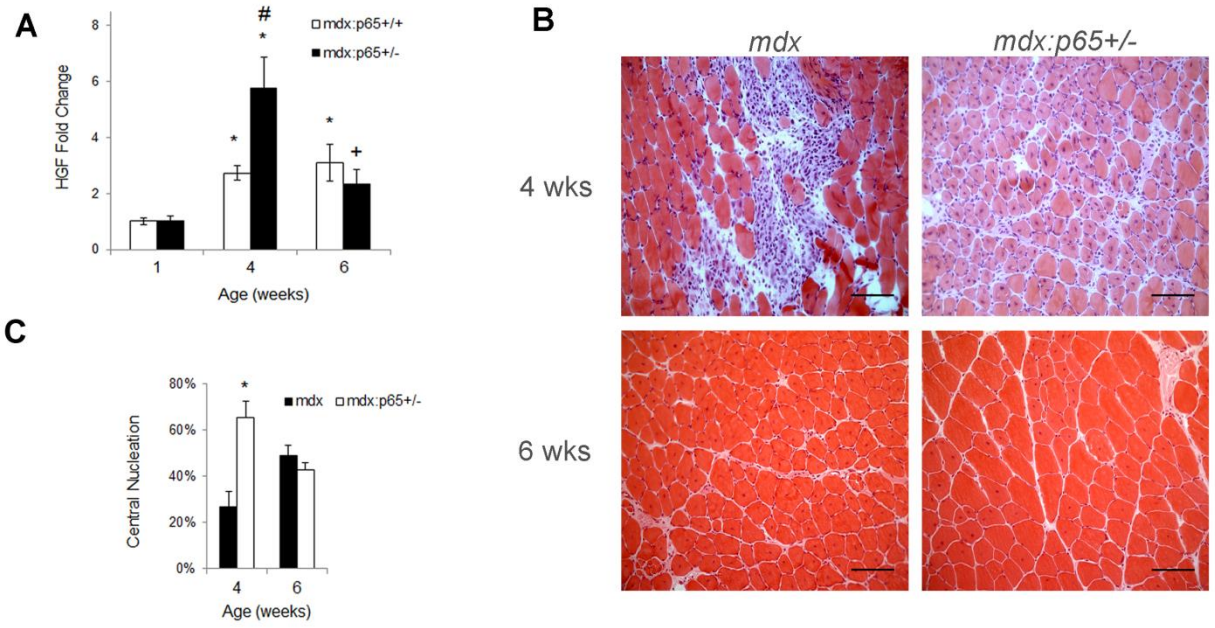

Figure 18. Improved regeneration of *mdx;p65*$^{+/-}$ skeletal muscle at 4 weeks of age correlates with significantly up-regulated HGF expression.

(A) Real time RT-PCR demonstrated that HGF expression was elevated in *mdx:p65*$^{+/-}$ muscle at 4 weeks, correlating with enhanced regeneration, quantified as the percent of nucleated fibers in (C). (B) Representative H&E staining of hind limb muscle from four week old *mdx* and *mdx:p65*$^{+/-}$ mice demonstrated that p65 haploinsufficiency is associated with fiber regeneration during what is typically the degenerative phase of the dystrophic phenotype. By six weeks of age, larger fibers are evident in *mdx*:*p65*$^{+/-}$ tissues. (*versus WT, $p \leq 0.05$; +versus WT, $p \leq 0.10$; #versus *mdx*, $p \leq 0.05$). Scale bar: 100 µm. n=4-6 mice per group.

In vivo silencing of HGF reverses the ameliorated phenotype of mdx;p65$^{+/-}$ *mice*

The question remained whether HGF upregulation in dystrophic skeletal muscle is a component of the mechanism of action for anti-NF-κB therapies. It is possible that HGF upregulation is actually secondary to regeneration, as it has been reported to be expressed by differentiating *mdx* muscle fibers (Honda, Abe et al. 2010). The link between NF-κB/p65 and HGF, however, was initially identified in uncommitted stem cells. Thus, we hypothesized that HGF upregulation precedes and subsequently promotes muscle regeneration. To test this hypothesis, shRNA targeting HGF was packaged in a musculotropic adeno-associated viral (AAV) vector. At 5 days of age, an AAV vector encoding the ZsGreen reporter gene and either

shRNA targeting HGF (HGF-shRNA) or scrambled, control shRNA (ct-shRNA), was delivered by i.p. injection to *mdx;p65*$^{+/-}$ mice and *mdx* littermates. Four weeks post-gene transfer, mice were sacrificed and their skeletal muscles and soft tissues were harvested. As indicated by ZsGreen expression, we observed high gene transfer efficiency in the fore limbs, hind limbs, and diaphragm muscle of treated mice. (**Figure 19**). To verify knockdown, we determined HGF expression by real time RT-PCR in both the diaphragm (DIA) and GAS muscles (**Figure 20A,**

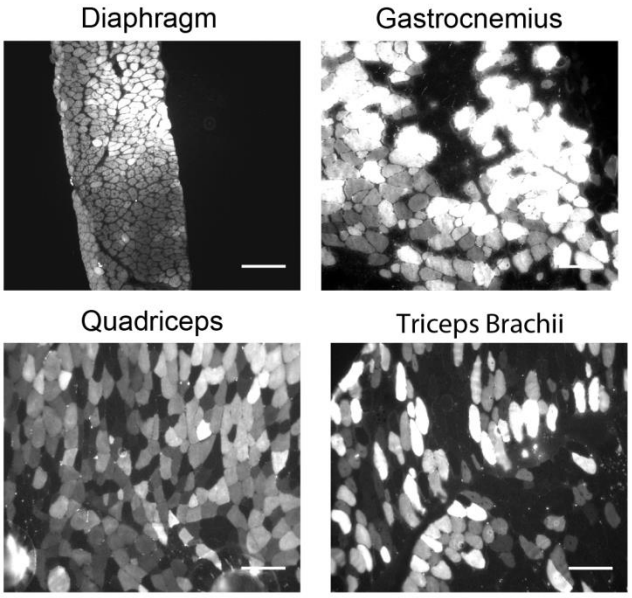

Figure 19. Transgene expression following AAV delivery.

IP injection of AAV efficiently delivered the reporter transgene to the musculature body wide. Four weeks post injection, transduced muscle fibers, identified by the expression of ZsGreen, were detected in muscles of the upper (triceps) and lower (quadriceps, gastrocnemius) limbs as well as the diaphragm. Scale bar: 200 µm.

upper). Similar to what we had found in untreated mice (**Figure 18 A**), HGF expression was up-regulated approximately +2 (p=0.08) and +3.3 (p=0.06) fold in the DIA and GAS muscles, respectively, of ct-shRNA *mdx*:*p65*$^{+/-}$ mice compared to ct-shRNA *mdx*. Treatment with HGF-shRNA reduced HGF by approximately -2 and -4 fold in *mdx;p65*$^{+/-}$ DIA and GAS, respectively

($p \leq 0.05$, HGF-shRNA vs ct-shRNA). Thus, HGF mRNA levels in the treated *mdx;p65*$^{+/-}$ DIA and GAS were similar to those of the ct-shRNA *mdx* mice (0.96 and 0.83, respectively). Paradoxically, HGF-shRNA treatment did not significantly decrease HGF expression in the DIA or GAS muscles ($p=0.80$ and $p=0.20$, respectively) of *mdx* mice. This may be due to a low level of HGF in *mdx* skeletal muscles, relative to those of *mdx*;*p65*$^{+/-}$, or more likely, given the higher amounts of myeloid cells in *mdx* versus *mdx;p65*$^{+/-}$ skeletal muscle, the escape of non-myogenic cells from AAV mediated transduction. Regenerating fibers, identified by central nucleation, also exhibited ZsGreen expression, indicating the transduction of skeletal muscle progenitor cells. Indeed, the lack of colocalization between CD68 and ZsGreen demonstrated that myeloid cells were not significantly transduced. Collectively, this indicates that we preferentially transduced skeletal muscle progenitor cells and muscle fibers (**Figure 20A, lower**).

relative to that of *mdx*;*p65*$^{+/-}$, in combination with the escape of non-myogenic cells from transduction.

HGF-shRNA treated skeletal muscle demonstrated striking morphological changes compared to control tissues expressing only ZsGreen. In particular, increased degeneration of both the DIA and GAS indicated a reversal of histological improvements associated with loss one *p65* allele (**Figure 20 B, D**). Despite that the decrease in HGF was not statistically significant in the *mdx* group, we still detected histological changes in treated skeletal muscle (**Figure 20 D**). We next carried out a closer examination of the diaphragm muscle, as respiratory failure is a leading cause of DMD patient death. We measured the extent of degeneration and found that HGF-shRNA treatment significantly increased the lesion area of *mdx*;*p65*$^{+/-}$ diaphragm, such that the size of lesions resembled those of *mdx* mice (**Figure 20 C).** Similarly,

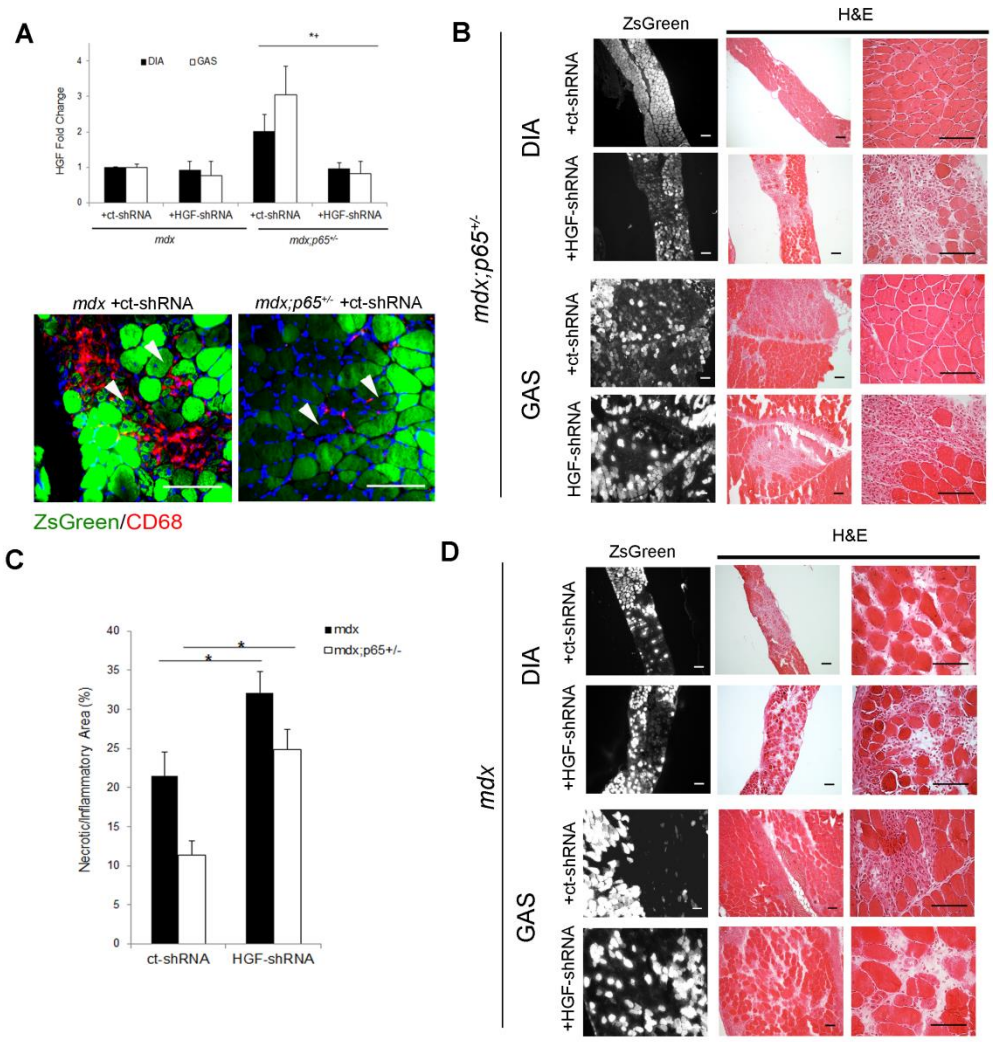

Figure 20. Silencing of HGF worsens the histopathology of *mdx;p65*$^{+/-}$ skeletal muscle.

(A) After four weeks, HGF expression was significantly reduced in the DIA and GAS muscles of HGF-shRNA treated *mdx;p65*$^{+/-}$ mice compared to the ct-shRNA treated group. (top,$p \leq 0.05$, +shHGF vs. ct-shRNA; *GAS,$^{+}$DIA). ZsGreen-expressing centrally nucleated fibers indicate that progenitor cells were successfully transduced (bottom, arrow). Muscle tissue sections of treated *mdx;p65*$^{+/-}$ mice (B) and *mdx* mice (D) demonstrated an exacerbation of dystrophic pathology in both DIA (top) and GAS (bottom). (C) 100x H&E images of the diaphragm were used to determine the necrotic/inflammatory lesion area (expressed as percent of total) within muscle cryosections. This revealed that silencing of HGF significantly increased the non-muscle area in the diaphragm of both *mdx* and *mdx;p65*$^{+/-}$ mice (*$p \leq 0.05$). Scale bar: 100 µm. n=4-6 mice per group.

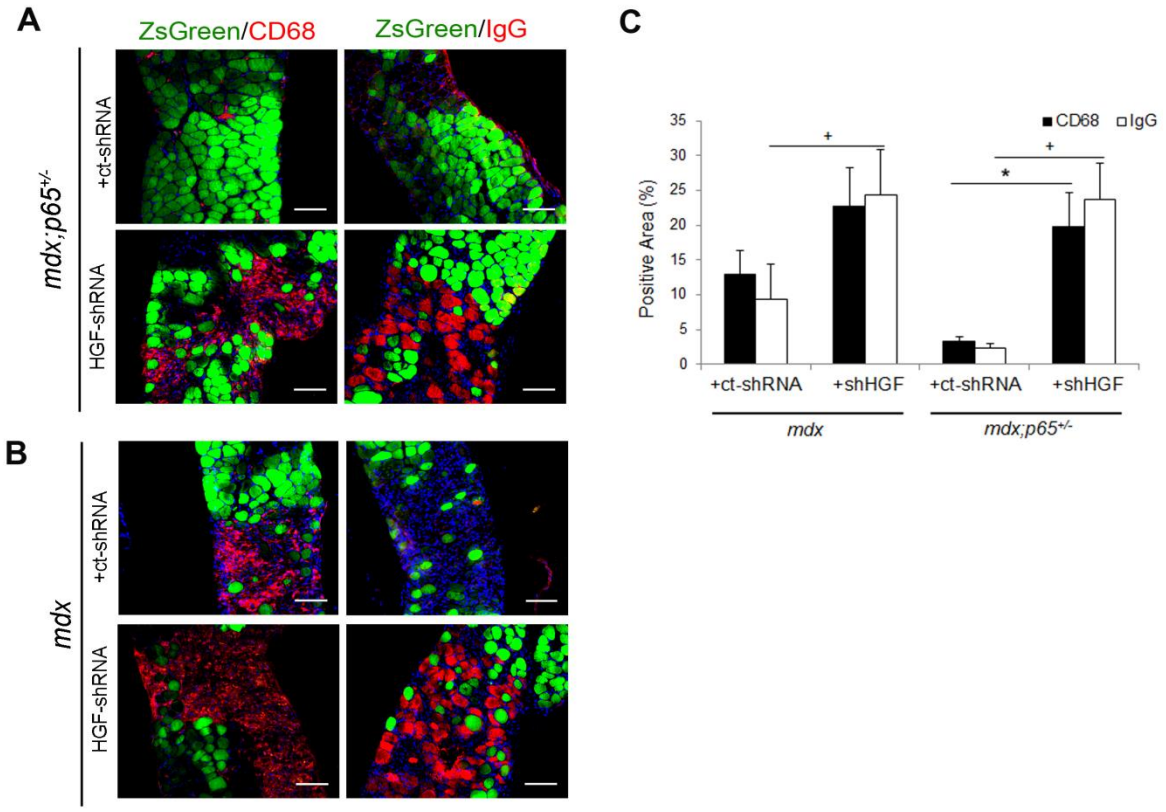

Figure 21. Silencing of HGF exacerbates inflammation and necrosis of *mdx* and *mdx;p65*$^{+/-}$ mice.

(A) Immunofluorescent staining revealed a striking increase in CD68+ macrophages (left) and IgG+ necrotic fibers (right) in *mdx;p65*$^{+/-}$ treated with HGF-shRNA compared to ct-shRNA *mdx;p65*$^{+/-}$. (B) In contrast, HGF-shRNA treated *mdx* mice did not demonstrate a statistically significant increase in inflammation (left), but did demonstrate a significant increase in fiber necrosis (right), indicated in (C). (*p≤0.05 vs CD68 in ct-shRNA group; +p≤0.05 vs. IgG in ct-shRNA group). Scale bar: 100 µm. n=4-6 mice per group.

the lesion area of *mdx* diaphragm also increased following HGF-shRNA treatment. These regions were found to contain both necrotic fibers and infiltrating macrophages, indicated by IgG and CD68 immunostaining, respectively (**Figure 21 A, B**). Notably, we found that the CD68+ positive area in HGF-shRNA treated *mdx* diaphragm lesions increased, but not significantly; however, necrotic areas significantly increased following treatment with HGF-shRNA (p≤0.05) (**Figure 21 B, C**). Both inflammatory and necrotic lesions significantly increased in *mdx;p65*$^{+/-}$

diaphragm following HGF silencing ($p \leq 0.05$) (**Figure 21 A, C**). Therefore, it is unlikely that HGF expression is secondary to regeneration. On the contrary, HGF would seem to be critical for muscle repair. Taken together, the results of this study indicate that HGF is critical for the beneficial effects of NF-κB/p65 blockade on dystrophic skeletal muscle.

3.5 DISCUSSION

The preponderance of evidence suggests that the maintenance of a chronic inflammatory state exacerbates muscle injury and accounts for a significant portion of muscle damage in DMD. The NF-κB pathway is central to inflammation and has been found to be dysregulated in muscular dystrophy (Monici, Aguennouz et al. 2003). In an earlier investigation, Archaryya and colleagues (2007) found that deleting one *p65* allele significantly alleviated the dystrophic phenotype of *mdx* mice (Acharyya, Villalta et al. 2007). Through the conditional knockout of IKKβ in either myeloid cells or skeletal muscle, they attributed this improvement to a reduction in inflammation and an enhancement in myogenesis by progenitor cells, respectively. Indeed, the p65 subunit of NF-κB has been found to be a potent suppressor of myogenic progenitor cell differentiation (Guttridge, Albanese et al. 1999; Acharyya, Villalta et al. 2007; Wang, Hertlein et al. 2007; Dahlman, Wang et al. 2009; Lu, Proto et al. 2012). In this study, using primary MDSCs, we investigated whether the beneficial effects of NF-κB inhibition on skeletal muscle results from more than enhanced myogenesis. By using AAV-mediated expression of targeting shRNA, we were able to demonstrate that HGF is critical for alleviating muscular dystrophy in mdx mice haploinsufficient for *p65*. Based on our results and summarized in **Figure 22**, we propose that NF-κB blockade on skeletal muscle not only promotes myogenic differentiation by

aiding lineage progression, but also by increasing cell survival and increasing HGF expression, which acts locally to modulate inflammation. Additionally, HGF might also directly or indirectly influence muscle fiber integrity, as HGF silencing significantly increased fiber necrosis (mouse IgG+ muscle fibers, **Figure 21**).

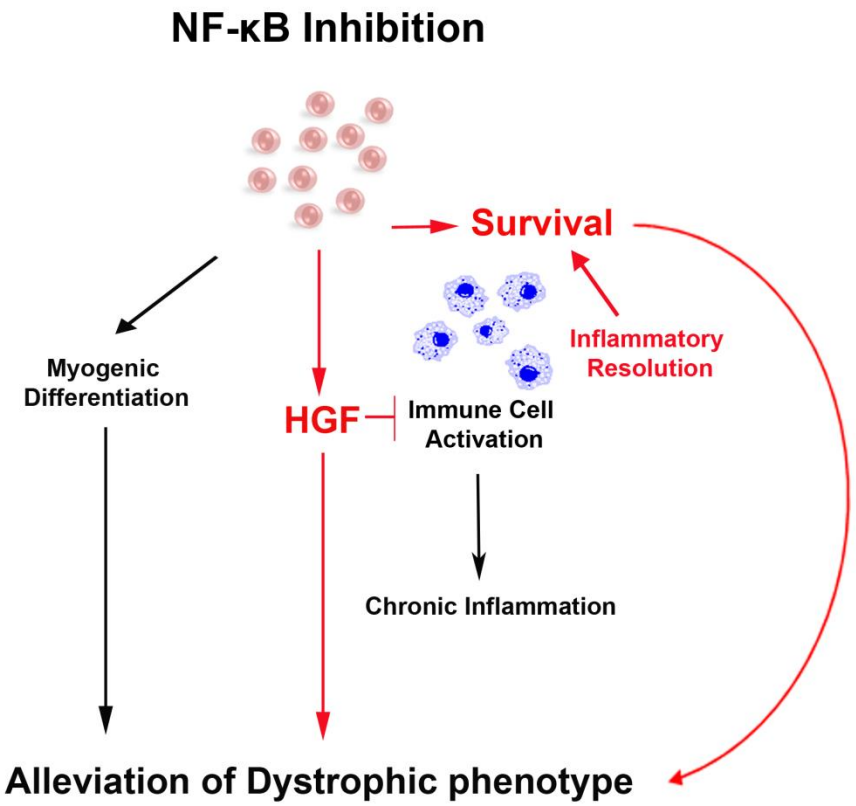

Figure 22. Model of the effects of NF-κB inhibition on muscle stem and progenitor cells.

We found that blocking classical NF-κB by either pharmacologic IKKβ inhibition or genetic depletion of p65 improved MDSC survival under oxidative stress *in vitro* and following i.m. transplantation *in vivo*. Paradoxically, NF-κB target genes are typically classified as pro-survival (Karin and Lin 2002). However, several reports have indicated that depending on the

stimulus, the p65 subunit of NF-κB can repress, rather than induce, anti-apoptotic gene expression (Campbell, Rocha et al. 2004; Wu and Miyamoto 2008). Although speculative, it is possible that a related mechanism mediates muscle cell death during oxidative stress. This is of particular relevance to DMD, as signs of oxidative stress can be observed in *mdx* mice even prior to the onset of muscle necrosis (Whitehead, Yeung et al. 2006). This is of particular relevance to DMD, as signs of oxidative stress can be observed in *mdx* mice even prior to the onset of muscle necrosis. Additionally, during inflammation, lysosomal enzymes and free radicals produced by activated leukocytes can escape inflammatory cells, damaging surrounding cells and exacerbating necrosis. Reactive oxygen species can be also be generated by muscle cells in response to TNFα, which may in turn mediate muscle ring finger 1 (MuRF1) and atrogin expression, two muscle specific E3 ubiquitin ligases that induce myofibrillary protein degradation (Li, Schwartz et al. 1998).

In this investigation, we have identified HGF as an important candidate for immunomodulation in skeletal muscle. HGF is secreted from cells in a pro-form, requiring cleavage via serine proteases, such as urokinase plasminogen activator (uPA), to produce an alpha and beta chain, which bind to form the active HGF heterodimer (Mars, Zarnegar et al. 1993; Gong, Rifai et al. 2008; Sisson, Nguyen et al. 2009). In skeletal muscle, both the active and inactive forms of HGF are stored in the extracellular matrix and are crucial for the activation of satellite cells following injury (Sheehan, Tatsumi et al. 2000; Tatsumi and Allen 2004). Furthermore, *uPA*$^{-/-}$ mice demonstrate defects in muscle regeneration (Sisson, Nguyen et al. 2009). Although relatively little is known about the regulation of HGF expression, the importance of repressor proteins has been found to be critical for cell type-specific regulation (Liu, Beedle et al. 1994; Ma, DeFrances et al. 2009), which makes it possible to speculate that

such a protein could be an NF-κB target gene in muscle cells. In a previous study, we found that host muscle inflammation and necrosis was reduced by donor *p65*$^{+/-}$ MDSC engraftment (Lu, Proto et al. 2012). In the present study, we found that in addition to reduced inflammation, host regeneration appeared to be enhanced, an observation that might be explained by the local actions of elevated HGF.

The anti-inflammatory effect of HGF appears to be dependent on MET-induced phosphorylation and inactivation of the serine/threonine protein kinase GSK3β in target cells (Martin, Rehani et al. 2005; Steinbrecher, Wilson et al. 2005; Gong, Rifai et al. 2008; Coudriet, He et al. 2010). Ubiquitously expressed, GSK3β is constitutively active under basal conditions, but is inactivated by phosphorylation at Ser9. Originally identified and named for its role in glucose metabolism, GSK3β has been found to have a much larger role in cell signaling than previously thought. For example, over 50 target substrates have been identified. More importantly, GSK3β activity has been reported to be elevated in a canine model of DMD, implicating its dysregulation in the dystrophic pathology (Feron, Guevel et al. 2009). Gong and colleagues (2008) have demonstrated that a subset of inflammatory genes, including *IL-6* and macrophage chemotactic protein-1 (*MCP-1*), requires GSK3β activity for efficient transcription (Martin, Rehani et al. 2005; Gong, Rifai et al. 2008; Coudriet, He et al. 2010). This may account for our finding that *p65*$^{+/-}$-CM suppresses IL-6 expression more so than WT-CM, but not IL-1β or TNFα. The specific consequences of IL-6 suppression are hard to pinpoint, as IL-6 has both pro- and anti-inflammatory roles. For example, pro-inflammatory roles of leukocyte-derived IL-6 include the induction of proliferation and differentiation of killer T cells and the differentiation of B cells. It has also been implicated in rheumatoid arthritis, and a humanized monoclonal neutralizing antibody for the IL-6 receptor, tocilizumab, is currently available for treatment

(Nishimoto and Kishimoto 2006). In skeletal muscle, IL-6 has also been implicated in the pathology of cachexia by inducing myofiber atrophy (Bonetto, Aydogdu et al. 2012). On the other hand, IL-6 deficient mice have an impaired muscle regeneration capacity, and muscle-derived IL-6 is known to be critical for muscle hypertrophy in response to overloading via activating and promoting satellite cell proliferation (Serrano, Baeza-Raja et al. 2008; Zhang, Li et al. 2013). Given the varied roles for this cytokine, it is reasonable to suggest that a pro- or anti-inflammatory role may be based not only on what cell type is releasing it, but also what cell type is being acted upon, as well as the duration of that interaction.

A relationship between NF-κB and HGF has implications for the treatment of muscular dystrophy. Roles for HGF have been found in the regeneration of diverse adult tissues. Perhaps HGF's most well described role is its stimulation of hepatocyte proliferation and regeneration of the injured liver (Tajima and Nakamura 1992; Pediaditakis, Lopez-Talavera et al. 2001). Similarly HGF promotes repair and renal tubule formation during acute renal failure (Ohnishi, Mizuno et al. 2008; Zhou, Tan et al. 2013). A positive role for HGF has also been described in the regeneration of musculoskeletal tissues, including bone, tendon, and cartilage (Takebayashi, Iwamoto et al. 1995; Nakase, Kitaoka et al. 2010; Goshima, Nakase et al. 2012). We found that at one week after birth, before the onset of the dystrophic phenotype, HGF expression was similar between *mdx* and *mdx;p65*$^{+/-}$ mice. However, at 4 weeks of age HGF expression was significantly higher in *mdx;p65*$^{+/-}$ muscle and correlated with a significant decrease in leukocyte infiltration and an increase in regeneration. Silencing HGF reversed the phenotypic improvements of *mdx;p65*$^{+/-}$ skeletal muscle. In particular, the diaphragm muscle showed dramatic degeneration following HGF silencing.

The current gold standard for DMD treatment, corticosteroid therapy with prednisone or deflazacort, blocks inflammation, but comes with a wide range of unwanted side effects, including cataracts, weight gain, growth impairment, and reduction in bone mineral density (Biggar, Harris et al. 2006). A number of drugs have been designed to target NF-κB activity, with varying degrees of specificity, but that treatment option remains problematic (Baud and Karin 2009). Given the ubiquitous nature of NF-κB and its many functions, this type of therapy can only be temporary, at best. For example, patients taking infliximab, a TNF antagonist, have an increased risk of infection and cancer (Keystone 2011). Based on this investigation, the beneficial effect of anti-NF-κB therapy might also be achieved by targeting HGF/MET. Such an approach may well be feasible, as clinical trials are underway testing HGF mimetics for treating heart attack and preventing delayed kidney graft function following transplant (Health 2012; Health 2013). Based on our findings, activation of HGF/MET might be a new approach to reduce inflammation and prolong fiber integrity in dystrophic muscle.

4.0 GENERAL DISCUSSION

4.1 Significance

Glucocorticoids are currently administered to DMD patients to delay the progression of muscle disease. Although the mechanism of action is not entirely understood, patients taking prednisolone show a stabilization of muscle degeneration and an improvement in muscle strength from 6 months up to two years following treatment. However, these improvements come with undesirable side effects, including weight gain, behavioural changes, and a cushingoid appearance (Manzur, Kuntzer et al. 2008). The success of steroid treatment has demonstrated that dystrophin does not need to be restored in order to improve patient life and healthspan. Thus, therapies that treat DMD symptoms, namely muscle inflammation and wasting, are vital to improving DMD patient quality of life.

NF-κB inhibition has been demonstrated to attenuate dystrophic pathology in mouse models of DMD. Some evidence for the benefits of anti- NF-κB therapy have also been demonstrated in the golden retriever model of muscular dystrophy, although the method of inhibition in this case was non-specific (Araujo, Bonuccelli et al. 2013). Due to the ubiquity of NF-κB, direct inhibition of this transcription factor remains problematic for human patients. NF-κB plays an important physiological role. This is made most obvious by the finding that *p65* or *IKKβ* deletion results in embryonic lethality in mice (Beg, Sha et al. 1995). In addition to the

obvious concern for hepatotoxicity, systemic administration of specific NF-κB inhibitors is likely to have many side effects. For example, given the critical role of NF-κB in innate immunity, this type of therapy can only be temporary. The work presented in this dissertation demonstrates that the success of anti-NF-κB therapy in the *mdx* mouse is due in large part to its effects on muscle progenitor cells, which include enhanced resistance to oxidative stress, enhanced myogenic capacity, and upregulation of HGF. In particular, HGF was shown to be critical for reducing inflammation and necrosis in *mdx;p65*$^{+/-}$ mice. These data suggest that a therapy activating HGF/MET may prove effective for delaying the progression of DMD in human patients.

Direct delivery of HGF protein may prove difficult due to the short half-lives typical of growth factors. For example, in a clinical trial investigating the efficacy of vascular endothelial growth factor (VEGF) for coronary artery disease, investigators found that a VEGF bolus delivered intravenously has a half-life of only approximately 30 minutes (Eppler, Combs et al. 2002). Researchers have developed innovative approaches to overcome this limitation, such as biomaterials-based delivery or a gene therapy approach. Alternatively, a small molecule HGF mimetic or MET agonist may be a successful approach to treatment. As with any drug, there are indeed concerns about unwanted side effects. HGF/MET has been implicated in cancer development and metastasis, and MET inhibitors are currently in Phase III trials as anti-cancer treatments (Goyal, Muzumdar et al. 2013). Despite this concern, the safety and efficacy of an HGF mimetic, BB3, is currently being evaluated in Phase II trials for the treatment of heart attack and delayed kidney graft function (Health 2012; Health 2013). The results from these current BB3 trials may provide the data necessary to support the safety of this approach. Thus, further investigation into the efficacy of HGF or MET agonists for the treatment of DMD is feasible and warranted.

Finally, HGF might also have potential as an adjuvant to dystrophin gene therapy. One of the largest obstacles to gene transfer in dystrophic muscle is the immune response. Due to chronic inflammation, dystrophic muscle is already infiltrated by immune cells, including monocytes and T-cells, prior to any type of gene delivery. Yuasa et al (2002) made the observation that immune reactions to transgenes delivered by AAV differed between *mdx* and wild type mice. Interestingly, they reported that the anti-AAV-IgG levels were equivalent between wild type and *mdx* mice, so the disparity was unlikely to be due to any difference in the humoral immune response to the viral capsid. The authors concluded that the enhanced response was due to higher levels of the transgene product in the extracellular space, allowing detection by antigen presenting cells. Such leakage is likely facilitated by the increased membrane permeability of the damaged, dystrophic fibers. (Yuasa, Sakamoto et al. 2002). Minimizing extracellular transgene product by reducing muscle necrosis may improve the efficiency of dystrophin gene delivery to injured muscle. It has been demonstrated that AAV-mediated delivery of a dominant negative mutant of IKKβ decreased necrosis in treated muscle of aged *mdx* mice (Tang, Reay et al. 2010). More recently, it has been reported that treatment with AAV-p65-shRNA reduced inflammatory cell infiltrate in *mdx* muscle and was associated with increased muscle membrane integrity. Based on the results contained in Chapter 3 of this dissertation, myofiber protection may also be mediated by HGF. Thus, "conditioning" dystrophic muscle with an HGF/MET therapy might increase the likelihood of successful dystrophin gene delivery.

4.2 Future Directions and Concluding Remarks

The data discussed in this dissertation indicate that NF-κB p65 is a major determinant of muscle stem cell trophic interactions and fate decisions. However, many questions remain unresolved, providing interesting avenues for future study. Continued research should begin with a more detailed evaluation of the major pathways discussed in this dissertation in the context of muscle injury and muscular dystrophy, using both primary MDSCs and mouse models. These pathways include NF-κB, GSK3β, and HGF/MET.

In Chapter 2, we identified NF-κB/p65 as a negative regulator of myogenic differentiation in MDSCs using cells isolated from $p65^{+/-}$ mice. We also made the observation that proliferation was accelerated in $p65^{+/-}$ MDSCs. This finding is contradictory to the known role of p65 in promoting cyclin D1 protein stability, and hence proliferation in myoblasts (Guttridge, Albanese et al. 1999; Dahlman, Wang et al. 2009). Examining the expression of cell cycle genes in wild type and $p65^{+/-}$ MDSC populations may shed light on this discrepancy. Although seemingly unusual, IKK/NF-κB has been demonstrated to negatively regulate cell proliferation in normal human fibroblasts, skin epithelial cells, and mouse embryonic fibroblasts. The authors of these studies reported that p65 physically disrupted the association of activating E2F transcription factors with histone acetyl transferase (HAT) complexes, and instead enhanced the DNA binding of a repressor E2F/p130 complex (Araki, Kawauchi et al. 2008). It would be interesting to see if the expression of E2F-responsive genes differs between wild type and $p65^{+/-}$ MDSCs. It is also possible that results could be obscured by the heterogeneity of the MDSC populations (Jankowski, Haluszczak et al. 2001). In the modified preplating isolation procedure, MDSCs are contained within the sixth preplate (pp6). These few slowly adhering cells attach to the flask and eventually form colonies. In order to reduce the probability of transcriptional

changes being obscured by population heterogeneity and/or clonal drift, single cell RT-PCR could be performed on passage one pp6 cells(Sacco, Doyonnas et al. 2008).

Aside from directly altering cell cycle gene transcription, NF-κB inhibition may affect proliferation in an indirect manner. As discussed in Chapter 3, we found that HGF was upregulated in *p65*$^{+/-}$ MDSCs. HGF has previously been reported to stimulate the proliferation of satellite cells and myoblasts in an autocrine manner (Sheehan, Tatsumi et al. 2000). Examining proliferation following knockdown of HGF or MET would be the first step towards determining the autocrine role for HGF in MDSC proliferation and survival *in vitro*. It is also important to determine whether the autocrine role of HGF is important for the enhanced engraftment capacity of *p65*$^{+/-}$ MDSCs *in vivo* and their regenerative effect on the host. To evaluate the importance of autocrine signaling for the success of donor MDSC transplant *in vivo*, MDSCs deficient for MET can be injected into injured muscle.

Finally, evidence from mouse models as well as studies on DMD patients, suggests that stem cell exhaustion plays a significant role in the progression of DMD (Webster and Blau 1990; Sacco, Mourkioti et al. 2010). Stem cell exhaustion is most often studied in the context of the natural aging process. In landmark experiments, the aged phenotype of murine satellite cells was shown to be reversible by exposure to young mouse serum (Conboy, Conboy et al. 2005; Brack, Conboy et al. 2007). This suggests that, in muscle at least, age-associated stem cell decline is not permanent and may result due to signals from the aged tissue environment. Perhaps a similar principle may apply to stem cell exhaustion in DMD. Of note, there has been no reported evidence that excessive DNA damage is responsible for stem cell senescence in skeletal muscle (Cousin, Ho et al. 2013). Thus far, research to determine the molecular pathways responsible for suppressing stem cell function during aging have identified Notch, Wnt, and fibroblast growth

factor 2 (FGF2) (Conboy and Rando 2002; Conboy, Conboy et al. 2003; Conboy, Conboy et al. 2005; Brack, Conboy et al. 2007). If we apply principles of stem cell "aging" to DMD-related stem cell exhaustion, progressive degeneration and chronic inflammation of skeletal muscle could lead to stem cell dysfunction in a feed-forward fashion. An investigation into whether p65 or even HGF plays a role in this process and whether it can be modulated to promote repair, is warranted.

In Chapter 3, we demonstrated a negative role for p65 in cell resistance to stress using an oxidative stress assay *in vitro* and a cell transplant model *in vivo*. Again, this finding contradicts the generally accepted notion of NF-κB as a pro-survival transcription factor (Karin and Lin 2002). There are some reports of NF-κB-induced expression of the pro-apoptotic proteins Fas and FasL in T-cells (Kasibhatla, Brunner et al. 1998). It has been indicated that depending on the stimulus, p65 can repress, rather than induce, anti-apoptotic gene expression (Campbell, Rocha et al. 2004; Wu and Miyamoto 2008). These reports are rare, but the majority of them involve NF-κB activation in response to DNA damage (Tilstra, Robinson et al. 2012). Thus, it may be interesting to test whether p65 deficiency enhances cell survival following exposure to a DNA damaging agent such as daunorubicin/doxorubicin or UV-C, both of which were reported to induce pro-apoptotic NF-κB activity (Campbell, Rocha et al. 2004).

We concluded that the anti-inflammatory effect of HGF in injured skeletal muscle is likely mediated by GSK3β inactivation in inflammatory cells. In this study, we did not examine GSK3β phosphorylation in myogenic cells. Presumably, HGF could inhibit its activity in these cell types as well. This may be relevant to muscle injury as it has been reported that GSK3β is required for muscle atrophy and is dysregulated in larger DMD animal models (Feron, Guevel et al. 2009; Verhees, Schols et al. 2011). Generally speaking, active GSK3β is associated with

catabolism, while inactivated GSK3β is associated with anabolism (Verhees, Schols et al. 2011). The metabolic consequences of the alterations in GSK3β activity could be evaluated in *mdx*;*p65*$^{+/-}$ mice, but as *mdx* mice do not demonstrate atrophy and wasting until a late age (18+ months), a more severe DMD model, such as the dystrophin-utrophin double knock out (dKO) mouse, may be useful here (Deconinck, Rafael et al. 1997).

We hypothesized that HGF was involved in the accelerated regeneration of *p65*$^{+/-}$ mice and in the therapeutic benefit of NF-κB inhibition in *mdx* mice. One of the drawbacks to this study was the lack of tissue specificity in *p65* allele deletion. It has been reported that conditional knockout of IKKβ in bone marrow was sufficient to reduce inflammation in *mdx* mice, but insufficient to enhance regeneration. Conversely, IKKβ deletion in muscle enhanced regeneration, but did not decrease inflammation (Acharyya, Villalta et al. 2007). On the contrary, we found HGF released by *p65* haploinsufficient muscle to both decrease inflammation and enhance regeneration. To verify that it is HGF derived from myogenic cells that is responsible for reducing inflammation after injury and disease, muscle regeneration should be analyzed in *p65*$^{+/-}$ or *mdx*;p65$^{+/-}$ mice that have received bone marrow transplants from wild type or *mdx* mice, respectively . Limiting *p65* deficiency to non-myeloid cell types will allow for a clearer understanding of the relationship of muscle-derived HGF and inflammation following injury.

In the *mdx*;*p65*$^{+/-}$ mice, we used a musculotropic AAV vector to deliver shRNA targeting HGF. After four weeks, we found that the reduction of HGF to levels similar to those in *mdx* mice exacerbated diaphragm histopathology. The next logical experiment would be to determine if HGF protein delivery to *mdx* mice improves their phenotype. Additionally, we posit that the primary sources of HGF in skeletal muscle are dividing progenitor cells and regenerating

myofibers. For a more direct confirmation, the temporal expression of HGF should be determined in *mdx*;*p65*$^{+/-}$ MDSCs as they progress down the myogenic lineage *in vitro*.

Finally, the mechanism of HGF upregulation following deletion of one p65 allele remains elusive. An NF-κB binding site has been identified in the 5' flanking region of *Hgf*, but its actual binding has not been demonstrated (Harrison and Farzaneh 2000). This was investigated using a cell line derived from human fetal lung tissue; therefore binding in other tissues remains a possibility. It is also reasonable to hypothesize that an NF-κB target gene product may be a repressor of HGF in the way that NF-κB inhibits MyHC expression via YY1 (Wang, Hertlein et al. 2007). Although an NF-κB dimer composed of p65/p50 is usually considered gene activating, p50 homodimers have been described as gene repressing (Tong, Yin et al. 2004; Elsharkawy, Oakley et al. 2010). Both p65/p50 and p50/p50 dimers translocate to the nucleus following classical activation (Acharyya, Villalta et al. 2007). Haploinsufficiency of p65 may serve to increase the proportion consisting of p50 homodimers. HGF may then be affected downstream of altered NF-κB target genes.

Answering these questions will begin to elucidate the molecular changes responsible for our observations, and may identify additional drug targets for the treatment of DMD. The success of glucocorticoid therapy has clearly demonstrated that treating the DMD pathologies secondary to dystrophin deficiency can improve patient quality of life and even lifespan. Anti-NF-κB therapy may be able to successfully treat DMD patients through increasing the regenerative and anti-inflammatory capacity of muscle stem cells. The danger of unwanted side effects is great enough that this approach is unlikely to hold up in clinical trials. Following the work presented in this dissertation, we now have a better understanding of the ways in which

NF-κB functions in muscle stem cells. The groundwork is now laid for future studies of HGF/MET activation as a treatment for DMD.

APPENDIX A: MODULATION OF NF-κB RESTORES THE MYOGENIC POTENTIAL OF AGED MDSCS *IN VITRO*

A.1 ABSTRACT

A decline in the regenerative capacity of adult muscle stem cells during aging is well documented. Globally, NF-κB is up regulated in aging tissues. Given the negative role that NF-κB plays in myogenesis, we investigated whether the aged phenotype of muscle-derived stem cells (MDSCs) was associated with over activated NF-κB. We isolated MDSCs from aged (24 month old) and young (14 day old) wild type (WT) mice. The decreased myogenic capacity of aged cells appeared to be associated with irregular cell cycle progression, indicated by high cyclin D1 levels post differentiation. Treatment with an inhibitor of NF-κB rescued the myogenic capacity of aged MDSCs, despite not decreasing cyclin D1 protein levels. A similar increase in differentiation was obtained in aged MDSCs isolated from 30 month old mice haploinsufficient for the NF-κB subunit p65 ($p65^{+/-}$), suggesting our observations were not due to off target effects of the pharmacologic inhibitor. Furthermore, we found that both aged MDSCs treated with an NF-κB inhibitor and aged $p65^{+/-}$ MDSCs have a higher resistance to oxidative stress than untreated aged cells. Finally, preliminary results with human skeletal muscle progenitor cells expressing shRNA against *p65* demonstrate an increase in cell fusion under differentiation conditions. Our results suggest that MDSC "aging" may be reversible, and that pharmacologic

targeting of pathways such as NF-κB may enhance the efficacy of cell therapies in aging patients, despite a mechanism that remains unclear.

A.2 INTRODUCTION

Stem cell based approaches for the treatment of disease and injury are often heralded as the future of regenerative medicine and tissue engineering. At present, clinical trials have taught us that many obstacles remain before cell therapies become common clinical practice. One such problem for both allogeneic and autologous cell sources is the impact of donor age on stem cell function and clinical efficacy, as substantial evidence suggests that stem cell dysfunction contributes to the aging process (Carlson and Conboy 2007; Dimmeler and Leri 2008). Transplantation of older, functionally impaired cell populations is likely to result in a reduced clinical efficacy compared to the transplantation of younger cell populations (Si, Zhao et al. 2010). This does not bode well for the future of cell therapy, given that diseases for which cell-based treatments seem the most promising have the highest prevalence among aging populations (Dimmeler and Leri 2008). For example, greater than 80 percent of those who die from coronary heart disease are over the age of 65 (Association 2010). Considering that the risks associated with immunosuppression may outweigh the benefits of allogeneic cell transplants, new strategies aimed at improving aged stem cell function must be investigated.

A decline in the function of tissue-resident stem cells is likely to contribute to the decreased wound healing and regeneration capacity that comes with age (Carlson and Conboy 2007). For example, skeletal muscle injury that would lead to the regeneration of functional tissue in children, instead leads to prolonged inflammation and fibrosis in older individuals

(Conboy and Rando 2005). This phenomenon has been demonstrated to be related to the functional decline of satellite cells. In landmark experiments, the aged phenotype of murine satellite cells was shown to be reversible by exposure to young mouse serum (Conboy, Conboy et al. 2005; Brack, Conboy et al. 2007). This suggests that, in muscle at least, age-associated stem cell decline is not permanent and likely results due to signals from the aged tissue environment. Thus, *ex vivo* priming or engineering to "rejuvenate" aged cells may be a viable approach for enhancing function following transplantation (Carlson and Conboy 2007). For these reasons, a better understanding of the processes controlling adult stem cell regenerative capacity are necessary before autologous cell therapies for aging patients can be used in the clinical setting.

Thus far, research to determine the molecular pathways responsible for suppressing stem cell function in skeletal muscle has identified both the Notch and Wnt signaling pathways (Conboy and Rando 2002; Conboy, Conboy et al. 2003; Conboy, Conboy et al. 2005; Brack, Conboy et al. 2007). In post-natal muscle, activation of the Notch pathway has been found to be required for satellite cell activation and cell cycle entry (Conboy and Rando 2002). In aged muscle, Notch activation in response to injury is impaired, and forced activation improves regeneration in aged muscle (Conboy, Conboy et al. 2003). Approximately twenty percent of aged satellite cell progeny undergo conversion to a non-myogenic, fibrotic cell type *in vitro*, which occurs in less than one percent of cell generated from young satellite cells. This process, mimicking the elevated fibrosis found in aged muscle, may be mediated through increased Wnt signaling, as inhibition of canonical Wnt decreases aged muscle fibrosis following injury (Brack, Conboy et al. 2007).

Increased NF-κB activity has been identified as a major regulator of gene expression programs associated with aging. Although total protein levels of NF-κB have not been found to change, increased amounts of NF-κB subunits have been found in nuclear extracts of aged mouse and rat skin, liver, kidneys, and brain (Salminen and Kaarniranta 2009). Local inhibition of NF-κB in aged murine skin led to tissue rejuvenation and changes in gene expression such that treated skin resembled younger skin more closely than it did untreated, age-matched controls (Adler, Sinha et al. 2007). Taken together, the current body of evidence suggests that pathways involved with normal post-natal myogenesis may become dysregulated during aging, and that targeting of molecular pathways associated with stem cell or tissue aging may be a viable approach for enhancing aged stem cell function (Brack, Conboy et al. 2007; Carlson, Suetta et al. 2009).

We investigated whether reversibility of the aged phenotype is possible in muscle-derived stem cells (MDSCs), as previous studies have demonstrated that MDSC-based therapies can be used for tissue repair following muscle, bone, cartilage, and cardiac injury. For our studies, we have isolated MDSCs from aged (24 month old) and young (14 day old) wild type mice and aged (30 month old) mice lacking one allele for the NF-κB subunit p65 ($p65^{+/-}$) using a modified pre-plate technique. We found that the declined myogenic capacity of aged MDSCs was associated with increased levels of the NF-κB regulated protein cyclin D1, but not YinYang1 (YY1), also regulated by NF-κB. Unexpectedly, we did not detect increased activation of NF-κB, measured by immunoblotting for phosphorylated p65. Despite this, treatment with a small molecule inhibitor of NF-κB restored the myogenic capacity of aged MDSCs to the same level as young non-treated MDSCs. Furthermore, pretreatment of aged MDSCs with an IKKβ inhibitor increased cell survival under oxidative stress. This led us to

hypothesize that NF-κB may represent a molecular target for enhancing the regenerative potential of aged MDSCs. To investigate this further, we isolated MDSCs from 30 month old transgenic mice haploinsufficient for p65 ($p65^{+/-}$). We found that aged $p65^{+/-}$ MDSCs retained myogenic potential *in vitro* and had a higher resistance to oxidative stress-induced cell death than aged wild type cells. The mechanism of myogenic enhancement following NF-κB inhibition remains elusive, as we detected no obvious changes in cyclin D1 or YY1 following treatment. Despite this remaining question, human skeletal muscle progenitors isolated from a 76 year old female appeared to have enhanced cell alignment for fusion following transfection with a p65 shRNA-encoding plasmid. Although leaving many questions to be answered, our results demonstrate that MDSC "aging" is at least partially reversible, and that targeting of NF-κB, whether through a genetic or pharmacological approach, may represent one strategy for enhancing the efficacy of autologous cell therapies in aging patients.

A.3 Materials and Methods

Murine cell isolation. Using the modified preplate technique, we isolated populations of muscle-derived cells from the leg muscles obtained from 24 month old (aged) and 2 week old (young) wt mice, and 20 month old p65 haploinsufficient (aged $p65^{+/-}$) mice. Rapidly adhering cells from early preplates, representing myoblasts (pp3-4) were also collected and cultured in growth medium consisting of 10% FBS, 10% horse serum, 1% Penn-strep, and 2.5 ng/mL basic fibroblast growth factor (bFGF) in DMEM.

Human skeletal muscle progenitor (hMPC) cell isolation. hMPCs were isolated from the muscle biopsy of a 76 year old female patient. This was done according to the modified preplate technique, with the hMPC population defined as pre-plate three.

Myogenic differentiation assay and fast Myosin Heavy Chain (MyHC) staining. After 15 passages, cells were plated on 24 well plates (20,000 cells per well) with DMEM supplemented with 2% FBS to stimulate myotube formation. At the indicated timepoints, cells were washed, fixed with ice cold 100% methanol, and stained for fast skeletal myosin heavy chain (MyHC) or vimentin. Briefly, cells were blocked with 10% horse serum for one hr and then incubated with a mouse anti-MyHC (1:250, Sigma) and rabbit anti-vimentin (1:100, Sigma) for 1 hr at RT. The primary antibody was detected with a secondary anti-mouse or anti-rabbit IgG antibody conjugated with 594 or 488 (1:500, Sigma) for 30 min. The nuclei were revealed by DAPI staining. The percentage of differentiated myotubes was quantified as the number of nuclei in MyHC positive myotubes relative to the total number of nuclei.

Transfection of hMPCs. Using the Xfect transfection reagent (Clontech, Mountain View, CA), hMPCs were transfected with 1.5 micrograms of plasmid DNA encoding shRNA against p65 (pP65-shRNA) or scrambled shRNA (pCtrl). The plasmids have been previously described elsewhere, and were kindly provided by Dr. Bing Wang.

Western Blot Cell and tissue lysates were prepared in RIPA buffer (Sigma) supplemented with protease and phosphotase inhibitors (#2 and #3, 1:100, Sigma) and quantified using the Bio Rad Protein Assay (Bio Rad). Immunoblotting was performed as previously described (Lu, Proto et al. 2012). Membranes were incubated with antibodies against phosphorylated p65 (Cellsignal) and the NF-κB targets YingYang1 (Santa Cruz Biotechnology)

and cyclin D1 (BD Pharmigen). at 4°C overnight in 5% milk or BSA in TBST. In order to ensure equal loading, membranes were probed with HRP-conjugated anti-GAPDH (Sigma).

Pharmacologic Inhibition of NF-kB Signaling. Aged murine MDSCs were treated with IKK-2 Inhibitor IV (Calbiochem) at varying doses during myogenic differentiation and cell survival assays.

Statistical analysis. All results are given as the mean ± standard error of the mean. Means were compared using the Students' *t*-test. Differences were considered statistically significant when the p-value was <0.05.

***In vitro* Measurement of Cell Survival under Oxidative Stress.** Cells were exposed to oxidative stress induced by treatment with 250 uM hydrogen peroxide. In order to visualize cell death, propidium iodide (PI), a DNA-binding dye, was added to culture medium according to manufacturer's protocols (BD Bioscience, San Jose, CA, USA). To block NF-κB activation, WT MDSCs were treated with the reversible ATP-competitive inhibitor of IKKβ, IKK-2 Inhibitor IV (EMD Millipore, 401481, Billirca, MA, USA) at 5uM. Using the LCI system described above, 10x brightfield and fluorescence images were taken in 10 minute intervals over 24 hours (Deasy, Jankowski et al. 2003). Identifying the number of PI+ cells per field of investigation out of the total cell number determined the percentage of cell death over time.

A.4 Results

***Aged MDSCs exhibit a resistance to cell cycle arrest under fusion conditions*.**

The myogenic potential of murine MDSCs declines with age (Lavasani, Robinson et al. 2012). Brack and colleagues (2007) have reported that a significant percentage of aged satellite

cell progeny undergo conversion to a non-myogenic, fibrotic cell type *in vitro* following isolation (Brack, Conboy et al. 2007). We speculated that a similar phenomenon may occur in MDSCs. We compared the capacity for myogenic differentiation of aged MDSCs, isolated from 24 month old mice, and young MDSCs, isolated from 2 week old mice. Within three days of culture under myogenic conditions, myotubes were evident in both young and aged MDSC cultures. Although equal numbers of cells were plated, there was a striking difference in cell density after 72 hours (Figure 23). We analyzed cell morphology by staining for fast skeletal myosin heavy chain (MyHC) and vimentin, an intermediate filament protein expressed in mesenchymal tissues. Unexpectedly, myotubes appeared as MyHC and vimentin double positive (Figure 23), but this has been reported before (Gallanti, Prelle et al. 1992). Several MyHC-vimentin+ cells demonstrated a fibroblast-like morphology (Figure 23, A inset), but this is insufficient evidence for phenotype conversion. What was clear from this assay, however, was that aged WT cells were proliferating after being plated in fusion medium. Before differentiation can occur, cells must undergo cell cycle arrest. NF-κB, specifically p65, has been implicated as a suppressor of myoblast cell cycle withdraw via stabilization of cyclin D1. As we hypothesized that NF-κB activity was implicated in aged MDSC dysfunction, we examined cyclin D1 and phosphorylated p65 (S536) levels in fusion cultures of young and aged MDSCs by western blot. We found significantly more cyclin D1 in aged fusion cultures compared to young cultures. Increased p65 activation, however, was not evident. As an additional indirect measurement of NF-κB activity, we looked at the NF-κB induced protein, YY1, a myofibrillary gene repressor, and found no obvious differences (Figure 23, B).

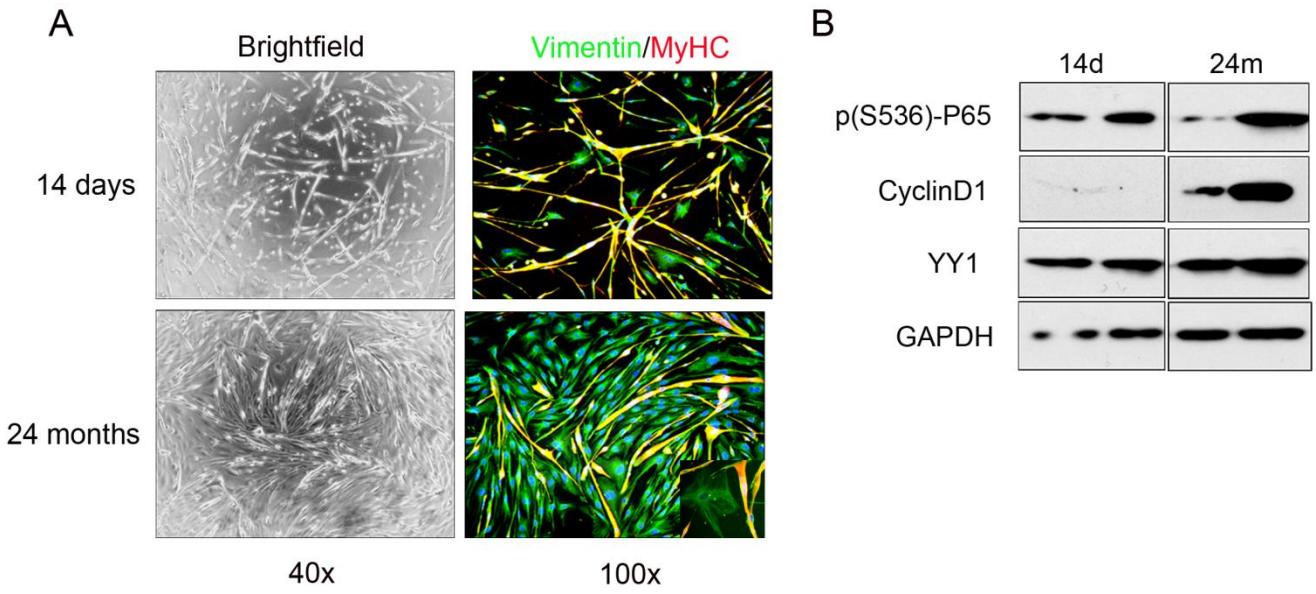

Figure 23. The coupling of cell cycle withdraw and differentiation is disrupted in aged MDSCs.

(a) MyHC (red) and vimentin (green) staining indicates that after 72 hours under fusion conditions, there is a much greater number of MyHC- cells in aged MDSC cultures. Some of these cells took on a fibroblast like morphology (inset). (b) Western blotting did not detect changes in p-p65, but did detect much higher levels of cyclin D1 in aged MDSCs.

Inhibition of IKKβ restores the myogenic potential of aged murine MDSCs

If NF-κB regulation is unaltered in aged MDSCs, that does not preclude it as a molecular candidate for enhancing myogenic potential. Therefore, we next investigated whether IKKβ inhibition would be sufficient to restore the myogenic potential of aged cells. Aged and young MDSCs were plated and cultured under myogenic conditions with or without the presence of IKK-2 inhibitor IV (IKKi) at 0, 1, 2, or 5 μM. Differentiation was monitored by brightfield microscopy. After 5 days, treated cells demonstrated an increase in myofiber formation and a decline in undifferentiated cell number. We quantified differentiation at 5 μM (the most effective dose) by immunofluorescent staining for MyHC. To help to rule out possible non-specific effects

of IKKi treatment, we also assessed the myogenic capacity of aged *p65*$^{+/-}$ MSDCs isolated from 30 month old mice. As shown in Figure 24, the percent of nuclei in myotubes was significantly increased by IKKβ inhibition and to a lesser degree by *p65* deficiency. IKKi treatment of aged

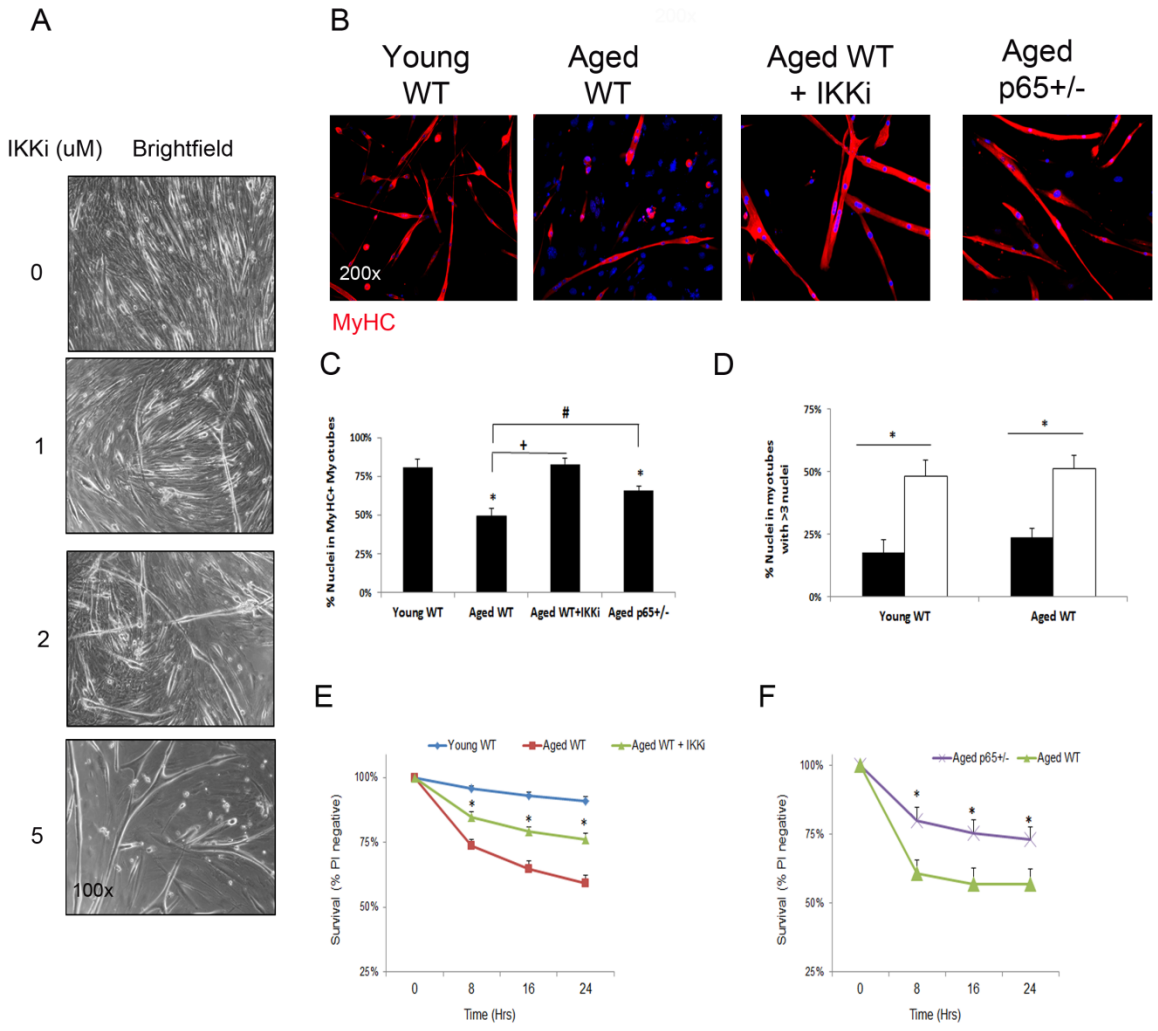

Figure 24. Inhibition of IKKβ or genetic loss of one p65 allele enhances myogenic differentiation and stress resistance in aged MDSCs.

(a) Brightfield images demonstrate a dose dependent increase in myotube formation following IKKi administration. (b) MyHC positivity was quantified at the dose of 5 μM. (C) This revealed that differentiation was significantly increased following NF-κB inhibition and this was not due to (c) increased myofiber maturation. (E) Under oxidative stress conditions, cell survival is increased in IKKi treated or (F) *p65* $^{+/-}$ aged MDSCs.

MDSCs increased their myogenic potential to levels similar to those of young cells. While aged *p65*$^{+/-}$ MDSCs did demonstrate an improvement, differentiation was still significantly lower compared to young MDSCs (Figure 24 B, C). As we did not detect any differences in NF-κB/p65 phosphorylation, we thought that perhaps NF-κB inhibition did not correct overactive transcription factor activity, but instead accelerated myogenesis. To investigate this, we analyzed fiber maturity by determining the number of nuclei per myotube. For our purposes, we defined "mature" myotubes as those containing greater than three nuclei. Interestingly, we found that the percent of nuclei in mature myotubes was similar between young and aged cells (Figure 24 D). This percentage increased after treatment, but again, was not different between young and aged cells. Although far from conclusive, these results suggest that NF-κB inhibition does not accelerate differentiation but rather increases the myogenic capacity of aged MDSCs. Furthermore, this appears to occur despite the absence of apparent NF-κB/p65 over activation compared to young cells.

NF-κB inhibition increases MDSC resistance to hydrogen peroxide induced oxidatve stress. Previous studies from our group have shown that MDSC resistance to oxidative stress, which declines with age, is an important characteristic that distinguishes them from myoblasts and correlates with high regenerative capacity *in vivo* (Urish, Vella et al. 2009; Lavasani, Robinson et al. 2012). As NF-κB is known to be a stress responsive pathway (Luo, Kamata et al. 2005), we examined if IKK/NF-κB inhibition would improve cell survival under hydrogen-peroxide (H_20_2) induced oxidative stress. Briefly, cells were triplicate plated in twenty-four well plates and exposed to 250 μM H_20_2 in proliferation medium containing propidium iodide, a fluorescent DNA stain. We monitored cell death and survival by capturing brightfield and fluorescent

images once every 10 minutes for twenty-four hours of multiple locations per well by LCI. As predicted, young MDSCs displayed a higher percentage of cell survival compared to aged MDSCs (Figure 24 E). We observed that pretreatment with 5uM IKKi significantly increased aged MDSC survival. To help rule out non-specific IKKi targets, we next compared survival between aged WT and aged $p65^{+/-}$ MDSCs. We found that genetic deficiency of *p65* had a similar effect to IKKi treatment (Figure 24 F).

NF-κB inhibition does not decrease cyclin D1 levels during aged MDSC differentiation

Now that we have identifed NF-κB as a target for enhancing cell survival and myogenic differentiation, we returned to the question of its regulation during aging. As we had found cyclin D1 to be significantly higher in aged fusion cultures compared to those of young cells, we re-examined the levels of cyclin D1, p-p65, and YY1 with and without IKKi treatment after 5 days of culture in fusion medium. Surprisingly, Cyclin D1 levels remained the same and YY1 levels were only modestly decreased (Figure 25 A). Similiarly, we did not see significant differences in p-p65 levels. We next examined p-p65 levels under normal proliferation conditions and were surprised to find that p-p65 was lower in aged, rather than young, MDSCs (Figure 25 B). It is possible that this is an artefact due to multiple passages *in vitro* during experiments. To test this, we examined the p-p65 levels in myoblasts from young and old muscle at passage 1 or 2 and detected no differences (Figure 25 C).

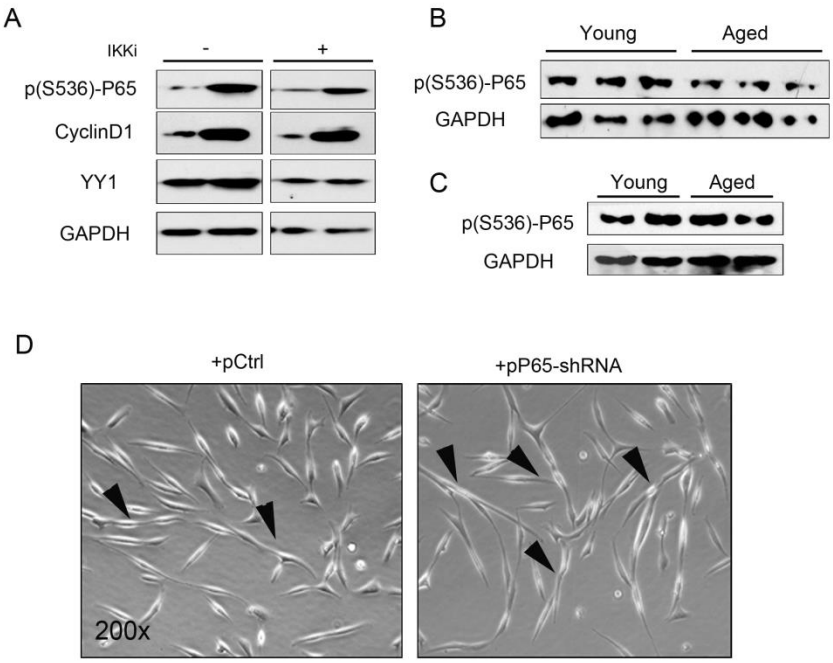

Figure 25. NF-κB activity does not appear to be altered under fusion conditions, but it may still be a target to improve the fusion of aged human muscle progenitor cells.

(a) Western blot of phosphorylated p65 and NF-κB target proteins after culture in fusion conditions. (b) Under normal proliferation conditions, steady state levels of p-p65 are decreased in aged MDSCs compared to young MDSCs, but this is (c) not seen in myoblasts. (d) Transfection with a plasmid encoding shRNA targeting p65 appears to increase pre-fusion alignment (arrows) of human skeletal muscle progenitor cells from a 76 year old female.

Preliminary evidence indicates that NF-κB suppression enhances the alignment of aged human skeletal muscle progenitor cells under myogenic differentiation conditions.

Despite these paradoxical findings, the enhanced differentiation and survival of aged MDSCs following NF-κB inhibition suggests that this may remain an effective strategy for improving the aged stem cell phenotype. Thus, we tested the efficacy of NF-κB inhibition on aged human skeletal muscle progenitor cells (hMPCs) isolated from a muscle biopsy taken from a 76 year old female. Treatment with IKKi proved to be toxic at high doses and ineffective at low doses (data not shown). Thus, we transfected the aged hMPCs with a plasmid encoding shRNA targeting *p65* (Yang, Tang et al. 2012). After 10 days, we examined differentiation

qualitatively by brightfield microscopy and found evidence of increased cell alignment following *p65* silencing, which is the earliest step towards cell fusion. Similar to the results with aged murine MDSCs, these preliminary results suggest that NF-κB inhibition may enhance the myogenic potential of hMPCs.

A.4 Discussion

We examined whether NF-κB is over activated in aged murine MDSCs and whether this contributes to the aged stem cell phenotype. This short and preliminary study yielded some confusing, yet interesting, results. We did not detect higher levels of NF-κB in aged MDSCs, as measured by p-p65. Rather, we found no overt differences in p-p65 levels after differentiation and even reduced levels during growth in proliferation medium. Despite this observation, NF-κB inhibition, whether by IKKi treatment or *p65* deficiency, improved the differentiation of aged MDSCs. Similarly, progression towards differentiation was detected in aged hMPCs following *p65* silencing.

During differentiation, we detected high levels of cyclin D1, which were not detected in young MDSC fusion cultures. Cyclin D1 activity can reduce the differentiation of myogenic cells by blocking MEF2, a transcription factor important for differentiation, from association with coactivators (Azmi, Ozog et al. 2004). Although it is attractive to hypothesize that over expression of cyclin D1 is responsible for blocking cell cycle exit and differentiation, NF-κB inhibition enhanced myogenesis without decreasing cyclin D1 levels. The presence of cyclin D1 in terminally differentiated cells is intriguing, but does not directly indicate its activity. Additionally, IκBα binds to and inhibits cyclin dependent kinase 4 (CDK4), the target of cyclin

D1. Presumably, blocking IKKβ activity would prevent the phosphorylation and degradation of IκBα. It would be interesting to see if CDK4 levels and activity differ between young and aged MDSCs during differentiation and it this changes following NF-κB inhibition.

We also found that resistance to oxidative stress was elevated in *p65* deficient or IKKi-treated aged cells. Although typically considered pro-survival, NF-κB can also be activated in response to DNA damage. In a mouse model of accelerated aging induced by DNA damage, NF-κB activity was associated with cell senescence. In this same model, blockade of NF-κB also reduced oxidative stress-induced DNA damage (Tilstra, Robinson et al. 2012). However, a direct relationship between NF-κB and resistance to stress is made complicated by the finding of lower levels of p-p65 in aged MDSCs compared to young populations. Due to the extended culture of MDSCs that is inherent in the pre-plate isolation technique, it is possible that this is an artefact. Given that we did not detect lowered levels in freshly isolated myoblasts, this needs to be investigated further. Rather than MDSCs, this could be investigated in another population of muscle progenitors, such as satellite cells, which can be rapidly isolated by FACS (Sacco, Doyonnas et al. 2008). These preliminary results indicate the NF-κB may play a role in aging, and warrant further investigation.

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