In Vitro Differentiation of Muscle Side Population Cells from Dystrophic Muscle Reveals Absence of Myogenesis and Implications for Hedgehog Signaling

DISSERTATION

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disorder affecting 1 in 3500 live male births per year. The disease is caused by mutations in the dystrophin gene resulting in a lack of functional dystrophin protein at the membrane of both skeletal and cardiac muscles. Lack of dystrophin results in muscle fiber destabilization and breakdown, leaving the muscle in a chronic state of degeneration and regeneration. As the disease progresses however, regeneration ultimately fails resulting in the progressive replacement of muscle with fibrosis that correlates with patient loss of mobility in the second decade of life. Cell therapy is one of multiple therapeutic strategies being investigated for treatment in DMD to counteract these effects by increasing the regenerative capacity of the muscle and slowing the progression of fibrosis. While satellite cells and myoblasts have been used extensively in human trials, their efficacy has been limited. To overcome this, recent studies have turned to alternative cell populations residing in the muscle that have superior migratory and cell survival characteristics upon injection into limb muscles of the DMD mouse model, mdx. The muscle side population cells are a population of these alternative cells that have been shown to successfully engraft and regenerate dystrophic muscle in animal models. However, our understanding of muscle side population biology is limited due to a lack of specific in vivo markers and in vitro culture systems.
Here we show a novel in vitro system capable of supporting side population cell growth and differentiation. Using this system, we show that muscle side population cells lacking expression of extracellular markers CD31 and CD45 but expressing the fibro-adipogenic progenitor marker, Pdgfra, are highly myogenic giving rise first to pax7 expressing satellite cells and later into differentiated α-actinin expressing myotubes. We utilized this novel in vitro culture system to determine the myogenic capacity of SP cells isolated from either acutely injured or dystrophic SP cells. In contrast to wild type cells, side population cells isolated from dystrophic or acutely injured muscle display no myogenesis while differentiating into fibroblasts, adipocytes, and fibro-adipogenic progenitor cells. These findings indicate that muscle damage and the state of the muscle environment direct the lineage commitment of the side population cells. Additionally, our results suggest that the cell fate switch of the muscle side population cells (SP) may play a role in dystrophic pathology. For example, cell fate switching of the side population may contribute to a reduced number of myogenic cells available for regeneration and in turn increasing numbers of fibro-adipogenic progenitors and fibroblasts responsible for fibrosis formation.

In order to better understand the mechanism responsible for the loss of myogenesis in mdx^{5cv} SP cells, we analyzed gene expression of multiple signaling pathways functioning in cell fate specification. Our studies revealed that the hedgehog pathway (Hh) was down regulated in SP cells isolated from mdx
mice. Using the chemical Hh agonist, purmorphamine, we show that stimulation of Hh signaling successfully restores the myogenesis of \textit{mdx}^{5cv} SP cells.

Our findings in the SP cells suggested that Hh signaling may not be induced, as is the case during normal muscle regeneration. Analysis of dystrophic muscle revealed global down-regulation of the Hh pathway in the diaphragm muscle of dystrophic \textit{mdx}^{5cv} mice. Due to multiple positive effects of Hh signaling during myogenesis these findings suggesting the lack of Hh induction may play a role in \textit{mdx}^{5cv} pathology. In order to test whether restoration of Hh signaling results in positive effects on \textit{mdx}^{5cv} pathology we treated \textit{mdx}^{5cv} mice with purmorphamine and analyzed multiple histological parameters. Treatment resulted in improvements in multiple disease parameters including increased resistance to fatigue, reduced immune cell infiltrate in limb muscles, and reduced central nuclei and Collagen 1 deposition in the \textit{mdx}^{5cv} diaphragm.

Collectively, the results in this dissertation show that SP cells isolated from either an actively regenerating or dystrophic muscle lack myogenic capacity. These results have implications for the use of autologous SP cell transplantation as dystrophic SP cells may not provide an optimal source of myogenic cells. Furthermore, we identify that Hh signaling serves to promote SP cell myogenesis and represents a potential mechanism and future therapeutic strategy in \textit{mdx}^{5cv} pathology.
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List of Abbreviations

CEBPα: CCAAT/enhancer binding protein
CTX: Cardiotoxin
DAPC: Dystrophin Associated Protein Complex
DMD: Duchenne Muscular Dystrophy
ECM: Extracellular Matrix
EMHC: Embryonic Myosin Heavy Chain
EMT: Epithelial to Mesenchymal Transition
FAP: Fibro-adipogenic Precursor
FGF: Fibroblast Growth Factor
Hh: Hedgehog
IGF: Insulin-like Growth Factor
IgG: Immunoglobulin G
MDSCs: Muscle Derived Stem Cells
MMP: Matrix Metalloproteinase
MP: Main Population
nNOS: Neuronal Nitric Oxide Synthase
PDGF: Platelet Derived Growth Factor
PDGFRα: Platelet Derived Growth Factor Alpha
SP: Side Population
TGFβ: Transforming Growth Factor Beta
VEGF: Vascular Endothelial Growth Factor
Chapter 1

Introduction

1.1 Introduction to Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is an X-linked progressive muscle degenerative disease affecting 1 in 3500 live male births per year (Emery 1993). DMD is normally diagnosed in the first decade of life at which time deficits in motor milestones are observed. Loss of mobility as a result of limb weakness occurs in the second decade of life, averaging at 12 years of age (Emery 1993). Dilated cardiomyopathy arising due to the loss of dystrophin in the heart, affects nearly all DMD patients by the age of 18 and is responsible for death in 10-40% of DMD patients (Baxter 2006). The majority of DMD patients die in their twenties from respiratory complications due to muscle weakness of the diaphragm muscle (Nowak and Davies 2004). Currently the standard of care is prednisone therapy which is successful at extending mobility but does not significantly increase lifespan (Mendell, Moxley et al. 1989; Griggs, Moxley et al. 1991; Connolly, Schierbecker et al. 2002).

DMD is caused by mutations in the dystrophin gene resulting in absence of functioning dystrophin protein (Hoffman, Brown et al. 1987). Dystrophin is a
large 427 kDa protein that associates with the Dystrophin Associated Protein Complex (DAPC) (Koenig, Hoffman et al. 1987). The complex functions to provide a physical connection between the extracellular matrix (ECM), the sarcolemma, and F-actin (Amann, Guo et al. 1999). Through this connection, the muscle maintains force transduction and membrane stability upon muscle contracture (Petrof, Shrager et al. 1993). Loss of dystrophin and destabilization of the DAPC complex disrupts multiple processes leading to myofiber dysfunction and necrosis (Straub and Campbell 1997; Kumar, Khandelwal et al. 2004). While muscle regeneration occurs it is insufficient in maintaining muscle mass resulting in a progressive loss of muscle fibers accompanied by fibrosis and fat deposition (Duance, Stephens et al. 1980; Stephens, Duance et al. 1982; Hantai, Labat-Robert et al. 1985; Klingler, Jurkat-Rott et al. 2012).

### 1.2 Mechanisms of Disease

DMD pathophysiology is a complex process that is currently only partially understood. Disease manifests due to a combination of factors including both intrinsic defects in the myofiber and secondary factors in the muscle microenvironment. This section will review both the mechanisms of myofiber breakdown caused by loss of dystrophin and the secondary factors contributing to disease including inflammation, fibrosis, and an insufficient regenerative response.
**Myofiber Dysfunction**

Dystrophin is thought to serve primarily as a structural protein responsible for dissipating force transduction of the myofiber during contraction and relaxation (Petrof, Shrager et al. 1993). Loss of dystrophin causes destabilization of the myofiber membrane increasing membrane permeability and causing micro-tearing at areas of stress (McNeil and Khakee 1992; Petrof, Shrager et al. 1993; Chen, Zhao et al. 2000). These proposed mechanisms have been supported by myofiber uptake of impermeable dyes such as Evan’s blue and the secretion of the myofiber enzyme creatine kinase detectable in patient serum (Okinaka, Ebashi et al. 1961; Percy, Chang et al. 1979; Matsuda, Nishikawa et al. 1995; Ozawa, Hagiwara et al. 1999; Hamer, McGeachie et al. 2002). Increased membrane permeability and micro-tearing is hypothesized to cause myofiber degeneration by allowing for increased calcium within the myofiber (Fong, Turner et al. 1990; Turner, Fong et al. 1991; Imbert, Cognard et al. 1995). It appears that increased calcium in *mdx* myofibers promotes degeneration by interfering with multiple processes including deregulation of mitochondrial genes, activation of calcium activated proteases such as calpain, and production of reactive oxygen species (Spencer, Croall et al. 1995; Disatnik, Dhawan et al. 1998; Kuznetsov, Winkler et al. 1998; Chen, Zhao et al. 2000).

In addition to structural roles dystrophin participates in cell signaling pathways that serve to regulate muscle function which are disrupted in DMD. For example, loss of dystrophin causes α-syntrophin to be absent from the DAPC
resulting in the mislocalization of neuronal nitric oxide synthase (nNOS). The absence of nNOS at the sarcolemma is thought to cause dysregulation of blood flow and cause ischemia contributing to disease (Lau, Grange et al. 1998; Thomas, Sander et al. 1998; Sander, Chavoshan et al. 2000). Additionally, loss of dystrophin has been shown to cause increased levels of Muscle-Specific RING Finger protein 1 and Muscle Atrophy F-box, both of which induce muscle atrophy by promoting proteasomal degradation of myofibers components (Acharyya, Butchbach et al. 2005).

Inflammation

Myofiber necrosis in DMD results in a chronic inflammatory response composed of multiple cell types that are recruited to clear debris to facilitate myofiber regeneration. While neutrophils are initially recruited by necrotic myofibers, they are replaced by macrophages that remain the predominant immune cell type in the dystrophic environment (Wehling, Spencer et al. 2001; Tidball and Villalta 2010). While macrophages are critical for the removal of necrotic debris, chronic macrophage infiltration is thought to accelerate myofiber breakdown, promote fibrosis, and worsen disease severity in DMD. For instance, macrophages induce muscle cell lysis in vitro through up-regulation of iNOS (Villalta, Nguyen et al. 2009). Additionally macrophages appear to play a significant role in the rate of myofiber necrosis in vivo, as macrophage depletion in young mdx mice significantly reduces myofiber degeneration (Tidball and Wehling-Henricks 2007). In addition to direct effects on muscle cell death,
macrophages secrete significant amounts of pro-fibrotic cytokines including TGFβ and PDGF factors that directly stimulate fibrotic gene expression in fibroblasts (Tidball, Spencer et al. 1992; Bernasconi, Torchiana et al. 1995).

**Fibrosis**

As the disease progresses, DMD patients lose muscle function and mobility due to significant development of fibrosis. Fibrosis is composed of excessive extracellular matrix that is deposited in the perimysium and endomysium compartments of the muscle (Duance, Stephens et al. 1980; Hantai, Labat-Robert et al. 1985). The process of fibrosis deposition begins early in disease before the onset of significant myofiber loss or degeneration (Duance, Stephens et al. 1980). However, once fibrosis deposition becomes significant in the severe stages of disease, it is it becomes an irreversible event in disease pathology resulting in significant muscle function deficit and loss of mobility. The degree of endomysial fibrosis has been shown to correlate strongly with poor motor outcome and age of loss of ambulation, more so than any other parameters including myofiber atrophy, necrosis, and fatty degeneration (Desguerre, Mayer et al. 2009). While both gene replacement and cell therapies are in development as treatments for DMD, fibrosis represents a significant barrier for their success. For this reason it is critical to identify the cellular sources and mechanisms responsible for fibrosis development.
The immune cytokines transforming growth factor beta (TGFβ) and platelet derived growth factor (PDGF) have been implicated in the development of fibrosis in multiple disorders including DMD and the *mdx* mouse (Zhao, Haginoya et al. 2003; Chen, Nagaraju et al. 2005). Multiple studies have investigated the blockage of these pathways in reducing fibrosis in the *mdx* mouse. For example, direct inhibition of TGFβ via neutralizing antibody is successful at reducing fibrosis deposition in *mdx* mice (Andreetta, Bernasconi et al. 2006). However a significant drawback to direct TGFβ ligand blockage is an exacerbated inflammatory response. On the other hand inhibition via TGFβ receptor antagonist, losartan, results in significant fibrosis reduction at 9 months of age without an observable increase in inflammation, but fails to inhibit fibrosis when animals reach 2 years of age (Cohn, van Erp et al. 2007; Bish, Yarchoan et al. 2011). Imatinib is a chemical compound that has been tested in *mdx* mice that successfully blocks PDGF signaling resulting in a moderate decrease in fibrosis and a significant reduction in inflammation (Huang, Zhao et al. 2009). Despite progress in identifying pro-fibrotic mechanisms and therapeutic targets, no anti-fibrotic treatments have progressed into effective therapies in human DMD patients. Furthermore, there is a critical need to investigate the basic mechanisms driving fibrosis deposition in DMD and identify the cellular effectors responsible to allow for optimal anti-fibrotic drug targeting and discovery.

The development of fibrosis in DMD has long been thought to derive from expression of excessive ECM components by muscle fibroblasts. However, the
identification and cellular origin of fibroblasts has remained elusive due to the lack of specific *in vivo* markers. Recently, a population of interstitial cells termed fibro-adipogenic progenitors (FAPS) or mesenchymal progenitors has been identified by their expression of PDGFRα, that are responsible as progenitor cells for both collagen expressing fibroblasts or fat producing adipose cells *in vitro* and *in vivo* (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010). In animal studies, FAPS appear to be the predominant population of cells capable of engrafting into the interstitial space and producing Collagen 1 in the *mdx* diaphragm (Uezumi, Ito et al. 2011). Additionally, FAPs represent a new cellular target for anti-fibrotic therapeutic development in DMD and other fibrotic disorders.

**Muscle Regeneration**

Multiple theories have been proposed in order to explain the deficit in muscle regeneration observed in dystrophic patients ranging from the exhaustion of the stem cell pool, to faulty differentiation of satellite cells and myoblasts, and lastly telomere shortening leading to satellite cell senescence due to extended periods of proliferation resulting from chronic damage. Telomere length has been measured in DMD patients and found to not be significantly altered (Oexle, Zwirner et al. 1997). Despite controversy surrounding these hypotheses, it appears that a block in myogenic differentiation is a primary cause for dysfunction of muscle regeneration in DMD. This is supported by studies on DMD biopsies by both electron microscopy staining as well as Pax7 staining, showing that satellite cell numbers are increased in DMD patients as compared
Further evidence for a defect in differentiation is supported by findings showing abnormal features of myofibers including caliber variation, and fiber branching in part thought to be due to incomplete fusion of myoblasts (Swash and Schwartz 1977; Schmalbruch 1984).

1.3 Mouse Models of DMD

The most common mouse model for studying DMD is the *mdx* mouse occurring in the BL10 background strain (Bulfield, Siller et al. 1984). The *mdx* mouse contains a naturally occurring premature stop codon on exon 23 leading to a lack of dystrophin expression (Bulfield, Siller et al. 1984). Signs of pathology begin at 3 weeks of age, at which time fiber degeneration and necrosis can be observed as well as elevated serum creatine kinase levels (Bulfield, Siller et al. 1984). The severity of fiber degeneration reduces to a low level by eight weeks of age in the limb muscles. However, in the diaphragm degeneration continues resulting in progressive fibrosis accumulation (Stedman, Sweeney et al. 1991). Due to the mild nature of pathology in the limb muscles, studies involving fibrosis deposition normally focus on the diaphragm muscle. Despite the relatively mild phenotype of the *mdx* mouse, the model has been invaluable in evaluating mechanisms of disease pathology and testing therapeutic strategies. For instance, pathological parameters are present in the *mdx* mouse model that reflects human pathology including elevated fiber size variability and presence of centrally nucleated fibers (Carnwath and Shotton 1987; Briguet, Courdier-Fruh et
al. 2004). Muscle fibers become centrally nucleated after they have undergone a cycle of degeneration and regeneration. Quantification of central nuclei provides both a measure of degeneration and the capacity of the muscle to regenerate (Briguet, Courdier-Fruh et al. 2004). Additionally, regenerative capacity can be measured by immunostaining for embryonic myosin heavy chain (eMHC) which is expressed in actively regenerating myofibers (Sartore, Gorza et al. 1982).

A number of alternative DMD mouse models were generated through the treatment of male mice with the mutagen N-ethyl-nitrosourea, generating the mouse lines \textit{mdx}^{2cv}, \textit{mdx}^{3cv}, \textit{mdx}^{4cv}, and \textit{mdx}^{5cv} (Chapman, Miller et al. 1989). These mice displayed elevated serum creatine kinase levels at similar levels to the \textit{mdx} mouse. Pathology was found to be similar in the \textit{mdx} mouse with a mild phenotype in the skeletal muscle and significant fibrosis in the diaphragm muscle. Interestingly, key differences were observed including increased fiber size variation and reduction in the number of revertant fibers, which are dystrophic fibers spontaneously expressing dystrophin protein (Danko, Chapman et al. 1992). In addition, \textit{mdx}^{5cv} mice display more significant motor function impairments, including more severe strength deficits in the diaphragm and more severe exercise induced fatigue (Beastrom, Lu et al. 2011). The more severe phenotype of the \textit{mdx}^{5cv} mouse represents a better model to study DMD pathology and evaluate the efficacy of therapeutics in increasing muscle strength and resistance to fatigue.
1.4 Cellular Therapies

There is a critical need to develop new therapeutic agents for treatment of DMD that significantly increase lifespan and restore mobility in patients. The current standard of care, prednisone, is only successful at extending mobility but has no significant effect on survival (Mendell, Moxley et al. 1989; Griggs, Moxley et al. 1991; Connolly, Schierbecker et al. 2002). A number of therapeutics are currently in development including both gene replacement and cell based therapies. Cellular therapies are a promising therapeutic strategy to increase regeneration in DMD patients through the transplantation of myogenic progenitors. Cell therapy has the potential to ameliorate disease pathology through two mechanisms. First, injection of myogenic cells will serve to increase muscle regeneration resulting in improved muscle strength and resistance to fibrosis development. Secondly, isolation of autologous patient cells represents a method to correct the loss of dystrophin. Cell therapy has the potential to be useful not only in restoring the primary defect, the loss of dystrophin expression, but also in generating additional muscle. The latter is especially important for late stage DMD patients, in which the muscle has been replaced by fibrosis; in those patients cell therapies could restore lost muscle. This section will review progress in cell therapy using traditional satellite cells and as well as introduce newer alternative populations of cells able to undergo myogenesis that show promise in cell therapy.
**Myoblast transplantation**

Myoblast transplantation was the first form of cell based therapy attempted in patients, involving the injection of myoblasts from unaffected donors into muscles of DMD recipients. This treatment showed great promise based on initial experiments in immunosuppressed *mdx* mice, in which dystrophin positive myoblasts successfully engrafted into recipient mice generating dystrophin positive myofibers (Partridge, Morgan et al. 1989). The resulting human trials yielded poor results (Karpati, Ajdukovic et al. 1993; Tremblay, Malouin et al. 1993), with a maximum of only 10% dystrophin expressing fibers in a fraction of patients despite injection of millions of myoblasts (Mendell, Kissel et al. 1995). This result was attributed to a combination of factors, including poor survival of cells post injection, poor immunosuppression, and poor migration of cells beyond the site of injection (Karpati, Ajdukovic et al. 1993; Mendell, Kissel et al. 1995). However, immune suppression studies on non-human primates show that multiple injection sites must be used 1mm apart in order to attain 25-65% engraftment rates (Kinoshita, Roy et al. 1996). There is concern regarding the long term use of immunosuppressants such as FK506 due to increased risk of significant adverse effects including nephrotoxicity, diabetes, and an increased risk of cancer (Radis, Kahl et al. 1995; Jindal, Sidner et al. 1997; Niethammer, Kummerle-Deschner et al. 1999). To circumvent these issues, interest has grown in the potential isolation and modification of a patient’s own myoblasts for autologous cell therapy. Transplantations of genetically modified cells with lentivirus to carry the micro-dystrophin construct have been successful in both
mice and non-human primate studies (Quenneville, Chapdelaine et al. 2007; Xiong, Xu et al. 2010; Benabdallah, Duval et al. 2013). However, myoblast therapy using genetically modified autologous myoblasts in patients has not yet been attempted.

Despite these promising results, myoblasts still exhibit poor survival and migration that prevent their clinical use. These shortcomings have been attributed to the more mature status of the myoblast in the myogenic cell hierarchy. It has been proposed that further upstream satellite cells represent a superior subset of cells for muscle regeneration and muscle cell transplantation.

While original studies involving myoblast therapy relied on crude methods for myogenic cell preparations resulting in impure populations, recent advances in cell sorting and the identification of extracellular markers of myogenic cells have made isolation of pure satellite cells possible. Commonly used surface markers for satellite cell isolation include positive selection for CD34, α7 integrin, β1 integrin, NCAM, c-Met, M-cadherin, and CXCR4 and negative selection for CD31, CD45, and Sca1 (Beauchamp, Heslop et al. 2000; Fukada, Higuchi et al. 2004; Cerletti, Jurga et al. 2008). Delivery of freshly isolated satellite cells into the mdx mouse results in increased engraftment as compared to earlier myoblast transplantation experiments (Cerletti, Jurga et al. 2008). However, satellite cell engraftment is still limited to the injection site. Furthermore, to be clinically successful satellite cell therapy would require a high number of intramuscular injections, similar to myoblasts. A significant drawback for the use of satellite cells is their relatively low abundance in vivo, limiting the number of cells
available for intramuscular injections. Additionally, *in vitro* culture of satellite cells reduces their engraftment potential, hindering *in vitro* expansion and *ex vivo* genetic modification for autologous studies.

**Alternative Myogenic Populations**

While satellite cells have long been considered the primary cell responsible for muscle regeneration, multiple populations in and outside of the skeletal muscle have emerged both that are capable of both migrating to the satellite cell position and regenerating damaged myofibers. These include side population cells, muscle derived stem cells, pericytes, and PW1 cells. A major benefit for cellular therapy using non-satellite stem cells is their ability to migrate to the muscle following intra-arterial injection. While satellite cells and myoblast therapy require high numbers of injections in each muscle, non-satellite stem cells have the potential to migrate to all of the muscles of the body.

**Side Population Cells**

Side population cells are a group of multipotent stem cells originally isolated from the bone marrow based on their ability to efflux the Hoechst 33342 dye (Goodell, Brose et al. 1996). The skeletal muscle SP are a heterogeneous population of cells composed of three major populations: CD45+ cells originating from the bone marrow, CD31+ endothelial cells, and CD31-/CD45- (Lin-) interstitial cells (Uezumi, Ojima et al. 2006). The Lin-SP cells have been localized to the interstitium in close proximity to the endothelium based on expression of
Sca-1 (Asakura, Seale et al. 2002). The Lin- fraction is myogenic upon transplantation in *in vivo* engraftment into regenerating cardiotoxin-injured muscle (Uezumi, Ojima et al. 2006). Moreover, CD45+ SP cells exhibit no engraftment potential and CD31+ SP cells engraft at very low levels in only a few fibers (Uezumi, Ojima et al. 2006).

In contrast to myoblasts and satellite cells, SP cells are successful upon intra-arterial delivery in migrating and engrafting in *mdx* skeletal muscle fibers (Bachrach, Li et al. 2004). However, only a maximum of 8% of myofibers were successfully engrafted (Bachrach, Li et al. 2004). These results may be due to a combination of factors. First, the total fraction of skeletal muscle SP cells included non-myogenic CD45+ and CD31+ cells. Secondly, similar to myoblast therapies, *in vitro* culture prior to injection likely reduced their myogenic activity leaving them in a more activated myoblast like state.

**Muscle Derived Stem Cells**

Another population of muscle stem cells, termed muscle derived stem cells (MDSCs), has been identified based on extended preplating techniques taking advantage of their low adherence characteristics and their ability to proliferate *in vitro* for an extended period of time (Qu-Petersen, Deasy et al. 2002; Deasy, Gharabeh et al. 2005). MDSCs have been characterized as CD45-/M-Cadherin-/CD34-/FLk-1+/Sca-1+/Desmin+ (Qu-Petersen, Deasy et al. 2002). These cells are not likely to be satellite cells due to the lack of M-cadherin expression. MDSCs have the potential to form multiple lineages *in vitro*
including, myogenic, adipogenic, osteogenic, chondrogenic, and hematopoietic cells (Peng and Huard 2004). MDSCs contribute to myogenesis *in vivo* upon intra-arterial delivery contributing to regenerated myofibers as well as the satellite cell position (Qu-Petersen, Deasy et al. 2002). A significant benefit of utilized MDSCs is that due to their stem cell nature, MDSCs are immune privileged and therefore would not require immune suppressants upon transplantation (Qu-Petersen, Deasy et al. 2002).

**Pericytes**

Pericytes are contractile connective tissue cells located in capillaries beneath the microvascular basement membrane. They function to regulate vessel contractility and division of endothelial cells (Tilton, Kilo et al. 1979; Hirschi and D’Amore 1996; Kutcher and Herman 2009). Pericytes are isolated from multiple tissues by the markers, CD45-/CD34-/CD56-/CD144-/CD146+/Pdgfrβ+/NG2+ (Dellavalle, Sampaolesi et al. 2007; Crisan, Deasy et al. 2008; Franco, Roswall et al. 2011). While they do not express satellite cell or myoblast markers such as Pax7, Myf5, or MyoD, pericytes isolated from multiple organs differentiate into muscle when cultured *in vitro* and upon injection into immunodeficient *mdx* mice (Dellavalle, Sampaolesi et al. 2007; Dellavalle, Maroli et al. 2011). In addition to myogenesis, pericytes also differentiate into chondrocytes, adipocytes, and osteocytes (Doherty, Ashton et al. 1998; Farrington-Rock, Crofts et al. 2004). Additionally, a small proportion of pericytes engraft into the satellite cell position *in vivo* (Dellavalle, Sampaolesi et al. 2007).
Pericyte engraftment into mdx mice shows improved physical performance, making this population an attractive target for therapeutic development in muscular dystrophy (Dellavalle, Sampaolesi et al. 2007). In addition, pericytes can be cultured in vitro without losing their stem cell potential allowing for expansion and potential insertion of micro-dystrophin transgenes for gene therapy in DMD (Westerman, Penvose et al. 2010).

**PW1 cells**

PW1 has been shown to be a marker of stem cells or progenitor cells in multiple organs in mice through the use of PW1 reporter mice (Besson, Smeriglio et al. 2011). In fact, there is a population of PW1+ cells located in the skeletal muscle interstitium (Mitchell, Pannerec et al. 2010). These interstitial cells are characterized by their expression profile, Sca-1+/CD34+/Pax7-, named PIC cells (Mitchell, Pannerec et al. 2010). Initial studies have shown the myogenic potential of PIC cells in vivo by intramuscular injection into regenerating muscle, generating both interstitial cells and satellite cells (Mitchell, Pannerec et al. 2010). The interpretation of these results is unclear however due to the surface markers used for isolation, Sca-1+/CD34+/CD45-. Sca-1 has been shown to be expressed on a subset of satellite cells/myoblasts (Mitchell, Mills et al. 2005; Bosnakovski, Xu et al. 2008), and Sca-1 and CD34 have been shown to be markers of fibro-adipogenic cells (Joe, Yi et al. 2010). Due to the lack of lineage tracing, the direct contribution of PIC cells in myogenesis remains unclear.
1.5 Myogenesis: From Satellite Cells to SP Cells

Cell based therapies are promising treatment strategies to increase regeneration and serve as a gene therapy vector in dystrophic tissue. As mentioned previously, myoblast transplantation has been met with many challenges due to poor migration and poor survival resulting in limited therapeutic benefit in patients. As a result, many new populations of myogenic cells distinct from satellite cells are under investigation for cell therapy. Although many of these populations display increased survival and migration, they are still not effective at alleviating pathology. This is likely due to our limited knowledge of the mechanisms regulating the myogenic commitment of these alternative populations and the effects of the dystrophic environment on their activity. This study is focused on understanding the biology of the side population cells in order to better understand their myogenesis and the role that the muscle environment may play in directing their lineage commitment. In order to gain an understanding of the mechanisms regulating the myogenesis of muscle SP cells, this section will review current knowledge pertaining to the developmental origin and molecular regulation of both satellite cells and muscle SP cells.

Embryonic Myogenesis

During embryonic development, skeletal muscles are derived from segments of paraxial mesoderm termed the somites (Christ and Ordahl 1995; Scaal and Christ 2004). The back and diaphragm muscles are derived from the epaxial segment of the somite while the abdominal and limb muscles form from
the hypaxial somite (Ordahl and Le Douarin 1992; Huang and Christ 2000; Gros, Scaal et al. 2004). Embryonic myogenesis occurs over two distinct stages (Gros, Scaal et al. 2004). Initially upon somite differentiation, post mitotic myocytes migrate from the dermomyotome (Gros, Scaal et al. 2004). These cells express Myf5 and Mrf4 forming muscles just beneath the dermomyotome, making up the primary myotome (Gros, Scaal et al. 2004). In the second stage, an epithelial to mesenchymal transition (EMT) occurs among cells expressing Pax3 and Pax7 localized in the central dermomyotome that migrate into the muscles formed during the initial stage of myogenesis (Kalcheim and Ben-Yair 2005). Satellite cells are thought to be derived from these migrating Pax3+/Pax7+ cells in the late developmental stages that become enveloped by the basal lamina of forming myofibers (Gros, Manceau et al. 2005).

The developmental origin for both satellite cells and the muscle side population has been investigated through the use of cellular labeling. Somitic cell labeling experiments have determined that the majority of limb muscle satellite cells and a significant proportion of limb muscle side population cells are derived from the hypaxial somite (Schienda, Engleka et al. 2006). Interestingly, these experiments show that somitically derived side population cells are significantly more myogenic in cell culture compared to non-somitically derived cells (Schienda, Engleka et al. 2006). These results illustrate that the developmental origin of SP cells greatly influences their myogenic potential.
Regeneration and the Intrinsic Myogenic Program

The major cell type responsible for adult muscle regeneration is the satellite cell. Satellite cells were originally identified by their location using electron microscopy as cells adjacent to myofibers but underneath the basal lamina (Mauro 1961). In healthy uninjured muscle, satellite cells are in a quiescent state. While in the quiescent state, satellite cells are characterized by expression of the transcription factor Pax7 while lacking expression of MyoD and Myogenin (Cornelison and Wold 1997; Seale, Sabourin et al. 2000). Additionally, expression of myf5 is present in about 90% of quiescent satellite cells (Beauchamp, Heslop et al. 2000). Upon muscle injury, satellite cells are activated, undergo proliferation, differentiate into myoblasts, and fuse to repair or replace damaged fibers. Once satellite cells are activated, they are characterized by expression of MyoD as well as increased expression of Myf5 (Grounds, Garrett et al. 1992; Cooper, Tajbakhsh et al. 1999; Zammit, Heslop et al. 2002). Interestingly, activation of satellite cells is not restricted to the location of muscle damage, as all satellite cells along a myofiber are activated and migrate to the area of damage (Schultz, Jaryszak et al. 1985). Following proliferation, the majority of activated satellite cells undergo terminal differentiation and fuse into damaged myofibers or fuse with each other to form new myofibers. The process of terminal differentiation and fusion begins with the expression of the transcription factors, Myogenin and Myf6 (Smith, Janney et al. 1994; Andres and Walsh 1996). The induction of Myogenin is dependent on MyoD expression and induces a number of genes activated by MyoD (Cao, Kumar et al. 2006). These
genes include a number of muscle specific structural and contractile genes including actins, myosins, and troponins (Lin, Yutzey et al. 1991; Berkes and Tapscott 2005; Meissner, Umeda et al. 2007).

The Role of the Microenvironment

The muscle microenvironment plays a critical role in regulating muscle regeneration and the activity of satellite cells. During regeneration, the muscle microenvironment changes dynamically, including remodeling of the extracellular matrix, activation and release of multiple growth factors, immune cell infiltration and cytokine release. In addition there is a growing role for the interaction between myogenic cells and cells of the interstitium. These processes work in cooperation to regulate all stages of myogenesis from initial activation, proliferation, and differentiation and fusion of myogenic cells.

The process of muscle regeneration involves significant remodeling of the extracellular matrix (ECM). Remodeling of the ECM is executed by matrix metalloproteinases (MMPs) that break down existing matrix and process inactive growth factors into active forms to stimulate regeneration (Carmeli, Moas et al. 2004). Hepatocyte growth factor (HGF) is one of the first stimuli activated during muscle regeneration and is responsible for driving muscle satellite cell activation and proliferation (Allen, Sheehan et al. 1995; Tatsumi, Anderson et al. 1998). Additional factors have been discovered that function during muscle regeneration including fibroblast growth factor (FGF) and insulin like growth factor (IGF).
(Edwall, Schalling et al. 1989; Lefaucheur and Sebille 1995; Philippou, Maridaki et al. 2007). These factors elicit multiple functions on satellite cells to drive the proliferation, differentiation, and fusion of satellite cells during the regenerative process (Allen, Dodson et al. 1984; Florini, Ewton et al. 1991; Magri, Ewton et al. 1991; Philippou, Halapas et al. 2007). They do this in part by regulating the expression and activity of the myogenic transcription factors mentioned previously, MyoD and Myogenin (Florini, Ewton et al. 1991; Fiore, Sebille et al. 2000).

During the process of muscle regeneration, multiple cytokines are produced by both muscle and inflammatory cells that regulate satellite cell proliferation and differentiation. These cytokines regulate myogenesis by influencing the activity of the myogenic regulatory factors previously mentioned. TNFα for example induces the proliferation of myogenic cells by activating NFκB signaling which in turn impacts levels of MyoD in myoblasts. NFκB promotes proliferation by inducing expression of cyclinD1, thus maintaining cell cycle activity and destabilizing MyoD, which is necessary to progress towards differentiation (Guttridge, Mayo et al. 2000). Transforming growth factor beta, TGFβ is expressed following muscle injury and in dystrophic mdx muscle (Gosselin, Williams et al. 2004; Li, Foster et al. 2004). TGFβ signaling promotes myogenic cell proliferation while inhibiting differentiation in C2C12 cells (Qing, Liu et al. 2004; Zhu, Goldschmidt-Clermont et al. 2004). Moreover, this process is in part mediated through downstream Smad3 inhibition of MEF2c and through

The muscle interstitium composes the stromal tissue between the basal lamina and epimysial sheath. The interstitium contains both connective tissue fibroblasts and FAPs. Fibroblasts act as support cells responsible for secreting growth factors and producing and modifying ECM components such as collagen, fibronectin, and laminin (Sasse, von der Mark et al. 1981; Sanderson, Fitch et al. 1986). Additionally, fibroblasts play a crucial role during muscle regeneration including regulating satellite cell differentiation and maintenance of the satellite cell pool (Murphy, Lawson et al. 2011). Ablation of fibroblasts significantly impacts regeneration resulting in reduced numbers of activated Pax7+/MyoD+ satellite cells and regenerating myofibers (Murphy, Lawson et al. 2011). Similarly FAPs from regenerating muscle promote myogenesis by inducing the up-regulation of several of the MRFs in myogenic cultures (Joe, Yi et al. 2010). While fibroblasts and FAPs are often viewed as contributors to fibrosis deposition, recent findings indicate that they are important components of the muscle regenerative process.
SP cell biology

The role of SP cells in myogenesis and regeneration is currently unclear. As previously mentioned, SP cells are identified by the efflux of the Hoechst dye which is facilitated by transporter pumps including Abcg2 and Mdr1 (Bunting, Zhou et al. 2000; Zhou, Schuetz et al. 2001). However, green fluorescent protein (GFP) labeling of cells expressing Abcg2 has shown that while approximately 22% of mononuclear muscle cells express Abcg2, less than 1% are associated with the SP phenotype (Doyle, Zhou et al. 2011). These results highlight that the expression of a transporter pump does not ensure a cell will efflux Hoechst or be an SP cell; but regulatory mechanisms are likely present that control the activity of the transporter that are currently unknown. Due to the lack of in vivo markers, lineage tracing and genetic ablation models are not currently available to test the contribution of SP cells to myogenesis. As a result, our understanding of SP cells is limited to transplantation studies using labeled cells, in vitro cell culture systems, and analysis of freshly sorted cells.

Despite the lack of direct evidence for the contribution of SP cells to myogenic regeneration multiple studies suggest that SP cells contribute to the regenerative process. For instance knockout of the Abcg2 pump in acutely injured muscle and knockout of the Mdr1 transporter in the mdx mouse both result in a hampered regenerative response (Israeli, Ziaei et al. 2007; Doyle, Zhou et al. 2011). Secondly, SP cells support the activity of satellite cells during regeneration. For example, co-injection of SP cells with satellite cells enhances
satellite cell engraftment by the production of MMP2 in SP cells, which in turn improves satellite cell migration (Motohashi, Uezumi et al. 2008). Additionally, human fetal SP cells support myogenic cell proliferation by production of BMP4 (Frank, Kho et al. 2006). These results demonstrate that while the direct contribution of SP cells to myogenesis is unclear, SP cells undoubtedly contribute to the process of muscle regeneration.

The cellular characterization of SP cells is currently unclear due to the heterogeneity of the muscle SP population. Due to the myogenic nature of SP cells in vivo (Uezumi, Ojima et al. 2006), it has been hypothesized that a portion of the SP pool are satellite cells. On one hand, recently a small fraction of satellite cells have been identified with the SP phenotype (Tanaka, Hall et al. 2009). On the other hand, multiple studies suggest that SP cells are a population of cells distinct from the satellite cells. Muscles from Pax7 knockout mice, while lacking functional satellite cells still contain an equivalent number of SP cells (Seale, Sabourin et al. 2000). In addition, SP cells lacking Pax7 undergo myogenesis when co-cultured with C2C12 myogenic cells (Asakura, Seale et al. 2002). Further evidence by RT-PCR demonstrated that SP cells do not express any of the early myogenic markers expressed by satellite cells including Pax3, Pax7, or Myf5 (Uezumi, Ojima et al. 2006). It remains difficult to clearly interpret these conflicting lines of evidence due to uncertainty whether the myogenesis observed upon injection of SP cells is due entirely or only in part to the satellite
cell sub fraction of the SP cells. Furthermore, it is possible that there are multiple populations of cells within the SP capable of producing myogenic cells.

As previously mentioned muscle SP cells have been investigated as a stem cell population for cell based therapy in order to enhance regeneration and potentially serve as a vector for gene therapy delivery in DMD. While multiple cell lineages including the SP cells are attractive for cell therapy, they are not committed to the myogenic lineage (Motohashi, Uezumi et al. 2008). Additionally, the dystrophic environment contains multiple cytokines that function in fibrotic development including TGFβ (Gosselin, Williams et al. 2004; Zhou, Porter et al. 2006) that may contribute to sub optimal myogenic commitment due to redirection towards alternative cell lineages such as fibroblasts or adipocytes. Currently, study of SP cells is limited to co-culture with the C2C12 myogenic cell line (Asakura, Seale et al. 2002). As a consequence, there is no in vitro system available to analyze either the intrinsic myogenic capacity of SP cells or the potential misdirection of SP cells towards non-myogenic lineages. While SP cells isolated form wild type muscle clearly engraft as myogenic cells, SP cells from acutely injured muscle fail to undergo myogenesis in vivo forming interstitial cells (Motohashi, Uezumi et al. 2008). Furthermore, the muscle environment clearly impacts SP myogenesis which has implications for the efficacy of the SP cells to be used for cell based therapies. For this reason, it is critical to develop tools to further investigate SP cell biology and determine the effects of the muscle
environment, including the dystrophic environment, on SP cell lineage determination.

### 1.6 Hedgehog signaling

In this study we have identified that the hedgehog pathway (Hh) is down-regulated in SP cells isolated from mdx mice compared to wild type. Our studies further show that Hh stimulation promotes myogenesis of SP cells isolated from $mdx^{5cv}$ mice and improves pathology in $mdx^{5cv}$ mice. This section will serve to review the Hh pathway and its functions in both early development of the skeletal muscle and in adult regeneration.

Hedgehog was discovered over 30 years ago as a factor regulating embryonic cuticle patterning in Drosophila (Nusslein-Volhard and Wieschaus 1980). Since its initial discover Hh signaling has been shown to contribute numerous functions throughout development in both Drosophila and mammals including limb and organ patterning, cell fate determination, and stem cell maintenance (Yang, Drossopoulou et al. 1997; Lai, Kaspar et al. 2003; Machold, Hayashi et al. 2003). While a single Hh ligand has been identified in Drosophila, three have been identified in vertebrates including Sonic hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) (Echelard, Epstein et al. 1993). Hh ligands function by binding to the receptor Ptc1 and relieving the inhibition Ptc1 normally elicits on the Smoothened (Smo) transmembrane protein (Chuang and McMahon 1999; Varjosalo and Taipale 2008). In the absence of Hh
signaling Gli2 and Gli3 are processed into transcriptional repressors (Wang, Fallon et al. 2000; Pan, Bai et al. 2006) (Figure 1.1). However Gli3 is recognized as the primary repressive effector while Gli2 has been regarded as only weakly repressing downstream genes (McDermott, Gustafsson et al. 2005). Hh signaling leads to activation of full length Gli2 and Gli3 into transcriptional activators (Wang, Fallon et al. 2000) (Figure 1.1). While both Gli2 and Gli3 are capable of activating transcription of target genes, studies have shown that Gli2 has a more potent activator function (Bai, Auerbach et al. 2002; Pan, Bai et al. 2006).

Following Gli2 activation, the pathway activates multiple regulatory feedback loops involving both Gli1 and Ptch1. Activation of Gli1 serves as a positive feedback loop that functions to extend and intensify Gli target gene expression (Hynes, Stone et al. 1997). On the other hand Ptch1 mRNA is induced which serves as a negative regulatory mechanism to reduce Hh pathway activation (Goodrich, Johnson et al. 1996). Both of these factors, Gli1 and Ptch1, have been used extensively to assay for Hh pathway activation status (Goodrich, Milenkovic et al. 1997; Bai, Auerbach et al. 2002).

Hedgehog pathway activity has been shown to be regulated by localization of pathway members to the cell membrane in Drosophila and the primary cilia in vertebrates. In Drosophila, in the absence of Hh Ptch1 inhibits Smo expression on the cell membrane by promoting Smo endocytosis and degradation (Denef, Neubuser et al. 2000; Zhu, Zheng et al. 2003). Upon Hh signaling, Smo accumulates on the cell membrane while Ptch1 is internalized.
Contrastingly in vertebrates, Ptch1, Smo, and Gli factors are localized in the primary cilia (Corbit, Aanstad et al. 2005; Haycraft, Banizs et al. 2005; Rohatgi, Milenkovic et al. 2007). Similar to Drosophila, without Hh signaling Ptch1 localizes to the cilia while preventing Smo localization; in response to Hh signaling Ptch1 moves out of the cilia resulting in Smo accumulation and further pathway activation (Rohatgi, Milenkovic et al. 2007). Small molecules have been identified that can either activate (purmorphamine and SAG) or repress (cyclopamine) the Hh pathway independent of Ptch1 or Hh ligands (Figure 1.1) (Chen, Taipale et al. 2002; Chen, Taipale et al. 2002; Sinha and Chen 2006). These molecules share their modes of action by directly acting and modulating the activity of Smo, independent of the presence of Ptch1, and by regulating the localization of Smo in the cilia (Chen, Taipale et al. 2002; Sinha and Chen 2006; Rohatgi, Milenkovic et al. 2009; Wang, Zhou et al. 2009). For example SAG has been demonstrated to stimulate Smo accumulation in the cilia where it can signal and promote Gli2/3 activators while cyclopamine prevents Smo localization in the cilia preventing pathway activation (Wang, Zhou et al. 2009).

Non-canonical Hh signaling has been reported in which cellular responses are observed without the induction of Gli transcription factor expression. While non-canonical Hh signaling is not a focus in this dissertation, it is presented here to provide a comprehensive review of Hh signaling and demonstrate its increasing functions in biology. In multiple cells ectopic expression of Ptch1
induces apoptosis while overexpression of Smo is incapable of preventing cell death (Thibert, Teillet et al. 2003; Mille, Thibert et al. 2009). Mechanistically this function appears to be mediated by the C-terminal domain of Ptch1 which serves as a substrate for caspase-3,-7, and -8 (Mille, Thibert et al. 2009). An additional non-canonical function has been described whereby Hh signaling regulates cytoskeletal organization. This has been demonstrated in endothelial cells whereby Hh signaling induces actin stress fiber formation and tubulogenesis without inducing gene expression of either Gli1 or Ptch1 (Chinchilla, Xiao et al. 2010). In addition to these functions reports have accumulated implicating Hh signaling in additional non-canonical functions including regulation of cyclin B1, Src activation in the axonal growth cone, and in calcium transients (Barnes, Kong et al. 2001; Yam, Langlois et al. 2009; Belgacem and Borodinsky 2011).

In skeletal muscle development, Hh has been shown to mediate its promyogenic effects through Gli2 binding to promoter elements of both Myf5 and MyoD, enhancing their activity (Gustafsson, Pan et al. 2002). Through this mechanism Hh controls expression and patterning of MyoD in the somite (Borycki, Mendham et al. 1998). The notochord has been found to be a critical source Hh ligand necessary for expression of MyoD and Myf5 in the developing myotome (Munsterberg, Kitajewski et al. 1995). Later in development during muscle formation, Hh has been shown to be an important factor in the formation of both epaxial and hypaxial muscles throughout the body. For example, in the developing epaxial muscle, Hh signaling is critical for the myogenic induction
activating both Myf5 and MyoD (Borycki, Brunk et al. 1999). Also Hh functions as a survival and proliferation factor for myogenic precursor cells in hypaxial muscle (Kruger, Mennerich et al. 2001).

While Hh signaling has been well investigated in embryonic myogenesis the role of the pathway in adult muscle regeneration has only recently been investigated. Expression of Shh is induced during muscle regeneration in response to both cardiotoxin and freeze injury (Straface, Aprahamian et al. 2009). In response to acute injury Hh promotes expression of MyoD and Myf5 as shown by Hh inhibition experiments using cyclopamine, whereby expression of MyoD and Myf5, and the number of satellite cells and myoblasts expressing these markers are reduced (Straface, Aprahamian et al. 2009). Interestingly, Hh signaling appears to be critical for the muscle regeneration process as cyclopamine injection into acutely damaged muscle results in fibrosis deposition (Straface, Aprahamian et al. 2009).

In vitro studies show that Hh increases proliferation in both primary satellite cells and C2C12 cells. However, the role of Hh signaling in muscle differentiation is currently unclear as there have been conflicting reports of Hh both promoting and inhibiting differentiation in vitro. A study by (Koleva, Kappler et al. 2005) has demonstrated that Hh stimulation during the differentiation phase of C2C12 cells and primary satellite cells reduces the number of differentiated myotubes and blocks expression of myogenin. On the other hand, studies by (Elia, Madhala et al. 2007), suggest that Hh promotes both proliferation and
differentiation of muscle cells by activating the MAPK/ERK and PI3K/AKT pathways, respectively. Additional support from these studies show that Hh ligands increase protein expression of Myosin Heavy Chain (MHC), a marker that is expressed in differentiating myoblasts. Further evidence for a differentiation promoting role has been presented in recent studies (Voronova, Coyne et al. 2013) revealing that Gli2 forms a protein complex with MyoD and inhibition of Hh signaling reduces expression of the MyoD promoter and well as reduced expression of downstream genes involved in differentiation including myogenin.

While there is a large amount of data implicating Hh as a critical factor in embryonic myogenesis, the role of Hh in adult regeneration is currently limited. While Hh inhibition studies clearly demonstrate a requirement for Hh signaling for maintaining satellite cell numbers, it is currently unclear whether increasing Hh pathway activity is capable of enhancing muscle regeneration following injury. Due to the pleiotropic functions of Hh signaling in satellite cell biology, fibrosis, and angiogenesis, modulation of the pathway may have the potential to enhance muscle regeneration by improving multiple processes in the muscle environment. Interestingly, despite the modulation of all of these Hh related parameters in DMD, there has yet to be a study investigating the state of Hh signaling in DMD or evaluation if increasing Hh signaling may represent a potential strategy to ameliorate disease pathology.
1.7 Summary

The genetic cause for DMD has been known for over 30 years yet there are currently no treatments available that stabilize disease progression. Despite intense research efforts to utilize traditional myoblasts and satellite cells for regenerative therapy, they are not effective in treating patients. Research in identifying alternative populations of cells within the skeletal muscle to be more efficacious for myogenic regeneration has increased. The side population cells are one example of these populations that have shown promising results in animal studies by engrafting into dystrophic muscle fibers. Recent evidence has suggested that the regenerating muscle environment may adversely affect the myogenic potential of the side population cells. Because of this, it is necessary to analyze the cell fate decisions of muscle side population cells from different muscle environments and determine what environmental factors govern their myogenesis. Currently, however there is no in vitro culture system available to study the intrinsic properties of the side population cells that would allow for analysis of their differentiation. In the present study, we provide an in vitro system capable of supporting side population cells in culture allowing for their spontaneous differentiation. By using this system, we analyzed the myogenesis of the side population and show that side population cells isolated from a dystrophic environment do not undergo myogenesis. Additionally, we identify Hh signaling as component down regulated in the dystrophic environment that functions to promote side population cell myogenesis and attenuate parameters of dystrophic pathology in the mdx mouse.
Figure 1.1: Schematic describing the regulation of the Hedgehog pathway in vertebrates. (A) In the absence of Hh ligand, Gli2 and Gli3 are processed into transcriptional repressors blocking expression of Gli target genes. (B) In the presence of Hh ligand, Hh inhibits the interaction between Ptch1 and Smo inducing a conformational change and activation of Smo. This leads to activation of the Gli2 and Gli3 factors into transcriptional activators and expression of downstream Hh target genes Gli1 and Ptch1. (C) Chemical pathway agonists SAG and Purmorphamine (Pur) induce activation of the pathway by binding directly to and activating Smo regardless of the presence of Ptch1. (D) Cyclopamine binds to and inhibits Smo signaling preventing activation of the pathway in the presence of Hh ligand.
Chapter 2

Muscle Side Population Cells from Dystrophic or Injured Muscle Adopt a Fibro-Adipogenic Fate.

2.1 Abstract

Muscle side population (SP) cells are rare multipotent stem cells that can participate in myogenesis and muscle regeneration upon transplantation. While they have been primarily studied for the development of cell-based therapies for Duchenne muscular dystrophy, little is known regarding their non-muscle lineage choices or whether the dystrophic muscle environment affects their ability to repair muscle. Unfortunately, the study of muscle SP cells has been challenged by their low abundance and the absence of specific SP cell markers. To address these issues, we developed culture conditions for the propagation and spontaneous multi-lineage differentiation of muscle SP cells. Using this approach, we show that SP cells from wild type muscle robustly differentiate into satellite cells and form myotubes without requiring co-culture with myogenic cells. Furthermore, this myogenic activity is associated with SP cells negative for immune (CD45) and vascular (CD31) markers but positive for Pax7, Sca1, and the mesenchymal progenitor marker PDGFRα. Additionally, our studies revealed that SP cells isolated from dystrophic or cardiotoxin-injured muscle fail to
undergo myogenesis. Instead, these SP cells rapidly expand giving rise to fibroblast and adipocyte progenitors (FAPs) and to their differentiated progeny, fibroblasts and adipocytes.

Our findings indicate that muscle damage affects the lineage choices of muscle SP cells, promoting their differentiation along fibro-adipogenic lineages while inhibiting myogenesis. These results have implications for a possible role of muscle SP cells in fibrosis and fat deposition in muscular dystrophy. In addition, our studies provide a useful in vitro system to analyze SP cell biology in both normal and pathological conditions.

2.2 Introduction

Adult skeletal muscle exhibits a robust regenerative response following injury. Impairment of this response with aging or due to genetic mutations leads to loss of muscle mass and ultimately loss of muscle function. Therefore, intense research efforts are aimed at understanding the cellular and molecular mechanisms that drive muscle regeneration, as they may reveal insights into muscle disease mechanisms.

The primary cellular effector of regeneration is the muscle satellite cell; a stem cell that resides in close apposition with the myofiber, underneath the basal lamina (Mauro 1961). Satellite cells respond to muscle damage by re-entering the cell cycle to both self-renew and to generate myoblasts that will eventually undergo terminal differentiation and fuse with myofibers to repair damage (Wang
and Rudnicki 2011). Although satellite cells represent the primary source of myogenic cells for regeneration, additional populations of cells have been identified that can undergo myogenic differentiation upon muscle injury (Peault, Rudnicki et al. 2007) and interest has grown towards understanding their roles in the highly coordinated process of muscle repair.

Among these populations are muscle side population (SP) cells. Transplantation studies using gender miss-matched or tagged donor SP cells have revealed that muscle SP cells can participate in muscle regeneration by giving rise to satellite cells (Gussoni, Soneoka et al. 1999; Asakura, Seale et al. 2002; Bachrach, Li et al. 2004; Muskiewicz, Frank et al. 2005; Bachrach, Perez et al. 2006; Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009). Importantly, muscle SP cells can engraft into damaged muscles following systemic delivery (Gussoni, Soneoka et al. 1999; Bachrach, Li et al. 2004; Bachrach, Perez et al. 2006) and they preferentially repopulate the satellite cell niche with the potential for long term muscle regeneration (Tanaka, Hall et al. 2009). Therefore, muscle SP cells are being investigated for their potential use in body-wide cell-based therapies for muscle diseases, such as muscular dystrophies where muscle regeneration progressively fails and satellite cells appear to be depleted (Blau, Webster et al. 1983; Webster and Blau 1990; Decary, Hamida et al. 2000). However, recent studies have cast doubt on the ability of muscle SP cells to contribute to myogenesis in injured muscle when they are not manipulated ex vivo for transplantation (Doyle, Zhou et al. 2011; Lepper, Partridge et al. 2011;
Murphy, Lawson et al. 2011). These studies do not invalidate the potential usefulness of SP cells in transplantations for cell-based therapies, but they indicate a need to develop tools to better understand the biology of SP cells.

SP cells are isolated by Fluorescence Activated Cell Sorter (FACS) based on their unique ability to efficiently efflux the DNA binding dye Hoechst 33342 (Gussoni, Soneoka et al. 1999; Montanaro, Liadaki et al. 2004). This property is primarily dependent on the activity of the Abcg2 transporter (Zhou, Schuetz et al. 2001; Doyle, Zhou et al. 2011). However, Abcg2 expression is not restricted to SP cells in muscle (Tanaka, Hall et al. 2009; Doyle, Zhou et al. 2011) and not all SP cells express Abcg2 (Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009). Indeed, muscle SP cells are heterogeneous with respect to the expression of several markers (Asakura, Seale et al. 2002; Montanaro, Liadaki et al. 2004; Uezumi, Ojima et al. 2006). The most abundant sub-population (about 80% of the SP fraction in non-injured adult mouse muscle) comprises SP cells associated with blood vessels that express the vascular endothelial marker CD31 (Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009). A second sub-population (2% to 10% of total muscle SP) is blood-derived and expresses the immune marker CD45 (McKinney-Freeman, Majka et al. 2003; Ojima, Uezumi et al. 2004; Rivier, Alkan et al. 2004). Their number increases in the presence of muscle damage (Gussoni, Soneoka et al. 1999; Asakura, Seale et al. 2002; Ojima, Uezumi et al. 2004; Uezumi, Ojima et al. 2006). CD31+ and CD45+ SP sub-populations express high levels of Abcg2 and in vivo studies suggest that they
might contribute to muscle regeneration by facilitating tissue vascularization and modulating the immune response (Doyle, Zhou et al. 2011). Finally, the myogenic activity of muscle SP cells is primarily accounted for by a third sub-population that comprises about 5% of the total SP and does not express CD31 or CD45. This sub-population is referred to as lineage negative SP (Lin- SP) and may express the satellite cell marker Pax7 as well as Abcg2 (Tanaka, Hall et al. 2009), although these findings are currently controversial (Uezumi, Ojima et al. 2006).

Lin- SP cells are particularly interesting because among all SP sub-populations they have the greatest muscle engraftment and myogenic differentiation potential (Uezumi, Ojima et al. 2006). Lin- SP cells proliferate in response to acute muscle injury and subsequently preferentially repopulate the satellite cell compartment but also contribute to fully differentiated myofibers (Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009). In addition, Lin- SP cells give rise to non-myogenic cells that reside in the interstitium (Motohashi, Uezumi et al. 2008). Interestingly, these non-myogenic cells interact with myogenic cells to enhance their engraftment and promote muscle regeneration. To date, the identity of the non-myogenic cells generated by Lin- SP cells is unknown and it is technically difficult to ascertain in vivo due to the lack of a unique Lin- SP marker allowing clear lineage tracing studies and to their very low abundance (Lin- SP cells comprise about 0.1% of muscle mononuclear cells). In addition, no single culture system currently supports the multi-lineage differentiation of muscle SP
cells. Instead, differentiation of muscle SP cells into hematopoietic, adipogenic, or osteogenic cells has only been demonstrated with media that contain potent inducers of these lineages (Asakura, Seale et al. 2002; Uezumi, Ojima et al. 2006). Even their in vitro myogenic differentiation is not spontaneous but typically requires co-culture with myogenic cells (Asakura, Seale et al. 2002; Schienda, Engleka et al. 2006; Uezumi, Ojima et al. 2006). To identify the non-myogenic cell types generated by Lin- SP cells in wild type and injured muscle, we developed a culture system that supports their spontaneous multi-lineage differentiation and closely mirrors in vivo findings from SP and Lin- SP cell transplantation studies.

2.3 Results

Muscle SP cells are capable of cell-autonomous myogenic differentiation in vitro.

We undertook a screening to identify cell culture conditions that would sustain the propagation of muscle SP cells isolated from wild type murine limb muscles. Similar to prior reports, muscle SP cells could not be maintained on a collagen or gelatin substrate in media that are commonly used for the growth and differentiation of primary muscle cells or of myogenic cell lines (Blau and Webster 1981; Montanaro, Gee et al. 1998). On a Matrigel substrate, a few muscle SP cells adhered but in our hands they did not form muscle as previously reported (Meeson, Hawke et al. 2004) but gave rise to small colonies of flat cells that were
morphologically similar to fibroblasts (data not shown). Even a commercially available medium specifically formulated for skeletal muscle cell growth (Lonza SkGM) did not support muscle SP cells but it did support the growth and differentiation of mouse primary muscle cells (data not shown). We also tested DMEM supplemented with 20% fetal bovine serum and 0.5nM basic FGF. This formulation is similar to that used by Tanaka et al. (Tanaka, Hall et al. 2009). We confirmed that this medium supports the proliferation of SP cells. However, differentiation was not observed as long as bFGF was present in the medium (data not shown).

As part of our screening, we included a medium formulated for the propagation of microvascular endothelial cells (Lonza EGM-2-MV). We tested this medium because the majority of SP cells from wild type muscle express several vascular endothelial markers, they bind the lectin Ulex europaeus agglutinin (UEA), and they incorporate acetylated LDL both in vitro and in vivo (Uezumi, Ojima et al. 2006), indicating that they constitute a sub-population of vascular endothelial cells that are lining blood vessels.

Interestingly, muscle SP cells cultured in microvascular EGM medium on either a gelatin or Matrigel substrate became adherent within 3 to 4 days and then rapidly proliferated (Figure 2.1 A and B). Because cells cultured on Matrigel appeared healthier and reached confluency faster, Matrigel was used as a substrate for all subsequent studies.
Unexpectedly, within 11 days of plating, muscle SP cells cultured in EGM medium differentiated into multinucleated myotubes that express α-actinin and spontaneously contract (Figure 2.1 C and D). Analysis of cultures at earlier time points revealed that muscle SP cells robustly gave rise to satellite cells (identified by expression of Pax7; Figure 2.1E) and to committed myoblasts (identified by expression of myogenin; Figure 2.1F). Terminal differentiation of muscle SP cells into myotubes did not require a switch to a medium with lower levels of serum or mitogens, as is the case for primary muscle cells and myogenic cell lines (Yaffe and Saxel 1977; Gunning, Hardeman et al. 1987; Sabourin, Girgis-Gabardo et al. 1999; Tomczak, Marinescu et al. 2004). Similarly, the EGM medium was capable of sustaining both the growth and spontaneous differentiation of primary muscle cells without requiring a switch to differentiation conditions (Figure 2.1G).

Overall these results indicate that the EGM-based in vitro system supports the expansion and differentiation of muscle SP cells into satellite cells, myoblasts and ultimately myotubes, thus mirroring in vivo results from SP cell transplantations (Gussoni, Soneoka et al. 1999; Asakura, Seale et al. 2002; Bachrach, Li et al. 2004; Muskiewicz, Frank et al. 2005; Bachrach, Perez et al. 2006). Importantly, muscle SP cells expressed their myogenic potential robustly in our in vitro system without requiring co-culture with myogenic cells, indicating that they are capable of cell autonomous muscle differentiation.
The Lin-SP sub-population is responsible for muscle SP myogenesis in vitro.

We next asked what SP sub-population (CD31+/CD45-, CD31-/CD45+, or Lin- SP) expanded in our in vitro system and differentiated into muscle. We first isolated by FACS three cell fractions: CD45 positive immune cells, CD31 positive vascular endothelial cells and cells negative for either lineage marker (Lin-; Figure 2.1H). Hoechst incorporation was then visualized for each pre-sorted cellular sub-fraction to further isolate CD31+/CD45- SP, CD31-/CD45+ SP, or Lin- SP cells (Figure 2.1I and data not shown) and culture them in EGM medium. CD31+/CD45- and CD31-/CD45+ SP cells did not adhere to Matrigel and died after a week in culture (Figure 2.1 J and K). By contrast, Lin- SP cells were indistinguishable from total muscle SP cell cultures generating small branched or spindle shaped cells that readily proliferated and then fused to form myotubes (Figure 2.1L). Interestingly, only one fifth of Lin- SP cells (500 cells per mm²) were needed for successful growth in culture compared to the total SP fraction (2,500 cells per mm²). This number agrees with the observation that Lin-SP cells represent about one fifth to one tenth of the total SP cell pool (10%-20%) based on marker expression by FACS (Table 2.1; (Uezumi, Ojima et al. 2006)). Therefore, the myogenic potential of cultured muscle SP cells can be attributed to the Lin- SP cell fraction.

We next tested for expression of Pax7 transcript in freshly isolated total muscle SP, Lin- SP and CD31+/CD45- SP cells. Pax7 mRNA was detected in total SP and in Lin- SP cells that have myogenic potential, but not in vascular
associated CD31+/CD45- SP cells. Therefore the Lin- SP sub-fraction that is myogenic expresses the satellite cell marker Pax7 when freshly isolated from wild type muscle.

**Lin- SP cells are activated in acutely injured and dystrophic muscle.**

Prior *in vivo* transplantation studies have suggested that acute muscle injury induced by cardiotoxin (CTX) increases the non-myogenic differentiation of Lin- SP cells (Motohashi, Uezumi et al. 2008). Since we are interested in identifying this non-myogenic cell type, we isolated and cultured Lin- SP cells from CTX injured muscle. Cells were isolated at day 3 post-CTX injection for consistency with the prior *in vivo* study (Motohashi, Uezumi et al. 2008). Day 3 also represents the peak of Lin- SP cell expansion following CTX injury (Uezumi, Ojima et al. 2006). We further extended our study to include Lin- SP cells isolated from dystrophic muscles of *mdx*<sup>5cv</sup> mice, a model for Duchenne Muscular Dystrophy (DMD), where skeletal muscles undergo chronic muscle damage (Danko, Chapman et al. 1992; Im, Phelps et al. 1996; Beastrom, Lu et al. 2011). Unlike CTX injured muscle that goes through a cycle of synchronized myofiber degeneration followed by regeneration, muscles of 8 week old *mdx*<sup>5cv</sup> mice show focal areas of degeneration and regeneration that co-exist within the same muscle (Figure 2.2 A). We therefore expected Lin- SP cells to show different responses to these two very different modalities of muscle damage.
We first determined whether chronic muscle injury in dystrophic muscles also activates Lin- SP cells similar to CTX injury. We quantified the total number of Lin-SP cells per gram of muscle tissue in 8 week old \( mdx^{5cv} \) mice and compared it to non injured and CTX injured wild type mice. We confirmed that CTX injury induces a significant increase (about 5-fold) in Lin- SP cells compared to non injured wild type muscle (Figure 2.2 B). In addition, we found that the number of Lin- SP cells was also significantly increased in \( mdx^{5cv} \) muscles by about 2-fold (Figure 2.2 B), indicating that Lin- SP cells are activated in dystrophic muscle. When cells were cultured in our \textit{in vitro} system, \( mdx^{5cv} \) Lin- SP cells and especially CTX Lin- SP cells showed significantly faster proliferation rates compared to Lin- SP cells isolated from non injured wild type muscle (Figure 2.2 C). \textit{In vitro} proliferation rates closely matched \textit{in vivo} cell quantification results, with Lin- SP cells from dystrophic muscle showing slightly lower proliferation rates than Lin- SP cells from CTX injured muscle. Differences in proliferation were also evident by visual observation of cells in culture. Wild type Lin- SP cells only became adherent after 3 days in culture and progressively increased in number from day 4 to day 11 without reaching confluence (Figure 2.2 D, WT column). By contrast, Lin- SP cells from CTX-injured and \( mdx^{5cv} \) muscles adhered to the culture substrate within 24 hours and rapidly began to proliferate reaching confluence by day 8 for CTX Lin- SP cells and by day 11 for \( mdx^{5cv} \) cultures (Figure 2.2 D, CTX and \textit{MDX} columns respectively).
These results indicate that Lin- SP cells are activated *in vivo* in both CTX-injured and dystrophic muscle. This activation results in faster adhesion to the substrate and a significant increase in proliferation rate *in vitro*.

**The Lin- SP cell fraction from CTX-injured and *mdx*\(^5cv\) muscles does not form muscle *in vitro*.**

Visual observation of the cultures at day 11 revealed a lack of multinucleated contracting myotubes in cultures of Lin- SP cells from CTX-injured and *mdx*\(^5cv\) muscles (Figure 2.2 D). Instead the predominant cell types generated by Lin- SP cells from damaged muscle were branched cells that appeared dark under phase, flat fibroblast-like cells, and small angular cells filled with lipid droplets (Figure 2.2 D). These cell types were present but rare in cultures of wild type Lin- SP cells where spindle shaped cells and myotubes were the predominant cell types, and myotubes were readily detectable by day 8 (Figure 2.2 D).

To confirm the lack of myogenic differentiation of CTX and *mdx*\(^5cv\) Lin-SP cells, day 11 cultures were immunolabelled for \(\alpha\)-actinin, a protein highly expressed in myotubes. No \(\alpha\)-actinin labeling was observed in three independent biological replicates of CTX or *mdx*\(^5cv\) Lin-SP cultures, while myotubes were covering the entire surface of wild type Lin-SP cultures (Figure 2.3). We then analyzed early cultures of Lin- SP cells for the presence of Pax7-positive satellite cells. At day 7, half the cells in cultures of wild type Lin-SP cells were Pax7-positive satellite cells (Table 2.2; Figure 2.3). By contrast, no Pax7 staining was
observed in cultures of CTX or \( mdx^{5cv} \) Lin-SP cells at day 7 (Table 2.2; Figure 2.3 or at earlier time points (days 1 and 5; data not shown). Therefore, lack of myogenesis in cultures of Lin-SP cells from CTX-injured or dystrophic muscle appears to be due to a lack of satellite cells in these cultures.

To further probe this question, we analyzed expression of Pax7 in freshly isolated Lin-SP cells, prior to any culture. As shown in Figure 4A, Pax7 mRNA was not detected in freshly sorted Lin-SP cells from CTX and \( mdx^{5cv} \) muscles. This result was confirmed by quantitative RT-PCR analysis where Pax7 mRNA was detected in wild type Lin-SP cells but was undetectable in CTX and \( mdx^{5cv} \) Lin-SP cells (data not shown). Of note, Pax7 expression in wild type Lin-SP cells was 2.7-fold lower than in a cell fraction enriched for satellite cells (the main population cell fraction that is negative for CD31, CD45, Sca1, and PDGFRα). These data show that Pax7 expression is lost in the Lin-SP cell fraction in response to muscle damage.

To determine whether other markers of satellite cells are expressed by Lin-SP cells and might be similarly affected by muscle damage, we analyzed expression of the early myogenic regulatory factor Myf5, and two cell surface markers expressed by satellite cells: integrin α7 and SM/C-2.6 (Fukada, Higuchi et al. 2004; Sacco, Doyonnas et al. 2008). RT-PCR analysis of Myf5 mRNA revealed that this transcription factor is present in freshly isolated CTX and \( mdx^{5cv} \) Lin-SP cells, albeit its levels of expression are decreased compared to wild type Lin-SP cells (Figure 2.4 A). Expression of integrin α7 and SM/C-2.6 was
assayed by FACS in freshly isolated Lin-SP cells. In wild type muscle, 12% of Lin-SP cells were double positive for integrin α7 and SM/C-2.6. In \( mdx^{5cv} \) muscle, Lin-SP cells double positive for integrin α7 and SM/C-2.6 were still present but their proportion was decreased to 6%. In addition, we found Lin-SP cells that were positive for one satellite cell marker but not the other. Lin-SP cells expressing only the integrin α7 marker represented about 45% of the total Lin-SP cell fraction. Lin-SP cells positive only for the SM/C-2.6 marker represented about 1% of the total Lin-SP cell population. Interestingly, the proportion of integrin α7 or SM/C-2.6 single positive Lin-SP cells did not change between wild type and \( mdx^{5cv} \) muscle.

Taken together, these results indicate that both acute (CTX injury) and chronic, focal muscle damage (\( mdx^{5cv} \) muscle) lead to a loss of \textit{in vitro} myogenic differentiation of Lin-SP cells. This is accompanied by a loss of expression of Pax7 and a decrease in Myf5 expression, as well as a decrease in the proportion of Lin-SP that express the satellite cell markers integrin α7 and SM/C-2.6.

**Muscle damage promotes \textit{in vitro formation} of fibro-adipogenic precursor cells by Lin-SP cells**

We next sought to identify the non-muscle cells generated by CTX and \( mdx^{5cv} \) Lin-SP cells. \textit{In vivo} studies have shown that upon transplantation Lin-SP cells isolated from injured muscle give rise to interstitial cells that facilitate myogenesis (Motohashi, Uezumi et al. 2008). The ability to support myogenesis and the interstitial location are reminiscent of the recently identified fibroblast and
adipocyte precursors (FAPs) (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010; Uezumi, Ito et al. 2011). FAPs can be identified based on expression of the platelet derived growth factor receptor alpha (PDGFRα), lack of Pax7 expression, and the ability to spontaneously give rise to both fibroblasts and adipocytes when cultured. We first performed immunostaining for PDGFRα in early (day 7) cultures of Lin-SP cells. In cultures of wild type Lin-SP cells PDGFRα was expressed in only a few cells (8.5% of total; Figure 2.3). By contrast, 48% and 67% of cells expressed PDGFRα in cultures of CTX and mdx<sup>5cv</sup> Lin-SP cells, respectively. Furthermore, PDGFRα was never co-expressed with Pax7 (data not shown), in agreement with reports that FAPs are not myogenic (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010; Uezumi, Ito et al. 2011). We next tested for the presence of fibroblasts and adipocytes in later cultures (day 11 and day 14, respectively) of Lin-SP cells. Fibroblasts were identified by immunolabeling for Collagen I, while adipocytes were recognized by immunolabeling for CEBP/α (Figure 2.3). Fibroblasts represented 12% of cells in day 11 wild type Lin-SP cultures compared to 39.5% in CTX and 60% in mdx<sup>5cv</sup> cultures. At day 14, adipocytes were over 2-fold more abundant in cultures of CTX (53% of total cells) and mdx<sup>5cv</sup> (55%) Lin-SP cells compared to wild type Lin-SP cultures (22%). Importantly, our culture medium does not contain factors that induce adipogenesis.

Taken together, these results indicate that both acute muscle damage and muscular dystrophy support the differentiation of Lin-SP cells to a fibro-
adipogenic lineage *in vitro*. Furthermore, our *in vitro* system suggests that cells within the Lin- SP cell fraction give rise to the recently identified FAPs or contain a small subset of FAPs.

**Freshly isolated Lin-SP cells express FAPs surface markers but are capable of myogenic differentiation**

We next asked whetherfreshly isolated Lin- SP cells expressed two markers of FAPs, namely PDGFRα and Sca1 (Joe, Yi et al. 2010). Transcripts for both PDGFRα and Sca1 were readily detected by RT-PCR in both wild type and *mdx*5°Cv Lin-SP cells right after isolation by FACS (Figure 2.4 A). To assay for protein expression, freshly isolated Lin- SP cells were double labeled with antibodies to PDGFRα and Sca1 and analyzed by FACS. As shown in Figure 2.4 B, Lin- SP cells are heterogeneous with about 40% of wild type Lin- SP cells expressing both PDGFRα and Sca1. The number of PDGFRα and Sca1 double positive Lin- SP cells increased to 77% in *mdx*5°Cv muscle, suggesting an expansion of this sub-population in dystrophic muscle.

We next tested which sub-population of wild type Lin- SP cells was responsible for myogenic differentiation *in vitro*. Since PDGFRα positive FAPs do not give rise to myogenic cells and since satellite cells do not typically express PDGFRα and Sca1, we hypothesized that PDGFRα positive Lin- SP cells would give rise to FAP cells, while PDGFRα negative Lin- SP cells would differentiate into myogenic cells. As control, we isolated PDGFRα positive Lin- main population (MP) cells that correspond to previously characterized FAPs, and
PDGFRα negative Lin- MP cells that are enriched in myogenic cells (Figure 2.4 C). Of note, FACS analysis indicated that PDGFRα positive Lin- MP cells (FAPs) were more abundant in mdx<sup>5cv</sup> muscle compared to wild type muscle. Purity of sorted cell populations was confirmed by RT-PCR analysis for PDGFRα in all 4 sorted cell fractions (Figure 2.4 D). PDGFRα transcript was correctly detected in PDGFRα+ Lin- SP and PDGFRα+ Lin- MP cells but not in PDGFRα- Lin- SP and PDGFRα- Lin- MP cells (Figure 2.4 D). As expected, PDGFRα- Lin- MP cells that are enriched for myogenic cells gave rise to myotubes while no myogenic differentiation was observed in cultures of PDGFRα+ Lin- MP cells (FAPs) from either wild type or dystrophic muscle (Figure 2.4 E). Interestingly, we observed about 4-fold less myotube formation in cultures of PDGFRα- Lin- MP cells from dystrophic muscle compared to wild type (Figure 2.4E). Contrary to our expectations, we found that PDGFRα+ Lin- SP cells from wild type muscle readily formed myotubes (Figure 2.4E) indicating that unlike FAPs these cells have myogenic potential. PDGFRα- Lin- SP cells did not survive in culture and could therefore not be assessed for their myogenic potential. Importantly, PDGFRα+ Lin- SP cells from mdx<sup>5cv</sup> muscle did not give rise to myogenic lineages (Figure 2.4E) but readily formed fibroblasts (Figure 2.4F) and adipocytes (not shown), indicating that the myogenic potential of the PDGFRα+ Lin- SP cell fraction is impaired in dystrophic muscle.

To further explore the unexpected myogenic potential of PDGFRα+ Lin-SP cells, we asked whether these cells expressed satellite cell markers.
Quantitative RT-PCR analysis revealed expression of Pax7 in freshly isolated wild type PDGFRα+ Lin- SP cells as well as PDGFRα- Lin- SP cells (Figure 2.4G). Pax7 expression in these populations was low compared to PDGFRα- Sca1- Lin- MP cells that are enriched in satellite cells. Pax7 expression was not detected in PDGFRα+ Sca1+ Lin- MP cells that correspond to FAPs (data not shown). We next analyzed by FACS the expression of the satellite cell markers integrin α7 and SM/C-2.6 in PDGFRα+ Sca1+ Lin- SP cells from both wild type and mdx5cv muscle. We found that about 70% and 60% of PDGFRα+ Sca1+ Lin- SP cells from wild type and mdx5cv muscle respectively are double positive for both integrin α7 and SM/C-2.6.

These results indicate that the myogenic differentiation potential within the Lin-SP cell fraction co-segregates with cells expressing markers typically associated with FAPs (PDGFRα and Sca1) and with satellite cells (Pax7, Myf5, integrin α7 and SM/C-2.6), suggesting that these Lin-SP cells are distinct from both FAPs and satellite cells. Furthermore, among these markers, only Pax7 expression becomes undetectable in dystrophic muscle, and appears to correlate with a loss of in vitro myogenic differentiation potential.

2.4 Discussion

Currently, the study of muscle SP cells is complicated by the lack of specific markers, their low abundance, their heterogeneity and the lack of a culture system that supports their multi-lineage differentiation. Here we have
identified culture conditions that promote the expansion and the multi-lineage (mesenchymal and myogenic) differentiation of the Lin- SP cell fraction. Our EGM-based culture conditions offer several advantages. First, they are compatible with the expansion and differentiation of myogenic cells, FAPs, fibroblasts and adipocytes rendering it particularly useful for the study of lineage choices. Second they closely mirror in vivo findings on the response of SP cells to muscle injury with regards to their cell autonomous differentiation into satellite cells, myoblasts and myotubes (Gussoni, Soneoka et al. 1999; Asakura, Seale et al. 2002; Bachrach, Li et al. 2004; Muskiewicz, Frank et al. 2005; Bachrach, Perez et al. 2006; Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009), their high myogenic potential (Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009), their increased proliferation in response to injury (Uezumi, Ojima et al. 2006; Tanaka, Hall et al. 2009), and their exclusive mesenchymal differentiation when isolated from CTX-injured muscle (Motohashi, Uezumi et al. 2008). Therefore, our culture conditions should prove extremely useful as a complement to in vivo experiments. Finally, the EGM medium can support the survival and differentiation of low numbers of SP cells, as low as 2000 cells per well in a 12 well plate. This allowed us to further sub-fractionate the Lin-SP cell population and to determine that the PDGFRα+, Sca1+, Lin-SP sub-fraction contains cells that can give rise to myogenic and mesenchymal progenitors.

One limitation of this culture system is that it cannot support the clonal growth of Lin-SP cells. Beneath 2000 cells per well, Lin-SP cell viability was
compromised and surviving cells adopted a fibroblast-like morphology and did not proliferate. Although we made several attempts at co-culturing single Lin-SP cells expressing green fluorescent protein (GFP) or LacZ with non-marked primary muscle cells, we could not test multi-lineage differentiation. We found that Lin-SP cells isolated from GFP+ transgenic mice (Jackson stock # 003291) have poor survival and myogenic differentiation in vitro even when cultured alone and at high density. We attribute this difference in the behavior of SP cells to the high level expression of GFP. Lin-SP cells isolated from LacZ expressing mice (Jackson stock# 002192) showed normal myogenic and mesenchymal differentiation when cultured alone. However the LacZ marker was not detectable in the mesenchymal lineages precluding tracing in mixed cultures of a single Lin-SP cell with myogenic cells. Therefore, at this time we cannot determine whether the PDGFRα+, Sca1+, Lin-SP sub-fraction contains cells with a dual mesenchymal and myogenic differentiation potential, or whether there are separate precursors for each lineage.

The relationship between SP cells and satellite cells is currently unclear. Although muscle SP cells and in particular Lin-SP cells give rise to myogenic cells both in vivo upon transplantation and in vitro [4-10], their participation in muscle repair in situ remains unclear. We have shown that a subset of freshly isolated Lin-SP cells shares with satellite cells expression of Pax7, Myf5, integrin α7 and SM/C-2.6. A prior study also found that myogenic cells within the Lin-SP occupy a typical satellite cell position at the muscle fiber but express a mix of
satellite cell (Pax7, syndecan 4) and non-satellite cell (Abcg2) markers (Tanaka, Hall et al. 2009). Taken together these data suggest that myogenic Lin-SP cells may represent a sub-population within the satellite cell pool. These two cell types may therefore be difficult to tease apart. Interestingly, Abcg2 knock-out mice show impaired regeneration with a 30% decrease in satellite cells after a single round of muscle degeneration/regeneration (Doyle, Zhou et al. 2011). Importantly, proliferation and differentiation of satellite cells is not affected by loss of Abcg2 expression, indicating that muscle regeneration is affected independently of the canonical Abcg2-negative satellite cell (Doyle, Zhou et al. 2011). While these findings support a role for Lin-SP cells in muscle regeneration and satellite cell replenishment, lineage tracing of Abcg2 expressing cells yielded conflicting results (Doyle, Zhou et al. 2011). This may be due to the very low level of Abcg2 expression in resting Lin-SP cells (Uezumi, Ojima et al. 2006) that may inefficiently activate the genetic tracing system. As a result, the question of the level of contribution of Lin-SP cells to the satellite cell compartment (and other mesenchymal cell lineages) remains unresolved and awaits the identification of a marker that is strongly expressed by Lin-SP cells and can differentiate them from other cell types.

One of our most interesting findings is that Lin-SP cells express Pdgfra and Sca1, markers previously associated with mesenchymal progenitors and FAPs but not myogenic cells. Culture of Lin-MP cells in our system agrees with previous studies (Uezumi, Fukada et al. 2010), showing Pdgfra+ cells generate
fibroblasts and adipocytes, while Pdgfrα- cells are highly enriched for myogenic cells. However, culture of Lin-SP cells positive for Pdgfrα yielded highly myogenic colonies when isolated from wild type muscle. These results suggest that while Pdgfrα is expressed by predominantly non-myogenic mesenchymal precursors in skeletal muscle, Lin-SP cells represent a unique sub population of Pdgfrα cells that have the potential to contribute to muscle regeneration. Future *in vivo* experiments are required in order to determine whether Pdgfrα+ Lin-SP cells contribute to *in vivo* muscle regeneration, potentially by replenishing the satellite cell pool.

Our culture system provides the tools necessary to study the myogenesis of Lin-SP isolated from multiple environments. By doing so, we have uncovered a critical finding that myogenic differentiation of Lin-SP cells is lost following muscle damage, either acute or chronic. It is unlikely that these results are an artifact of our *in vitro* system because Lin-SP cells isolated from CTX-injured muscle lose their myogenic potential *in vivo* following transplantation and give rise to mesenchymal cells that support myoblast engraftment (Motohashi, Uezumi et al. 2008), a property also attributed to FAPs (Joe, Yi et al. 2010). Overall these results support a model where following muscle damage Lin-SP cells may give rise to FAPs that promote the differentiation of myogenic cells. The loss of myogenic potential in Lin-SP cells from CTX-injured or dystrophic muscle can be interpreted in at least two different ways. The first scenario assumes that distinct cell populations within the Lin-SP are responsible for
mesenchymal and myogenic differentiation. Loss of Pax7 expression in Lin-SP cells from damaged muscle would be interpreted as a loss of the myogenic fraction from the Lin-SP. This could arise from Pax7+ Lin-SP cells being recruited to participate in muscle repair and differentiate into myogenic cells that no longer efflux Hoechst. The second scenario assumes the existence of bi-potent cells within the Lin-SP and a requirement for Pax7 expression in order to allow myogenic differentiation to occur. This agrees with our finding that some markers of satellite cells, namely Myf5, integrin α7 and SM/C-2.6 are still expressed by Lin-SP cells from CTX-injured or dystrophic muscle. Interestingly, total SP cells isolated form Pax7 null mice show a 2-fold decrease in myogenic differentiation but not a complete loss of myogenic potential when co-cultured with myogenic cells (Asakura, Seale et al. 2002). These findings suggest that while Pax7 is down regulated in Lin-SP cells from Ctx-injured and dystrophic muscle, there are likely multiple mechanisms responsible for their loss of myogenic potential.

Overall, our culture system reveals a change operated by the dystrophic muscle environment upon Lin-SP cells with potential implications for their use in cell-based treatments of DMD. It will be important to determine whether the presence of chronic muscle damage promotes a continuous production of FAPs, fibroblasts and ultimately adipocytes by Lin-SP cells. Interestingly, \textit{mdx}^{5cv} Lin-SP cells show a strong bias for the formation of fibroblasts compared to CTX Lin-SP cell cultures (60% versus 39.5%). It is therefore possible that a sustained production of fibroblasts by Lin-SP cells may over the long term significantly
contribute to the progressive fibrosis observed in dystrophic mice (Pastoret and Sebille 1995; Beastrom, Lu et al. 2011). It will therefore be important to determine how in vivo ablation of Lin-SP cells, once unique markers are identified, impacts disease progression in dystrophic muscles. In the meantime, our in vitro system may prove very useful in the identification of factors within dystrophic and damaged muscle that dictate the fate and behavior of Lin-SP cells.

2.5 Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the Animal Care and Use Committee of Nationwide Children’s Hospital.

Animals

Eight to twelve week old female wild type C57BL/6J mice (Jackson Laboratories; Stock 000664) and dystrophin-deficient B6Ros.Cg-Dmdmdx5cv mice (mdx5cv, a gift of Louis Kunkel, Harvard Medical School, Boston, MA) were used for all experiments. Mdx5cv mice have a mutation in exon 10 of the DMD gene that disrupts dystrophin expression and they were originally purchased from Jackson Laboratories (Stock 002379). All mice were bred in house.
Cardiotoxin injury

Acute muscle damage was induced by intramuscular injection of CTX (Sigma, St. Louis, MO) in wild type C57BL/6 mice. CTX (10μM in sterile PBS) was unilaterally injected into the tibialis anterior (25μl), quadriceps femoris (50μl), and gastrocnemius (25μl) muscles under anesthesia. Mice were sacrificed for cell isolation or histological analysis 3 days post-injection.

Cell Isolations

For all SP cell isolations, muscles from 6 to 8 mice were pooled to obtain enough cells for culture or analysis. Muscle cells were isolated from hindlimb skeletal muscles as previously described (Montanaro, Liadaki et al. 2004). Briefly, muscles were trimmed of fat and tendons and finely minced. The minced muscle was digested for up to 30 minutes at 37°C in 1.2U/ml Dispase (Worthington Biochemicals) and 5mg/ml Collagenase Type IV (Worthington Biochemicals) in phosphate buffered saline (PBS) containing 2.5mM CaCl₂. The enzymes were inactivated with DMEM/10% FBS, and the digest passed through 70μm and 40μm cell strainers. Cell suspensions were overlaid on Horse Serum (Gibco) and centrifuged at 160 xg for 10 min without brakes to remove debris.

For general isolation of myogenic cells, the pre-plating technique was used. Briefly, dissociated cells were centrifuged at 450 xg and resuspended in complete EGM2-MV medium (Lonza) without hydrocortisone. Cells were transferred to a 10cm tissue culture dish (BD Falcon) and incubated for 1 hour at
37°C, 5% CO₂ to allow fibroblasts to adhere. Non adherent cells were removed and re-plated on a fresh 10cm dish and incubated an additional hour at 37°C, 5% CO₂ to remove residual fibroblasts. Non-adherent myogenic cells were transferred to a Matrigel coated dish and cultured for 11 days prior to fixation for immunolabeling.

For isolation of SP cells our previously described protocol was followed (Montanaro, Liadaki et al. 2004). Cells were resuspended at 10⁶ cells/ml in PBS containing 0.5% (w/v) bovine serum albumin (Sigma; PBS/BSA) and labeled with 12.5 ug/ml Hoechst 33342 (Sigma) in the presence or absence of 100mM verapamil (Sigma) for 1 hour at 37°C. Cells were washed in PBS/BSA and counterstained with propidium iodide (Fisher Scientific) to label and exclude dead cells. Cells were visualized and sorted on a FACSvantge DIVA (BD Biosciences) cell sorter using our previously described configuration settings (Montanaro, Liadaki et al. 2004). Cells were collected in culture medium.

For isolation of SP and MP cell sub-fractions, cells were resuspended at 2x10⁶ cells per 100μl after Hoechst staining and pre-incubated on ice for 10 min in FC block (BD Biosciences). Cells were then labeled with the following primary antibodies for 15 min on ice: CD31-APC (MEC 13.3, BD Biosciences), CD45-PeCy7 (clone 30-F11, BD Biosciences), Ly-6A/E (Sca-1)-FITC (clone E13-167.7, BD Biosciences), and PDGFRα-PE (clone Apa5, Abcam,). Samples were counterstained with propidium iodide (Fisher Scientific) to label and exclude dead cells. The cell sorter was setup to first visualize propidium iodide, CD31-APC and
CD45-PeCy7. Live cells (propidium iodide negative) were sorted for each fraction (CD31+/CD45-, CD31-/CD45+ and CD31-/CD45-) using a 4-way sort head. Cells were collected on ice in PBS/BSA and immediately re-analyzed by FACS to visualize Hoechst for isolation of SP and MP cells. In some experiments, Sca1-FITC and PDGFRα-PE were co-visualized with Hoechst to further sub-fractionate Lin- SP and MP cells. For cell culture experiments, sorted cells were directly collected in culture medium. For RNA isolation, cells were collected in Trizol-LS (Invitrogen). From tissue pooled from 6 to 8 mice we routinely obtained 10,000 to 20,000 Lin-SP cells and 5,000 to 10,000 Lin- Pdgfra+/+ SP cells.

**Quantification of Lin- SP cells by FACS**

For each biological replicate, hind limb muscles from 3 wild type, CTX-injected or mdx<sup>5cv</sup> mice were pooled and weighed prior to cell isolation. The entire cell suspension was labeled for Hoechst, CD31-APC, CD45-PeCy7 and propidium iodide as described above. Lin- SP cells were sorted and automatically counted during collection. The number of cells sorted was divided by the initial muscle weight. Values from 4 to 6 independent biological replicates were used to derive means and standard deviations. Comparisons between WT, CTX, and MDX were performed using a one way ANOVA followed by a Bonferroni pairwise comparison. Significance threshold was set at p <0.05.
Analysis of vascular marker expression

For cell surface marker analysis, muscle cells were isolated and stained with Hoechst as described above. Cells were resuspended in PBS/BSA at 2x10^6 cells per 100μl and 100 μl aliquots were prepared for single antibody or lectin staining. Samples were pre-incubated on ice for 10 min in FC block (BD Biosciences) and then labeled with the following primary antibodies for 15 min on ice: CD31-FITC (clone MEC 13.3, BD Biosciences), VE-Cadherin (clone 11D4.1, BD Biosciences), Ulex europaeus agglutinin I (UEA)-biotin (Vector Labs), and Ly6C-biotin (clone AL-21, BD Biosciences). Cells were rinsed, resuspended in 100 μl PBS/BSA, and the appropriate samples were incubated with Goat anti Rat IgG-FITC secondary antibody (BD Biosciences) or with Streptavidin-FITC (BD Biosciences) for 10 min on ice. Samples were rinsed and counterstained with propidium iodide prior to FACS analysis.

To assay for Ac-LDL uptake in vivo, 100μg Ac-LDL conjugated to Alexa488 was injected in a total volume of 100μl into the tail vein of wild type mice. Animals were sacrificed 16 hours later and muscles were harvested for cell isolation. Cells were stained with Hoechst and then propidium iodide to eliminate dead cells from analysis. Alexa 488 fluorescence was co-visualized with Hoechst by FACS to quantify the number of live SP cells that had incorporated Ac-LDL. For some experiments, samples were also labeled with an antibody for CD31 directly conjugated to APC (BD Biosciences). In these experiments, all SP cells that had incorporated Ac-LDL in vivo were also positive for CD31.
For *ex-vivo* Ac-LDL uptake, cells were stained with Hoechst then incubated for 10 min in ice cold PBS/BSA containing 15 μg/ml Ac-LDL conjugated to Alexa 488. Cells were counterstained with propidium iodide and then analyzed by FACS.

**Cell Culture**

All cells were cultured in a 37°C incubator with 5% CO₂ in complete EGM-2 MV medium (Lonza) without hydrocortisone. Cells were plated in 12 well tissue culture treated plates coated with Matrigel (BD Biosciences) diluted 1:1 in complete EGM-2 MV medium. Cell density at plating was 2500 cells per cm² for total SP cells and 500 cells per cm² for SP and MP sub-fractionated cells. This resulted in about 4 to 6 cultures to be established per cell isolation from muscle pooled from 6 to 8 mice. Half of the medium was replenished every 4 days.

**Immunocytochemistry and cell quantifications**

Cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton-X100 for 10 min, incubated first for 1 hour at room temperature in blocking solution (PBS with 10% horse serum), and then overnight at 4°C with primary antibodies diluted in blocking solution. Primary antibodies are anti-Pax7 (DSHB), anti-myogenin (clone F5D, DAKO), anti-α-actinin (Sigma), anti-Collagen 1 (Cedarlane), anti-PDGFRα (clone APA5, BD Biosciences) and anti-C/EBPα (Santa Cruz). Cells were incubated with Alexa488 or Texas Red conjugated secondary antibodies (Jackson Immunoresearch) for 1 hour at room temperature
followed by incubation with DAPI (Invitrogen) to counterstain nuclei. Coverslips were mounted with n-propyl gallate (Sigma) mounting media. To measure the percentage of cells positive for each marker, 8 randomly selected fields were photographed per well. Images were taken from two to three independent experiments. The percentage of positive cells for each differentiation marker was determined by dividing the number of positively stained cells by the total number of nuclei as determined by DAPI counterstain. Comparisons between groups were performed using a one way ANOVA followed by Bonferroni pairwise comparisons. Results were considered significant if p <0.05.

Alamar Blue Cell Proliferation Assay

Cells were seeded at a density of 1000 cells per well in a 96 well plate coated with Matrigel (BD Biosciences) containing 180μl EGM-2 MV medium (Lonza). Twenty-four hours after plating, 20μl Alamar Blue (Invitrogen) reagent was added to each well. Absorbance measurements were taken at 570nm and 600nm on a SpectraMax M2 (Molecular Devices) microplate reader and recorded using Softmax Pro software. Readings were recorded once per day from the same wells until 8 days of culture. Proliferation calculations were performed to manufacturer specifications. Linear regression was performed on each group and pairwise comparisons were performed by comparing slopes using ANOVA and Bonferroni correction. Comparisons were considered significant if p<0.05.
Reverse transcription PCR

Cells were sorted directly into Trizol LS (Invitrogen) and RNA was isolated according to manufacturer specifications. Isolated RNA was further purified using the RNeasy Micro Kit (Qiagen). First strand cDNA was synthesized using Superscript III (Invitrogen) from 20ng of input RNA using oligo dT primers. Primer sequences used for RT-PCR are: Sca1: Forward-5'
TGGATTCTCAAACAAGGAAAGTAA AGA -3', Reverse-5'
ACCCAGGATCTCTACATTCTTCAATA -3', Pdgfrα: Forward- 5'
GACGAGTGTCCTTCGCCAAAGTG-3', Reverse-5'
CAAAATCCGACCAAGCAGGAGG-3', Pax7: Forward- 5'
CCCAACAGGTTTTCCTCAACTG-3', Reverse-5'
CGGCCTTTCTCTAGGTTCTGCT-3', Myf5: Forward-5'
TTAGCAAACCATGAACACGAAACA-3', Reverse-5'
AAGGGGGGCTTCATTACACCAGG-3', Gapdh: Forward-5'
CACGGCAAATTCAACGGGCACAGTCAAGG-3', Reverse-5'
GTTCACACCCATACAAACTG-3', and βactin: Forward-5'
ATGGAGGGGAATACAGCCC-3', Reverse- 5'-TTCTTTGAGCTCCTTGCCTT-3'.

Quantitative RT-PCR was performed on an Applied Biosystems 7500 using Sybr Green Master Mix (Fermentas). Gene expression fold change was determined by the delta-delta Ct method using TATA binding protein (TBP) as the housekeeping gene. Primer sequences for quantitative RT-PCR include Pax7
as previously mentioned, and *TBP*: Forward-5'

CCGTGAATCTTGCTGTAAACTTG-3', Reverse-5'

CAACGCAGTTGTCGCTGGCTCTCTCTCT-3'.
Table 1: Muscle SP cells express vascular endothelial cell markers and incorporate Acetylated-LDL \textit{in vivo}.

<table>
<thead>
<tr>
<th>% positive cells</th>
<th>CD31</th>
<th>Ly6C</th>
<th>VE-cadherin</th>
<th>UEA</th>
<th>Ac-LDL \textit{in vitro}</th>
<th>Ac-LDL \textit{in vivo}</th>
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<tr>
<td>Muscle</td>
<td>90.5 ± 7.1</td>
<td>75.3 ± 8.5</td>
<td>89.8 ± 9.2</td>
<td>78.6 ± 11.5</td>
<td>90 ± 7 (n=5)</td>
<td>70.3 ± 13 (n=7)</td>
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<tr>
<td>SP</td>
<td>7.1 (n=12)</td>
<td>8.5 (n=4)</td>
<td>9.2 (n=5)</td>
<td>11.5 (n=4)</td>
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Values represent mean ± standard deviation from the indicated number (n) of biological replicates.

Table 2: \textit{In vitro} lineage choices of Lin-SP cells isolated from wild type, \textit{mdx}^{5cv}, or cardiotoxin-injured muscle.

<table>
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<tr>
<th>Days in culture</th>
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<tr>
<td></td>
<td>Myogenic</td>
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<td>PDGFRα</td>
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<td></td>
<td>FAPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibroblast</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td></td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>% cells positive</th>
<th>Wild type</th>
<th>52 ± 6</th>
<th>8 ± 5</th>
<th>12 ± 3</th>
<th>22 ± 3</th>
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<tbody>
<tr>
<td>\textit{mdx}^{5cv}</td>
<td>0</td>
<td>67 ± 9</td>
<td>60 ± 7</td>
<td>55 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cardiotoxin</td>
<td>0</td>
<td>48 ± 8</td>
<td>40 ± 2</td>
<td>53 ± 4</td>
<td></td>
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Values represent mean ± standard deviation from 3 biological replicates.
Figure 2.1: *In vitro* cell-autonomous myogenic differentiation of muscle SP cells and Lin- SP cells. **A-C:** Phase pictures of wild type muscle SP cells cultured in EGM medium on Matrigel. Cells attached to Matrigel at days 3 (**A**), had significantly proliferated by day 7 (**B**), and differentiated into contracting multinucleated myotubes by day 11 (**C**). Scale bar = 50 μm. **D-F:** Immunostaining of muscle SP cultures for the myotube marker α-actinin (**D**; green) at day 11; for the myoblast marker myogenin (**E**; green) at day 5; and for the satellite cell marker Pax 7 (**F**; green) at day 3. Cells were counterstained with DAPI (blue) to visualize nuclei. Scale bar = 100 μm. **G:** Phase picture of day 11 cultures of primary myogenic cells in EGM medium on a Matrigel substrate showing formation of large numbers of myotubes. **H-I:** Isolation of muscle SP sub-populations by FACS. Total muscle mononuclear cells were labeled with antibodies to CD31 and CD45 and CD31+, CD45+ and Lin- cells were sorted first (**H**). Cells in each fraction were re-analyzed by FACS for incorporation of Hoechst and SP cells were isolated. The SP profile for the Lin- subset of cells in **H** is shown in **I**. MP cells are indicated. **J-L:** Phase pictures of day 11 cultures of CD45+ SP cells (**J**), CD31+ SP cells (**K**), and Lin- SP cells (**L**). Scale bar = 50 μm. **M:** Pax7 mRNA was detected by RT-PCR in RNA isolated from muscle SP and Lin-SP cells but not CD31+/CD45- SP cells, Lin- MP cells shown in **I** were used as a positive control since they contain satellite cells. RNA was isolated immediately after cell isolation by FACS from wild type muscles.
Figure 2.1
Figure 2.2: CTX-injury and muscular dystrophy activate Lin- SP cells in vivo and alter their in vitro proliferation and morphology. A: Histological comparison of tissue sections from wild type (WT), CTX-injured (CTX), and mdx^{5cv} (MDX) tibialis anterior muscle stained with Hematoxylin and Eosin. Wild type muscle shows closely apposed muscle fibers with peripherally located nuclei. CTX-injured muscle at 3 days post-injection has only a few degenerating myofibers surrounded by large numbers of mononuclear cells. Muscle from 8 week old mdx^{5cv} mice shows areas of active regeneration (white arrow), and areas of muscle degeneration with accumulations of inflammatory cells (green arrow). Scale bar = 50 μm. B: Comparison of the number of Lin-SP cells isolated by FACS per gram of muscle from wild type, CTX-injured and mdx^{5cv} mice. Asterisks indicate a significant difference (p<0.01, Student’s t-test) from wild type muscle. C: Quantification of in vitro Lin- SP cell proliferation using the Alamar Blue vital dye assay. Asterisks indicate a significant difference (p<0.001, linear regression analysis) from wild type muscle. C: Phase pictures of wild type, CTX and mdx^{5cv} Lin- SP cells at days 3, 8 and 11 in culture showing differences in cell adhesion, proliferation and morphology. Scale bar = 50 μm.
Figure 2.3: Muscle damage abolishes *in vitro* myogenesis of Lin- SP cells and favors their differentiation into FAPs, fibroblasts and adipocytes. Cultures of Lin- SP cells isolated from wild type (WT) CTX-injured (CTX), or *mdx*<sup>5cv</sup> mice were immunolabelled with antibodies to the myogenic (green) or mesenchymal (red) markers indicated. Labeling for Pax7 (satellite cells), PDGFRα (FAPs) and Collagen 1 (fibroblasts) was done on day 7 cultures. Labeling for α-actinin (myotubes) and C/ebpα (adipocytes) was performed at day 11. Scale bar for α-actinin pictures is 500 μm. Scale bar shown in WT Pax7 picture applies to all other pictures and is 100 μm.
Figure 2.4: Freshly isolated Lin-SP cells express FAPs surface markers but are capable of myogenic differentiation. A: RT-PCR analysis of freshly isolated Lin-SP cells from wild type (WT) and mdx5cv (MDX) muscle for myogenic markers (Pax7 and Myf5) and FAPs markers (PDGFRα and Sca1). Positive controls (PC) are sorted Sca1-positive cells for Sca1 and Lin- MP cells for Pax7, Myf5 and PDGFRα. Negative controls (NC) are sorted Sca1-negative cells for Sca1 and CD45-positive MP cells for Pax7, Myf5 and PDGFRα. B, C: FACS analysis of PDGFRα and Sca1 protein expression in Lin- SP cells (B) and Lin-MP cells (C) from wild type (WT) and mdx5cv (MDX) muscle. Percentages of cells double positive (red) and double negative (green) for PDGFRα and Sca1 are shown. D: Confirmation by RT-PCR for PDGFRα expression in Lin- SP and Lin-MP cells sorted into PDGFRα-positive (Pα+) and PDGFRα-negative (Pα-) subfractions. E: In vitro myogenic differentiation of Lin- SP and MP cells sorted based on PDGFRα (Pα) expression. Cells were fixed after 14 days in culture and immunolabelled for α-actinin (green) to reveal myotubes. Cultures were counterstained with DAPI (blue) to visualize nuclei. Lin- MP Pα+ cells correspond to the previously characterized FAPs. Lin- MP Pα- cells are enriched in myogenic cells and also contain fibroblasts. Cultures of wild type Lin- MP Pα- cells had an average of 2,261 myotubes while similar cultures from mdx5cv Lin- MP Pα- cells had only 541 myotubes. Lin- SP Pα- cells did not survive in culture and are not shown. Scale bar = 400 μm. E: Cultures of Lin- SP Pα- cells were double labeled with antibodies to α-actinin (green) and collagen I (red) to visualize myotubes and fibroblasts, respectively. Cultures of Lin- SP Pα- cells from dystrophic muscle (MDX) do not contain myotubes but give rise to fibroblasts. Scale bar = 100 μm. G. Quantitative RT-PCR for Pax7 expression in Pdgfra+ and Pdgfra- Lin-SP cells, and Pdgfra- Lin-MP cells. Data is presented as means +/- s.d. from 3 technical replicates.
Figure 2.4

A

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<th>WT</th>
<th>CTX</th>
<th>MDX</th>
<th>PC</th>
<th>NC</th>
<th>-RT</th>
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<tr>
<td>Pax7</td>
<td></td>
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<td></td>
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<tr>
<td>Myf5</td>
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</tr>
<tr>
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B

WT

MDX

C

WT

MDX

D

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<tbody>
<tr>
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<td>Pα-</td>
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<tr>
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E

WT

MDX

F

WT

MDX

G

Pax7 Fold Change

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<th>Pα+ SP</th>
<th>Pα- SP</th>
<th>Pα- MP</th>
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Chapter 3

Hedgehog Activation Restores Myogenic Potential of Dystrophic $Mdx^{5cv}$ SP Cells.

(*) This chapter presents a preliminary study where we discovered that Hh signaling is down-regulated in the Lin-SP cells isolated from $mdx^{5cv}$ muscle. This finding led us to further investigate Hh signaling in dystrophic muscle and the potential positive effects of stimulating the pathway on disease pathology which are both presented in Chapter IV.

3.1 Abstract

Muscle side population (SP) cells are a population of rare multipotent stem cells that participate in myogenesis and have been investigated for cell therapy in Duchenne Muscular Dystrophy (DMD). In Chapter II we confirmed the sub-population of SP cells, termed Lin-SP cells, have the greatest myogenic potential as shown by their activity in our culture system. Interestingly, our studies have shown that the myogenic potential of Lin-SP cells is influenced by the muscle environment. Specifically these cells lose the ability to form muscle when isolated from acutely injured or dystrophic muscle. In the current study we sought to identify the signaling pathway that is altered in Lin-SP by the dystrophic muscle environment and that regulates their myogenic potential. Here we show that Hedgehog (Hh) signaling, an important player in embryonic myogenesis, is active
in wild type Lin-SP cells and is down-regulated in Lin-SP cells isolated from dystrophic muscle. Upon stimulation with the Hh agonist purmorphamine \textit{in vitro} and \textit{in vivo}, Lin-SP cell myogenesis was restored in culture. These findings demonstrate that Hh functions to promote Lin-SP myogenesis and suggest that inhibition of Hh signaling by the dystrophic muscle environment may negatively influence the ability of these cells to participate in muscle regeneration.

\textbf{3.2 Introduction}

Muscle side population cells (SP) are a population of muscle stem cells that successfully engraft in cellular transplantations and have been investigated for their potential use in cell therapies in DMD. Initial experiments transplanting wild type donor Lin-SP cells into cardiotoxin injured muscle demonstrated that Lin-SP cells successfully engraft in to injured muscle incorporating into myofibers and contributing to the satellite cell position (Asakura, Seale et al. 2002; Uezumi, Ojima et al. 2006). However, subsequent experiments transplanting donor \textit{mdx}^{5cv} SP cells into \textit{mdx}^{5cv} muscle resulted in low amounts of engraftment beneath therapeutic levels (Bachrach, Li et al. 2004). The low abundance of SP cells in skeletal muscle remains an additional barrier for both analysis of their biology and their use for therapeutic purposes. Acute muscle injury using cardiotoxin injection has been used in studies in an attempt to expand the number of SP cells for transplantation studies however; this treatment ablated the myogenic activity of the Lin-SP cells resulting in only
interstitial cell engraftment (Motohashi, Uezumi et al. 2008). In combination these studies suggested that the state of the muscle environment may negatively regulate SP cell myogenesis and may prevent their use as a cell therapy. In chapter II, we presented a novel *in vitro* system capable of supporting SP cell growth and differentiation without the need for co-culture with C2C12 cells as previously required (Asakura, Seale et al. 2002). By utilizing this culture system, we have shown that while SP cells isolated from BL6 muscle display robust myogenic differentiation, cells isolated from acutely injured or dystrophic muscle fail to undergo myogenesis. These findings suggested that the dystrophic microenvironment negatively influences Lin-SP myogenesis which may in turn represent a mechanism in disease pathology and represent a barrier to the therapeutic use of SP cells.

In the current study, we analyze gene expression of SP cells isolated from wild type and dystrophic Lin- SP cells revealing that Hh signaling is down-regulated in *mdx*\textsuperscript{5cv} SP cells. The Hh pathway functions in multiple organ systems during development and has recently been studied in regards to adult regeneration in multiple organ systems (Lavine, Long et al. 2008; Straface, Aprahamian et al. 2009; Bond, Angeloni et al. 2013). During muscle development Hh signaling promotes expression of Myf5 and MyoD in the developing somite, a structure responsible for generating the skeletal muscle of the body (Borycki, Mendham et al. 1998; Borycki, Brunk et al. 1999). Additionally, reports have shown that downstream Hh target, Gli1, functions to promote Myf5 expression by
binding to an enhancer sequence upstream of the promoter (Gustafsson, Pan et al. 2002). The Hh effector, Gli2, has recently been shown to be required for MyoD expression in satellite cells and has been shown to form a complex with MyoD and MEF2C where it functions to enhance expression of skeletal muscle promoters (Voronova, Coyne et al. 2013). Due to the multiple functions of Hh signaling in myogenesis we hypothesized that Hh signaling may function to promote SP myogenic cell fate and may represent a mechanism for the lack of myogenesis of \( mdx^{5cv} \) Lin-SP cells. To test this we activated Hh signaling via the agonist, purmorphamine, to determine whether activation of Hh signaling is capable of restoring dystrophic Lin-SP cell myogenesis.

### 3.3 Results

**Mdx\(^{5cv}\) Lin-SP cell myogenesis is restored by Hh stimulation.**

In order to elucidate the mechanism responsible for the loss of myogenesis in \( mdx^{5cv} \) Lin-SP cells observed in chapter II, we performed gene expression screening using SA Biosciences Real Time PCR Pathfinder arrays comparing the activity of multiple signaling pathways by analyzing expression of downstream transcriptional targets. This analysis revealed that multiple downstream Hh pathway target genes were down-regulated in \( mdx^{5cv} \) Lin-SP cells compared to BL6 Lin-SP cells by more than 3 fold (Figure 3.1 A). These results suggested that Hh signaling is down-regulated in Lin-SP cells isolated from dystrophic muscle. Furthermore, due to the involvement of Hh signaling in myogenesis, from embryonic myogenic commitment to satellite cell biology
(Kruger, Mennerich et al. 2001; Koleva, Kappler et al. 2005), these results suggested that down-regulated Hh signaling may be a mechanism responsible for the lack of myogenesis in dystrophic Lin-SP cells. Next in order to further confirm the down-regulation of Hh signaling in dystrophic SP cells we developed a Real Time PCR assay for quantification of Hh transcriptional target, Gli1. Studies have suggested that Gli1 is a primary responder to Hh signaling functioning upstream of Hh target genes including Ptch1 and Hhip (Vokes, Ji et al. 2007; Vokes, Ji et al. 2008). Additionally while multiple Hh target genes were differentially regulated in our previously mentioned results utilizing the SA Biosciences Real Time PCR arrays, we chose to focus on Gli1 based on previous in vitro data obtained in our laboratory showing that Gli1 is a more reliable indicator of pathway activation compared to target genes Ptch1 or Hhip. In order to reduce experimental variability likely due to the low number of Lin-SP cells available for analysis (~5000-10000 cells), we optimized experimental protocols involved in RNA extraction and cDNA synthesis allowing for gene expression quantification without the requirement for pre-amplification. We performed two independent experiments comparing the expression of Gli1 in mdx<sup>5cv</sup> Lin-SP to wild type Lin-SP cells revealing that mdx<sup>5cv</sup> Lin-SP Gli1 expression is down-regulated by approximately -3.4 +/- .02 fold compared to wild type SP cells (Figure 3.1B). This result provides further evidence confirming that Hh signaling is down-regulated in dystrophic Lin-SP cells.
Considering these results we next sought to determine if stimulation of Hh signaling is capable of restoring myogenic activity in \( mdx^{5cv} \) Lin-SP cells. To accomplish this we treated freshly isolated \( mdx^{5cv} \) Lin-SP cells with purmorphamine, cultured the cells for eleven days, and performed immunostaining for α-actinin in order to detect myotube formation. Similar to previous findings presented in chapter II, vehicle treated cultures displayed no myotube formation (Figure 3.2). In contrast, purmorphamine treated cultures displayed significant myotube formation including multiple large myogenic colonies (Figure 3.2). We must note however that in addition to the observed myotubes, a significant number of non-myogenic cells were present in the treated cultures appearing to be of fibroblast morphology. Significantly these results indicate that \( mdx^{5cv} \) Lin-SP cell myogenesis is not permanently lost and is capable of restoration, however Hh stimulation is not capable of inducing complete myogenic cell differentiation in all Lin-SP cells.

**In vivo stimulation of Hh signaling via purmorphamine restores \( mdx^{5cv} \) Lin-SP myogenesis.**

Considering our data showing down-regulation of Gli1 in freshly isolated \( mdx^{5cv} \) Lin-SP cells we next aimed to test whether stimulation of Hh signaling *in vivo* in the \( mdx^{5cv} \) mouse is capable of restoring downstream SP cell myogenesis in culture. Studies in our laboratory have shown that a single injection of purmorphamine induces Gli1 expression 8 hours post-injection but this induction is not maintained out to 24 hours (Figure 3.3 A, B). Because of this, subsequent
in vivo studies with purmorphamine include injections twice per day to maintain Hh induction. We treated mdx<sup>5cv</sup> mice for one week with purmorphamine by intraperitoneal injection and isolated cells to assay for myogenesis. Cells were grown in vitro for 9 days followed by immunostaining for both Pax7 and Myogenin in order to identify both satellite cells and differentiating myoblasts. We immunostained for these markers as opposed to α-actinin because cultures were monitored visually for myogenesis but no visual myotubes were starting to form by nine days as previously observed. Moreover, staining for these markers expressed earlier in the myogenic program allows for detection of myogenesis even in the event that terminal differentiation might not take place. We observed significant myogenic cell formation in cultures isolated from purmorphamine treated mice while control cultures displayed no myogenic activity in agreement with our previous studies (Figure 3.4).

Taken together these results show that restoration of Hh signaling in vivo via purmorphamine successfully restores myogenesis in a portion of mdx<sup>5cv</sup> Lin-SP cells. Furthermore, these results support our hypothesis that Hh is a pro-myogenic factor in the muscle environment and may represent a mechanism responsible for their loss of myogenesis in mdx<sup>5cv</sup> muscle.

**In vivo stimulation of Hh signaling induces downstream Hh target Gli1 gene expression without effect on myogenic transcription factor expression.**

Hh signaling has been demonstrated to promote myogenesis through the downstream interaction of Gli transcription factors with both Myf5 and MyoD
myogenic regulatory factors. We reasoned that Hh may be restoring \( mdx^{5cv} \) Lin-SP myogenesis by promoting expression of myogenic transcription factors including Myf5 and Pax7, shown in chapter II to be expressed in wild type Lin-SP cells. To test this hypothesis we treated \( mdx^{5cv} \) mice with purmorphamine for 1 week, isolated fresh Lin-SP cells, and performed qRT-PCR to measure expression of Gli1 to confirm up-regulation of Hh signaling. However, we observed no significant increase in Gli1 expression level in Lin-SP cells isolated from purmorphamine treated \( mdx^{5cv} \) compared to vehicle injected \( mdx^{5cv} \) mice (Figure 3.5 A). We attributed the lack of Gli1 induction to be due to the time between the last injection until the cell isolation, including up to 24 hours. This finding agrees with our previous studies in the diaphragm where we observed purmorphamine is not capable of maintaining up-regulation of Gli1 after 24 hours. Shortly following completion of this study, a new report was released using the more potent Hh agonist, SAG (smoothened agonist), in \textit{in vivo} experiments (Xiao, Hou et al. 2012). SAG has been shown to induce Hh signaling while requiring significantly lower concentrations and exhibiting significantly higher binding affinity for the Smoothened receptor than purmorphamine; considering this we reasoned that SAG may be more capable of maintaining Hh signaling in SP cells until the time of cell isolation (Wang, Lu et al. 2010). We treated \( mdx^{5cv} \) mice with SAG for one week, isolated Lin-SP cells and compared Gli1 expression between treated and vehicle injected mice. QRT-PCR analysis of freshly isolated Lin-SP cells showed that SAG treatment induced Gli1 expression compared to \( mdx^{5cv} \) control reaching BL6 SP cell levels (Figure 3.5 B). Despite the increase in
Hh signaling in SAG treated $mdx^{5cv}$ Lin-SP cells, none of the myogenic transcription factors including Pax7, Myf5, or MyoD, were significantly increased in response to Hh up-regulation (Figure 3.5 C). Intriguingly, we observed an increase in the levels of Myogenin expression in $mdx^{5cv}$ Lin-SP cells compared to BL6 (Figure 3.5 D). SAG treated $mdx^{5cv}$ Lin-SP cells expressed Myogenin at a level between BL6 and $mdx^{5cv}$ Lin-SP cells (Figure 3.5 D). However, these results are preliminary including only a single biological replicate for each experimental group and will need to be repeated to confirm these findings.

**In vitro modulation of Wnt and BMP signaling results in limited $mdx^{5cv}$ Lin-SP myogenesis.**

Due to our findings presented here revealing that $mdx^{5cv}$ Lin-SP cell myogenesis can be restored, we performed a small preliminary study testing whether modulation of additional developmental/signaling pathways could similarly restore myogenesis in $mdx^{5cv}$ Lin-SP cell cultures including Wnt and BMP. Wnt and BMP have been shown to contribute significant functions during muscle regeneration and in satellite cell biology by serving to regulate the balance between myogenic cell proliferation and differentiation (Seale, Polesskaya et al. 2003; Brack, Conboy et al. 2008; Ono, Calhabeu et al. 2011). Additionally, both of these pathways have been shown in numerous developmental studies to modulate cellular responses to Hh signaling by interacting and regulating levels of Gli1 (Liu, Massague et al. 1998; Borycki, Brown et al. 2000; Mullor, Dahmane et al. 2001; Watt 2004).
Data generated from the SA Biosciences Stem Cell Signaling Real Time PCR Array revealed that Lef1, a transcriptional target of canonical β-catenin/Wnt signaling, was down-regulated in \(mdx^{5cv}\) Lin-SP cells compared to wild type (-10.80 fold). In order to test whether activation of Wnt signaling is capable of restoring myogenesis in \(mdx^{5cv}\) Lin-SP cells we treated cells with a common Wnt agonist LiCl. Immunostaining for α-actinin revealed that LiCl treatment resulted in myotube formation, although there was only a single small observable colony (Figure 3.6).

Experiments utilizing SA Biosciences TGFβ/BMP Real Time PCR arrays comparing expression TGFβ/BMP signaling members between \(mdx^{5cv}\) and BL6 quadriceps muscles revealed that the BMP inhibitor, Noggin, was found to be down-regulated (-2.8 fold) in dystrophic quadriceps muscle. This result in combination with recent findings showing an antagonistic relationship between BMP signaling and muscle differentiation, suggested that reduced Noggin in dystrophic muscle may in turn translate into increased activity of BMPs and inhibition of SP myogenesis (Ono, Calhabeu et al. 2011). To test whether inhibition of BMPs would be capable of restoring \(mdx^{5cv}\) Lin-SP myogenesis, we treated cells with Noggin. Treatment of \(mdx^{5cv}\) Lin-SP cells with Noggin resulted in a small amount of myogenesis similar to LiCl treatment with cultures containing a small number of myotubes (Figure 3.6).
3.4 Discussion

The findings provided here expand upon our previous studies in which we have shown that the *mdx* microenvironment inhibits the myogenic differentiation of Lin-SP cells. We have identified Hh signaling as a potential new regulator of myogenesis in Lin-SP cells that is inhibited in dystrophic muscle. Stimulation of Hh signaling resulted in significant myogenic differentiation of *mdx* \(^{5cv}\) Lin-SP cells *in vitro*. Despite these positive results, the mechanism by which Hh promotes myogenic differentiation in SP cells is currently unclear. Previous developmental studies have shown Hh to elicit pro-myogenic effects in cells of the epaxial somite by enhancing expression of Myf5 and MyoD myogenic transcription factors (Borycki, Brunk et al. 1999). We have treated *mdx* \(^{5cv}\) mice with purmorphamine and an additional more potent Hh agonist, SAG, but we do not detect significant induction of Pax7, MyoD, or Myf5, in Lin-SP cells. These results suggest that the action of Hh on adult SP cells may differ from previous developmental studies and further that Hh induction of SP myogenesis may involve additional factors other than the traditional myogenic regulatory factors. For example, Hh mediated myogenesis of *mdx* \(^{5cv}\) cells may be facilitated by induction of Six1/2 or Eya transcription factors which have been found to function upstream of Pax7, Myf5 and MyoD during the early stages of myogenesis (Fougerousse, Durand et al. 2002). In future studies we will compare expression of these early markers to determine if they are differentially expressed between wild type and *mdx* \(^{5cv}\) Lin-SP cells and determine if they are induced upon Hh
stimulation. An alternative mechanism however may be that Hh may influence SP myogenesis not through expression of myogenic regulatory factors but instead by modulating their activity on downstream promoters. For example, Gli2 forms a complex with MyoD and functions to modulate its activity on downstream transcriptional targets (Voronova, Coyne et al. 2013). Future experiments will be aimed at examining whether Hh stimulation induces expression of identified downstream targets of MyoD that function in myogenic differentiation. The results of these studies will not only enrich our understanding of SP myogenesis but may also supply mechanisms which may be conserved with other myogenic stem cell populations upstream of the satellite cells.

An important consideration regarding Hh stimulation in Lin- $mdx^{5cv}$ SP cultures is that only a portion of SP cells undergo myogenesis while a large proportion of cells still differentiate into fibroblasts or adipocytes. One potential reason for this finding could be that only a portion of the SP cell compartment is able to receive and respond to the Hh signal. An alternative mechanism may be that all of the cells receive and respond to Hh signaling, but do so in different manners resulting in some cells becoming myogenic and others differentiating into fibroblasts and adipocytes. There are two currently available mouse model systems that may be utilized to distinguish between these two scenarios. The Gli1-Lacz reporter mouse has been used in numerous animal studies to label and identify cells that are actively receiving and responding to Hh signaling (Bai, Auerbach et al. 2002; Ahn and Joyner 2004). The strength of this model is that it
will allow us to measure the proportion of SP cells via flow cytometry/FACS that respond to Hh signaling \textit{in vivo} in response to Hh agonists. This will allow us to determine if treatment with different Hh agonists stimulate the entire population of SP cells or if only a portion respond. In the case where not all of the SP cells respond, we can utilize FACS to fractionate the SP population into Hh responding (Lacz+) and non Hh responding (Lacz-) populations and subsequently assay for their myogenic activity in our culture system. By conducting this study this will allow us to quantify the number of SP cells responding to different Hh agonists and determine if all cells responding to Hh signaling are directed towards myogenesis. While this study will be useful for \textit{in vivo} studies, it will not be suitable for \textit{in vitro} study of effects of Hh agonists in SP myogenesis. To study the effects of Hh agonists on SP cell fate decisions, cells are treated after isolation and analyzed for expression of cellular markers after a week or longer in culture. Because this system reports signaling in real time, we would be limited to analyzing downstream progeny of SP cells, not SP cells directly \textit{in vitro} as they quickly differentiate. In order to perform this analysis it will be necessary to utilize a Gli1 promoter driven Cre recombinase lineage tracing model. To develop this, the tamoxifen inducible Gli1-Cre mouse will be bred with R26R-GFP mice (Mao, Fujiwara et al. 2001; Ahn and Joyner 2004). By using this model we will induce the reporter immediately when SP cells are cultured along with purmorphamine treatment; afterwards the cells responding to Hh signaling, expressing Gli1, will irreversibly express GFP. This analysis will allow us to determine the direct effects of Hh signaling on SP cell differentiation and the
proportion of downstream cells (satellite cells, fibroblasts, etc.) generated. Moreover, we will be able to determine if Hh signaling stimulates myogenesis in all responding SP cells or if Hh signaling only elicits its effects on a portion of SP cells. Additionally, this model will prove useful in vivo if we are not able to visualize lacZ expression by FACS for technical reasons; in this case we will inject mice with tamoxifen along with Hh agonists and evaluate the cellular fate of both downstream cells expressing and not expressing GFP.

Our experiments clearly show that Hh signaling is capable of promoting Lin-SP myogenesis in dystrophic SP cells but it is unclear if Hh signaling is a required component of myogenesis in wild type uninjured Lin-SP cells. One method to address this question would be to treat cultured SP cells with the Hh inhibitor, cyclopamine, and assay for myogenic differentiation. However, cyclopamine has been documented to exhibit off-target effects and induce apoptosis through non-canonical Hh mechanisms by mediating sphingomyelinase 2/ceramide induction (Meyers-Needham, Lewis et al. 2012). To best address whether Hh is required for SP myogenesis, we will breed the tamoxifen activated R26R-Cre recombinase mouse to the Smo floxed transgenic mouse (Long, Zhang et al. 2001). This model will allow us to induce the knockout of Smo rendering all cells insensitive to Hh ligands. Isolation and culture of Lin-SP cells from this transgenic model will determine if SP myogenesis is dependent on Hh signaling. An important consideration regarding tamoxifen induced Cre systems is they do not result in recombination in 100% of cells.
(Hara-Kaonga, Gao et al. 2006; Takata, Kondo et al. 2011). A method to overcome this will be to cross the R26R floxed eGFP reporter mouse into this model (Mao, Fujiwara et al. 2001). In this case in cells that receive tamoxifen, Cre will knockout Smo function and simultaneously activate an eGFP reporter label. This will allow for purification of Lin-SP cells lacking Smo expression through isolation of cells positive for GFP. A potential outcome of this experiment would be the observation that wild type Lin-SP cells don’t undergo myogenesis in our in vitro system. However, this result would be very interesting and would require further investigation determine the mechanism for this outcome. One possibility is that failure of myogenesis is due to redirection towards FAPs or fibroblasts. A second possibility is that the lack of Hh signaling may prevent Lin-SP cells from undergoing commitment to the satellite cell lineage. In the case where cells do not express markers of FAPs, fibroblasts, or myogenic cells at the protein level, RNA can be isolated from cultured cells to determine whether early myogenic markers are expressed such as Eya and Six or if there is any detectable expression of Myf5, Pax7, or MyoD at the mRNA level.

The partial myogenic restoration of dystrophic Lin-SP cells clearly demonstrated that myogenesis is not permanently lost and can be rescued by modulation of the Hh pathway. This finding is interesting and significant because it demonstrates that dystrophic SP cells have myogenic capacity but this ability has been temporarily inhibited due to effects by the dystrophic microenvironment.
Preliminary data generated in the laboratory led us to conduct a preliminary study to determine whether modulation of additional pathways may be similarly successful in restoring \( mdx^{5cv} \) myogenesis. We focused on testing the modulation of the Wnt and BMP signaling pathways. Due to our results observing a decrease in Lef1 (a Wnt transcription target) in \( mdx^{5cv} \) Lin-SP cells, we chose to test whether stimulation of the Wnt pathway via LiCl treatment would be capable of restoring myogenesis. Because of the large number of Wnt ligands, we chose to utilize LiCl to stimulate canonical Wnt signaling for a preliminary study despite its off target effects. Additionally, through comparison of BMP/TGFβ pathway expression profiling of the quadriceps muscles we found that the BMP antagonist, Noggin, was down-regulated in \( mdx^{5cv} \) muscle compared to wild type. This result suggested that \( mdx^{5cv} \) Lin-SP cells may be interacting with increased amounts of BMPs in the microenvironment, which considering previous studies showing that BMPs inhibit myogenic differentiation, may in turn represent a mechanism responsible for inhibition of \( mdx^{5cv} \) Lin-SP myogenesis (Ono, Calhabeu et al. 2011). Both stimulation of canonical Wnt signaling activation via LiCl and BMP inhibition via Noggin generated a small amount of myogenesis \( \textit{in vitro} \), albeit much less compared to the amount of myogenesis observed after pumorphamine treatment. Nevertheless considering our data in combination, our data have identified three signaling pathways that function in Lin-SP cell myogenesis; a research area previously lacking information regarding myogenic regulation. An important consideration for interpreting these results is that signaling pathways often interact to generate unique responses. Due to the
observed interactions between Wnt, BMPs, and Hh in the literature it will be important to determine if these pathways interact with each other and potentially display synergistic or antagonistic effects on Lin-SP myogenesis (Silva-Vargas, Lo Celso et al. 2005; Dennler, Andre et al. 2007; Katoh and Katoh 2009; Javelaud, Alexaki et al. 2011). In order to better understand the function of these pathways in SP myogenesis we will test different combinations of Hh, Wnt, and BMP modulation to determine how they may cooperate to regulate SP cell differentiation. These analyses will determine if modulation of multiple pathways simultaneously is capable of improving upon the myogenesis we have observed with purmorphamine alone. In the case where multiple treatments are capable of enhancing myogenic differentiation of dystrophic Lin-SP cells beyond purmorphamine alone, it will be important to address whether these pathways are active in the same cell or eliciting responses on different populations of cells. To address this, I would cross the previously mentioned Gli1-LacZ reporter mouse with an additional reporter for Wnt or BMP signaling. For Wnt signaling, I would use the Lef-GFP reporter (Ferrer-Vaquer, Piliszek et al. 2010) and for BMP signaling I would utilize the Id1-GFP reporter (Perry, Zhao et al. 2007). Next, I would perform multiple in vivo treatments; (1) first including LiCl systemic injection for activating the Wnt pathway in combination with SAG system administration, (2) second involving Noggin intramuscular injection for inhibiting BMP accompanied by SAG systemic administration. These experiments will determine if Hh induction (observed by LacZ expression) and Wnt/BMP modulation (observed by EGFP fluorescence) occur in the same SP cell or in
different populations. Additionally, these reporters will be useful to determine if there is crosstalk among pathways which can be determined by performing a single treatment, for example SAG treatment only, and analyzing whether Wnt signaling is induced or BMP signaling is altered.

3.5 Materials and Methods

Ethics statement
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the Animal Care and Use Committee of Nationwide Children’s Hospital.

Animals

$Mdx^{5cv}$ mice (a gift of Louis Kunkel, Harvard Medical School, Boston, MA) were used in all experiments. $Mdx^{5cv}$ mice have a mutation in exon 10 of the DMD gene that disrupts dystrophin expression. C57BL/6J mice were used as background matched wild type controls, originally purchased from Jackson Laboratories. All mice were bred in house.

SP Isolation

For all SP cell isolations, muscles from six to eight mice were pooled to obtain enough cells for culture or analysis. Muscle cells were isolated from
hindlimb skeletal muscles as previously described. Briefly, muscles were trimmed of fat and tendons and finely minced. The minced muscle was digested for up to 30 minutes at 37°C in 1.2 U/ml Dispase (Worthington Biochemicals) and 5 mg/ml Collagenase Type IV (Worthington Biochemicals) in phosphate buffered saline (PBS) containing 2.5 mM CaCl2. The enzymes were inactivated with DMEM/10% FBS, and the digest passed through 70 μm and 40 μm cell strainers. Cell suspensions were overlaid with Horse Serum (Gibco) and centrifuged at 160 xg for 10 min without brakes to remove debris. Cells were resuspended at 10^6 cells/ml in PBS containing 0.5% (w/v) bovine serum albumin (Sigma; PBS/BSA) and labeled with 12.5 ug/ml Hoechst 33342 (Sigma) in the presence or absence of 100 mM verapamil (Sigma) for 1 hour at 37°C. Cells were resuspended in 100ul of PBS/BSA and incubated with FC block (BD Biosciences) for 10 min on ice. Afterwards, cells were stained with antibodies to CD31-APC(MEC 13.3, BD Biosciences) and CD45-PECy7(30-F11, BD Biosciences) for 15 minutes on ice in the dark. Just prior to analysis, cells were counterstained with Propidium Iodide (Fisher Scientific) for exclusion of dead cells. CD31-/CD45- cells were collected on ice in PBS/BSA and reanalyzed by FACS to visualize Hoechst for isolation of SP cells. Cells were visualized and sorted on a FACSVantage DIVA (BD Biosciences) cell sorter. For cell culture experiments, cells were collected in culture medium. For RNA isolation, cells were collected in Trizol-LS (Invitrogen) while maintaining 3:1 Trizol-LS to buffer ratio.
**In Vitro Cell Treatments**

Cells were treated 24 hours after culture with a single dose of purmorphamine (Toronto Research Chemicals # P840300) at a final concentration of .25uM to stimulation hedgehog signaling, a single dose of Noggin (Peprotech # 250-38) at a final concentration of 500 ng/mL to inhibit BMP signaling, or LiCl at a final dosage of 20mM to stimulate canonical Wnt signaling. Cells were grown for eleven days on matrigel coated coverslips in EGM2-MV media.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton-X100 for 10 min, incubated first for 1 hour at room temperature in blocking solution (PBS with 10% horse serum), and then overnight at 4°C with primary antibodies diluted in blocking solution. Primary antibodies include anti-Pax7 (DSHB), anti-myogenin (clone F5D, DAKO), and anti-αactinin (Sigma). Cells were incubated with conjugated secondary antibodies (Jackson Immunoresearch) for 1 hour at room temperature followed by incubation with DAPI (Invitrogen) to counterstain nuclei. Coverslips were mounted with n-propyl gallate (Sigma) mounting media.

**RNA Isolation**

For sorted cells, samples were collected in Trizol-LS (Invitrogen). Chloroform was added per manufacturer specifications, and the samples were
spun in a microcentrifuge at 4°C. Afterward, the aqueous top layer was extracted and combined with an equal volume of 70% EtOH. This mixture was then purified through a RNeasy MinElute Column (Qiagen) using the manufacturer protocol. RNA was eluted in nuclease free water.

**CDNA Synthesis and Real Time PCR**

First strand synthesis was performed by using the Superscript Vilo Kit (Life Technologies) according to the manufacturer’s protocol. Primers used for real time RT-PCR include: Gli1: F 5’-ACTTGCCAACCATCATATCCAG-3’, R 5’-GTGTACCACATGACTCTACTCG-3’, Probe FAM 5’-
TGCAACCTGCCAGCTGAAGTCA-3’; Pax7 : F 5’-
CTCAGTGAGTTTCAGCAGC-3’, R 5’- AGACGTTCCCTTTGTCGC-3’, Probe FAM 5’- AGAGGACGACGAGGAAGGACAA-3’; Myf5: F 5’-
CCTGTCTGTTCCGAAAGAAC-3’, R 5’-GACGTGATCCGATCCACAATG-3’, Probe FAM 5’-TCCTGATGATCTCAATGCGTGC-3’; Myogenin F 5’-
GCAATGCACTGAGGATCTCG-3’, R 5’-ACGATGAGGACTGAAGGAGTG-3’, Probe FAM 5’-TCCAACCGAGGAGATCATTGTG-3’; MyoD F 5’
GCTCTGATGGCATGAGTATCC-3’, R 5’-GACACAGCCGCAACTTCC-3’, Probe FAM 5’-CAGAATGGCTACGACGCGC-3’; TBP: F 5’-
ACCCCCACTCTCTCCATTCC-3’, R 5’-AGCCAAAGATTCACGTCG-3’, HPRT: F 5’-
CCTCATGGACTTATGGACAG-3’ R 5’-TCAGCAAAAGAATTATAGCC-3’, Probe MAX 5’-AGATGTCATGAGGAGATGGAGGC-3’. Real time PCR was
performed using Taqman Fast Advanced Master Mix (Life Technologies) using 10ul reaction volumes in an ABI 7500 (Life Technologies). PCR reactions were conducted either in duplex or triplex using TBP or both TBP and HPRT as housekeeping genes respectively. Efficiency of each PCR primer set was validated to amplify with 90% or greater efficiency. Validation experiments were conducted with these primer sets confirming that dynamic range, efficiency, and limit of detection are unaffected in multiplexed reactions compared to singleplex. Fold changes were calculated using the delta-delta Ct method using Data Assist software (Life Technologies).

**Purmorphamine Injections**

Mice were injected with purmorphamine (Toronto Research Chemicals #P840300) at a dosage of 10mg/kg twice a day for 1 week for SP cell studies. Stock purmorphamine solution was prepared at a concentration of 30mg/mL in DMSO. Working solutions were made at a concentration of 1mg/mL in vehicle solution composed of 10% DMSO, 18% Cremaphore, and 20% glucose in distilled water.

**SAG Injections**

Mice were injected with SAG (smoothened agonist, Calbiochem #566660) at a dosage of 5mg/kg/day injected once per day for 1 week. Stock solution was prepared using distilled water at a concentration of 1mg/mL and sterile filtered through a 0.2um filter.
SA Biosciences Arrays

RNA was extracted using Trizol and Qiagen purification as previously mentioned. CDNA was synthesized using the SA Biosciences First Strand CDNA synthesis kit according to manufacturer specifications. For the Signal Transduction Pathfinder PCR Array (PAMM-014) and the Stem Cell Signaling PCR Array (PAM-047) preamplification was performed prior to running the arrays according to manufacturer specifications using the SA Biosciences RT² PreAMP cDNA Synthesis Kit (#330451) with specific primers for each array PBM-014 and PBM-047, respectively. For the TGFβ/BMP Signaling Pathway PCR Array (PAMM-035) preamplification was not performed. Afterwards, cDNA was diluted and added to SA Biosciences Real Time Array plates and Real Time PCR was performed on an ABI 7500 following manufacturer specifications. Gene expression changes were calculated using the SA Biosciences web based software which calculates fold changes using the delta delta ct method. Normalization was performed using the housekeeping genes contained on the plates.

Statistical Analysis

Statistical tests for pairwise comparisons were performed using the non-parametric Mann-Whitney test. For multiple comparisons ANOVA was utilized followed with the Kruskal-Wallas post-hoc test for non-parametric analysis. Calculations were made using Graphpad Prism software.
Figure 3.1: Gene expression analysis of freshly isolated Lin- SP cells from $Mdx^{5cv}$ and BL6 reveals down-regulation of multiple downstream Hh signaling components in cells from dystrophic mice. (A) SA Biosciences Real Time PCR array data comparing expression of Hh target genes between SP cells isolated from $mdx^{5cv}$ and BL6 mice. Multiple target genes are down-regulated suggesting Hh signaling is reduced in $mdx^{5cv}$ SP cells. Data shown for two independent experiments. (B) Real Time PCR comparing expression of Hh target gene Gli1 between SP cells isolated from $mdx^{5cv}$ and BL6 mice. Cells isolated from $Mdx^{5cv}$ mice show reduced Gli1 expression indicating down-regulation of the Hh pathway. Significance was evaluated by performing the non-parametric Mann-Whitney test; while Gli1 was reduced in Mdx compared to BL6 this result was not determined to be statistically significant. Data shown for two independent experiments.
Figure 3.2: *In vitro* activation of Hh signaling induces myogenesis in \textit{mdx}^{5cv} SP cultures. SP cells were isolated from Mdx^{5cv} mice and treated with the Hh agonist, purmorphamine resulting in \textit{mdx}^{5cv} SP myogenesis. Myogenesis was determined by immunostaining for the myotube marker, a-actinin (green). Nuclei were counterstained with DAPI (blue). Scale bar: 500um.
Figure 3.3: Purmorphamine significantly stimulates Hh signaling in the \(mdx^{5cv}\) diaphragm muscle. QRT-PCR comparing Gli1 expression between \(mdx^{5cv}\) control mice, BL6, and purmorphamine injected \(mdx^{5cv}\) mice. (A) Purmorphamine significantly induces Gli1 expression in the \(mdx^{5cv}\) diaphragm to BL6 levels 8 hours after a single purmorphamine injection. N= 5 BL6, 4 \(Mdx^{5cv}\), and 2 Pur injected \(Mdx^{5cv}\) mice. (B) Hh signaling is not significantly up-regulated in the Diaphragm 24 hours after a single purmorphamine injection. N= 2 BL6, 4 \(Mdx^{5cv}\), and 3 Pur injected \(Mdx^{5cv}\) mice. * indicates Pval <.05. Significance determined by one-way ANOVA, pairwise comparisons were performed using Kruskal-Wallis posthoc. * indicates Pval < .05. Lack of asterisk indicates lack of significance.
Figure 3.4: Restoration of signaling in vivo rescues downstream in vitro mdx\textsuperscript{5cv} SP cell myogenesis. In vivo administration of purmorphamine for one week prior to isolation successfully restores the myogenesis of cultured mdx\textsuperscript{5cv} SP cells. Myogenesis was detected by immunostaining SP cells isolated from vehicle-control and purmorphamine-injected mdx\textsuperscript{5cv} mice with antibody cocktail of Pax7 and myogenin (green) labeling satellite cells and myoblasts, respectively. Nuclei were counterstained with DAPI (blue). Scale bar: 100um.
Figure 3.5: *In vivo* stimulation of Hh signaling induces Hh signaling without effect on myogenic transcription factor expression in *mdx*\(^{5cv}\) SP cells. (A) Real Time RT-PCR comparing Hh target Gli1 expression in SP cells isolated from *mdx*\(^{5cv}\) control, purmorphamine treated *mdx*\(^{5cv}\) mice, and BL6 mice. Gli1 expression is not increased in SP cells isolated from *mdx*\(^{5cv}\) mice receiving purmorphamine treatment. (B) A more potent Hh agonist, SAG, increases Gli1 expression in Lin- SP cells isolated from treated *mdx*\(^{5cv}\) mice. (C) Comparison of expression of myogenic transcription factors, Myf5, MyoD, and Pax7, in *mdx*\(^{5cv}\) control, SAG treated *mdx*\(^{5cv}\) mice, and BL6 mice. SAG does not induce expression of Myf5, MyoD, or Pax7 in *mdx*\(^{5cv}\) SP cells. (D) Comparison of expression of myogenin in SP cells isolated from *mdx*\(^{5cv}\) control, SAG treated *mdx*\(^{5cv}\) mice, and BL6 mice. Expression of myogenin is increased in SP cells isolated from *mdx*\(^{5cv}\) control and SAG treated *mdx*\(^{5cv}\) mice compared to wild type. Data is from a single experiment. Error bars represent standard deviation for 3 technical replicates.
Figure 3.6: *In vitro* modulation of Wnt and BMP signaling results in limited *mdx*<sup>5cv</sup> SP myogenesis. SP cells were isolated from *Mdx*<sup>5cv</sup> mice and treated with pathway modulators for Wnt and BMP signaling. LiCl was used to stimulate canonical Wnt signaling and Noggin was administered to inhibit BMP signaling. Myogenesis was determined by immunostaining for the myotube marker, α-actinin (green). Nuclei were counterstained with DAPI (blue). Scale bar: 500um.
Chapter 4

Hh Signaling: a New Factor in MDX Pathology

4.1 Abstract

Hedgehog (Hh) signaling functions during development to specify the fate of multiple organ systems. While primarily studied for its role in developmental biology, Hh signaling has only recently been implicated in regenerative processes in multiple organisms including the skeletal muscle. Hh signaling is induced during acute muscle injury where it functions to promote the expansion of satellite cells. Inhibition of Hh signaling during acute injury leads to fibrosis and a reduced regenerative response. While Hh signaling has previously been demonstrated to play a role during muscle regeneration, study of Hh in muscular dystrophy has not been characterized. In chapter III, we showed that mdx^{5cv} Lin-SP cells display reduced Hh signaling which is unexpected considering the up-regulation observed during acute injury. This finding suggested that Hh signaling may not be induced in dystrophic muscle prompting us to analyze Hh signaling in the mdx^{5cv} diaphragm and limb. In the current study we reveal that in contrast to previous regeneration studies, Hh signaling is not induced in the mdx^{5cv} diaphragm or quadriceps muscles potentially representing a mechanism contributing to disease pathology. Activation of the pathway by in vivo
purmorphamine treatment of $mdx^{5cv}$ mice improved multiple parameters of disease pathology including reduced Collagen 1 deposition, a decrease in central nuclei, and improvement in exercise-induces fatigue. Additionally, our studies demonstrate that Hh signaling serves to promote the expression of myogenic transcription factors associated with satellite cell proliferation and differentiation, suggesting a positive role for Hh signaling in muscle regeneration. These data suggest that insufficient Hh signaling may play a role in DMD pathology and stimulation of Hh signaling may represent a new strategy to counteract collagen deposition and maintain functional mobility.

4.2 Introduction

The hedgehog signaling (Hh) pathway is an evolutionarily conserved pathway that functions in tissue morphogenesis of multiple organ systems including skeletal muscle (Ingham, Nakano et al. 2011). Beyond its roles in developmental biology, Hh signaling has been shown to function in adult tissue regeneration of multiple organ systems including, heart, skin, bone, nerve, and liver (Lavine, Long et al. 2008; Mak, Bi et al. 2008; Ochoa, Syn et al. 2010; Bond, Angeloni et al. 2013). Only recently have studies begun to investigate the role of Hh signaling in adult skeletal muscle regeneration following acute injury by ischemia or cardiotoxin injection (Pola, Ling et al. 2003; Straface, Aprahamian et al. 2009). Additionally, multiple studies have revealed key contributions of Hh signaling in the muscle regeneration process, regulating proliferation and differentiation of cultured myogenic cells (Koleva, Kappler et al. 2005), promoting
angiogenesis (Straface, Aprahamian et al. 2009), and halting fibrosis in skeletal muscle (Kusano, Pola et al. 2005; Straface, Aprahamian et al. 2009; Ahmed, Haider et al. 2010). Because progressive failure of regeneration, fibrosis and impaired myogenic cell differentiation have all been reported in DMD, we examined the status of Hh signaling in the dystrophic mdx$^{5cv}$ mouse model of DMD. We report here for the first time that Hh signaling is not activated in mdx$^{5cv}$ muscle in spite of the presence of chronic muscle injury in young 12 week old mice. Furthermore, we found a significant down-regulation of Hh signaling in the diaphragm, the most affected muscle in mdx$^{5cv}$ mice.

In agreement with our SP studies in chapter III, Hh signaling is reduced in the diaphragm muscle and unchanged in the limb. We hypothesized that failure to activate Hh signaling in mdx muscles may represent an important mechanism in dystrophic pathology by potentially affecting multiple disease parameters. To test this, we utilized an Hh agonist, purmorphamine, to stimulate Hh signaling in mdx$^{5cv}$ mice and assayed for effects on multiple histological parameters associated with pathology. Our results show that stimulation of the Hh pathway ameliorates multiple parameters of mdx$^{5cv}$ pathology including reduced Collagen 1 deposition, effecting muscle regeneration/degeneration observed by reductions in central nuclei, and improved resistance to exercise-induced fatigue.
4.3 Results

*Mdx*<sup>5cv</sup> diaphragm and quadriceps muscle do not exhibit Hh induction.

Previous studies have shown that Hh signaling is significantly up-regulated during muscle regeneration in acute injury and muscle ischemia models (Pola, Ling et al. 2003; Straface, Aprahamian et al. 2009). Since dystrophic tissue is in a chronic state of degeneration and regeneration, it is reasonable to hypothesize that Hh signaling should be similarly up-regulated in dystrophic muscle. To directly test this we isolated RNA and measured Gli1 expression levels from both diaphragm and quadriceps muscles by qRT-PCR. Interestingly, we observed no induction of Hh signaling in the quadriceps muscle while Hh signaling was actually decreased in the *mdx*<sup>5cv</sup> diaphragm muscle where Gli1 displays a significant 2-fold down-regulation compared to wild type muscle (Figure 4.1). Considering the multiple roles of Hh signaling in muscle regeneration these results suggested that an inability to induce Hh signaling may play a role in dystrophic pathology.

In order to test whether stimulation of Hh signaling affects dystrophic pathology we designed two studies to evaluate different time frames of treatment in the *mdx*<sup>5cv</sup> mouse (Figure 4.2). *Mdx* mouse pathology has been characterized as consisting of an early stage (4-8 weeks of age) characterized by significant muscle degeneration and regeneration, followed by a later stage (8-12 weeks of age) at which point degeneration and regeneration occur but stabilize at a lower level followed by detectable fibrosis at 12 weeks of age (Stedman, Sweeney et
al. 1991; Andreetta, Bernasconi et al. 2006). We chose to perform the first treatment in \( \textit{mdx}^{5\text{cv}} \) over eight weeks extending in the mice from four weeks to twelve weeks of age (Group A). The first treatment addresses whether purmorphamine can significantly alter the process of regeneration and degeneration in the early phase that would in turn translate to improvement in histological parameters at 12 weeks of age. The second treatment began later, starting at eight weeks of age and ending at twelve weeks of age (Group B). The second treatment only including the later stage addresses whether Hh stimulation significantly impacts the development of fibrosis in the \( \textit{mdx}^{5\text{cv}} \) mouse. By testing purmorphamine administration over these two time frames we are able to address multiple questions regarding the drug’s effect on disease pathology. Experiments were designed to incorporate purmorphamine injections twice per day in order to maintain Hh signaling induction based on previous studies from chapter 3 (Figure 3.3).

**Purmorphamine improves resistance to fatigue.**

During the course of treatment in group A, we assayed the effect of purmorphamine treatment on the fatigability of \( \textit{mdx}^{5\text{cv}} \) mice. Post-exercise fatigue is a physical performance assay that has previously been shown to be a sensitive indicator of disease pathology in \( \textit{mdx}^{5\text{cv}} \) mice and a sensitive assay to evaluate potential therapeutics in dystrophic mice (Kobayashi, Rader et al. 2008). In order to measure this parameter we quantified physical activity both before
and after treadmill exercise by using a Photobeam Activity System (Beastrom, Lu et al. 2011). At both 4 weeks and 8 weeks after treatment (8 weeks and 12 weeks of age, respectively), rearing events were significantly increased following exercise in treated mice compared to control. After 4 weeks of purmorphamine treated mice displayed an improvement in the reduction in rearing events (-76.06% +/- 7.57), compared to control mice (-96.96% +/- 4.44, P<.01) (Figure 4.3). Similarly following 8 weeks of purmorphamine treated mice displayed an improvement in the reduction in rearing events of (-88.28% +/- 9.03) compared to control (-98.55% +/- 2.17, P<.05) (Figure 4.3). These results suggest that purmorphamine treatment successfully improves one of the observed physical parameters associated with \(mdx^{5cv}\) mice and may improve disease pathology.

**Purmorphamine treatment reduces Collagen 1 deposition in the \(mdx^{5cv}\) diaphragm.**

A relationship has been observed between Hh signaling and fibrosis accumulation whereby inhibition of Hh signaling during acute muscle regeneration results in significant fibrosis accumulation (Straface, Aprahamian et al. 2009). Due to these findings we next tested whether Hh stimulation via purmorphamine can decrease fibrosis in the \(mdx^{5cv}\) diaphragm. Our studies focus on the diaphragm muscle because it is the skeletal muscle that most closely recapitulates the progressive fibrosis accumulation observed in patients (Stedman, Sweeney et al. 1991). In order to determine this we performed immunostaining for Collagen 1 in the diaphragm muscles from mice in both
Group A and Group B. We visually observed a reduction the amount of area staining for Collagen 1 in both Group A (Figure 4.4 A, B) and Group B. To confirm this finding we quantified the immunoreactive area positive for Collagen 1 using ImageJ. In Group A, Collagen 1 deposition was reduced in the treated mice (23.4% +/- 2.6) compared to controls (30.6% +/- 3.0) (Figure 4.4 C). Similarly, in diaphragms from the shorter treatment, Group B, treated mice displayed reduced amounts of Collagen 1 (22.0% +/- 4.5) as compared to control mdx5cv mice (31.4% +/- 3.6, ) (Figure 4.4 D). While Collagen 1 was clearly decreased in both of these treatment groups, due to variability and small sample sizes, statistical significance was not established. Additional studies will be required in order to determine if these findings are statistically significant.

**Fibroblasts display induced Gli1 expression following delivery of Purmorphamine and SAG.**

As we demonstrated, purmorphamine treatment reduces Collagen 1 deposition in the mdx5cv diaphragm muscle. Next we aimed to determine whether this result is a direct effect of Hh stimulation on collagen gene expression. In order to determine whether Hh stimulation directly reduces collagen gene expression we measured the expression of Collagen 1 and Collagen 3 by qRT-PCR from BL6, mdx5cv, and treated mdx5cv diaphragms from Group A. Neither expression of Collagen 1 nor Collagen 3 was significantly reduced in treated mdx5cv diaphragms compared to control (Figure 4.5 A). This finding suggests that collagen mRNA expression is not significantly decreased at 12 weeks of age.
in the diaphragm of treated mice. Due to the fact that fibroblasts are a major contributor of collagen in fibrotic disorders, we next performed a series of experiments to determine whether \textit{in vivo} Hh stimulation decreases collagen gene expression in fibroblasts. To accomplish this we performed a one week treatment of \textit{mdx}^{5cv} mice, and isolated fibroblasts by the preplating method. To confirm Hh stimulation in fibroblasts we measured Gli1 expression by qRT-PCR. Fibroblasts isolated from purmorphamine treated \textit{mdx}^{5cv} mice displayed Hh induction by expressing Gli1 at approximately two fold greater than cells isolated from vehicle injected \textit{mdx}^{5cv} mice (Figure 4.5 B). Interestingly, fibroblasts from BL6 mice expressed Gli1 approximately 7 fold higher than vehicle injected \textit{mdx}^{5cv} mice (Figure 4.5 B). This shows that while purmorphamine does induce Hh signaling in dystrophic muscle fibroblasts, it does not restore expression to wild type levels. Despite the observed increase in Hh signaling in response to purmorphamine, neither expression of Collagen 1 nor Collagen 3 was significantly changed in response to purmorphamine (Figure 4.5 C). Next we performed an additional 1 week treatment with SAG in order to reach higher levels of Hh induction as observed in BL6 fibroblasts. SAG treatment in \textit{mdx}^{5cv} mice resulted in expression of Gli1 in isolated fibroblasts by approximately 15 fold greater than \textit{mdx}^{5cv} control fibroblasts (Figure 4.5 D). We next measured expression of Collagen 1 and Collagen 3 by qRT-PCR in fibroblasts isolated from SAG treated mice. Similarly to purmorphamine treatment, we observed no significant decline in the expression of either Collagen 1 or Collagen 3 in response to SAG (Figure 4.5 E). Taken together these results suggest that the
observed decreased in Collagen 1 deposition *in vivo* is not due to Hh mediated down-regulation of collagen gene expression.

**Analysis of Hh stimulation on parameters of regeneration and degeneration.**

Studies have shown a positive correlation between Hh signaling and the expression levels of myogenic regulatory factors and numbers of activated satellite cells in injured muscle (Stratface, Aprahamian et al. 2009). Considering these findings we hypothesized that stimulation of Hh signaling may be beneficial for dystrophic pathology by enhancing the regenerative response. To determine this we next performed a series of assays to determine if Hh stimulation significantly improves parameters of muscle pathology focusing on regeneration. DMD and *mdx*\textsuperscript{5cv} muscle undergo continuous cycles of degeneration and regeneration resulting in significantly increased fiber size variation and altered fiber size distribution. Therefore, fiber diameter quantification is a sensitive assay that can be used to determine if muscle regeneration or degeneration are significantly altered. Fiber diameter was measured between purmorphamine treated and control diaphragms in Group A and Group B but no significant differences were found in the distribution of fiber sizes (Figure 4.6 A, B). As an additional assay for determining potential changes in regeneration, we next quantified the proportion of centrally nucleated muscle fibers. Interestingly in Group A, we observed a reduced proportion of centrally nucleated fibers in treated mice (33.1% +/- 2.6) compared to *mdx*\textsuperscript{5cv} control (47.7% +/- 7.6,) (Figure
While there was a trend towards decreased central nuclei, statistical significance was not met due to small sample size. However, in Group B we did not observe a decline in the proportion of centrally nucleated fibers between treated mice (41.0% +/- 10.3) and control (47.7% +/- 7.6); Figure 4.6 C). The observed trend towards reduced centrally nucleated fibers in Group A suggested that purmorphamine treatment impacted either muscle regeneration or degeneration during the course of treatment. However, interpretation of a change in central nuclei is difficult because a change in the rate of either fiber degeneration or regeneration can shift the proportion of centrally nucleated fibers. To overcome this limitation we performed additional assays in diaphragms from Group A that are more specific for regeneration and degeneration. Because muscle regeneration is a multi-step process involving both, the activation and proliferation of satellite cells and their eventual fusion and differentiation generating regenerated myofibers, we assayed whether purmorphamine treatment improves either of these processes in the \(mdx^{5cv}\) diaphragm. To assay the number of newly regenerating myofibers in the diaphragm we performed immunostaining for embryonic myosin heavy chain (eMHC), a marker specifically expressed in newly regenerating myofibers. The number of regenerating fibers was found to be similar in both treated (5.2% +/- 0.2) and \(mdx^{5cv}\) control (4.2% +/- 1.9) diaphragm muscles, (Figure 4.6 D) suggesting that new muscle fiber formation is not significantly increased in response to purmorphamine. Lastly, in order to quantify the amount of fiber degeneration in the diaphragm, we performed staining for immunoglobulin G (IgG). The percentage of IgG
immunoreactive area was not significantly reduced in treated mice (19.69% +/- 3.95) compared to \textit{mdx}^{5cv} control (20.18% +/- 3.69) suggesting that muscle degeneration is not significantly reduced (Figure 4.6 E).

**Hh signaling promotes expression of myogenic regulatory factors.**

Previous studies in regeneration models show that Hh signaling functions \textit{in vivo} to increase the number of satellite cells and myogenic transcription factor expression (Straface, Aprahamian et al. 2009; Piccioni, Gaetani et al. 2013). These findings combined with our studies showing a lack of Hh induction in \textit{mdx}^{5cv} mice led us to hypothesize that satellite cells may not be receiving Hh signal in dystrophic muscle. To test this we isolated myogenic cells by the pre-plating method and compared Gli1 expression levels between \textit{mdx}^{5cv} and wild type cells. Similarly to Lin-SP cells, \textit{mdx}^{5cv} myogenic cells display down-regulation of Gli1 by \~2 fold compared to wild type cells (Figure 4.7 A). During collection of the Lin-SP cells in our previously mentioned study in chapter III in which SAG successfully induced Hh signaling, we isolated RNA from Lin- main population cells (Lin-MP) which includes satellite cells and myoblasts. Analysis of the Lin-MP cells revealed induction of Gli1 in Lin-MP cells isolated from SAG treated mice compared to vehicle control (Figure 4.7 B). We next compared expression of myogenic regulatory factors, Pax7, Myf5, MyoD, and myogenin, to compare expression between \textit{mdx}^{5cv} and wild type Lin-MP cells and to determine if Hh stimulation influenced expression of these factors. Pax7 and Myf5 were
both reduced in $mdx^{5cv}$ Lin-MP cells while myogenin was increased slightly by 1.7 fold compared to wild type Lin-MP cells (Figure 4.7 B). MyoD expression was unchanged between $mdx^{5cv}$ and wild type Lin-MP cells (Figure 4.8 B).

Interestingly Hh induction via SAG up-regulated Pax7, Myf5, and myogenin transcription factors compared to vehicle control Lin-MP cells (Figure 4.7 B). Taken together these results suggest that myogenic cells in dystrophic muscle may not be receiving Hh signal and pharmacological up-regulation of Hh via SAG may be able to enhance muscle regeneration in dystrophic muscle.

Our laboratory has generated preliminary data using the myogenic C2C12 cell line to further investigate the relationship between Hh signaling and myogenic transcription factor expression. In order to test whether Hh stimulation or inhibition significantly changes myogenic factor expression, C2C12 cells were cultured in the growth phase whereby purmorphamine and cyclopamine were added in vitro to test the effects of Hh stimulation and inhibition, respectively. Next, qRT-PCR was conducted to compare expression of Pax7, Myf5, MyoD, and myogenin (Figure 4.7 C). We observed an effect in purmorphamine treated cultures with a trend towards increased expression of Pax7, Myf5, and myogenin. Importantly, treatment of C2C12 cells with cyclopamine, an Hh antagonist, led to a trend towards reduced expression of Pax7, Myf5, and myogenin (Figure 4.7 C). These results suggest that Hh signaling appears to function in multiple stages of myogenesis, both in the proliferative and differentiation stages, and may be a critical component involved in myogenic transcription factor expression.
**Inflammation**

Inflammation plays multiple roles in dystrophic pathology by impacting muscle regeneration/degeneration as well as contributing to fibrosis development (Grounds and Torrisi 2004; Andreetta, Bernasconi et al. 2006; Tidball and Wehling-Henricks 2007). While a direct role of Hh signaling in muscle inflammation has not yet been established, multiple studies have shown connections between Hh signaling and immune system function and development (Outram, Varas et al. 2000; Stewart, Lowrey et al. 2002; Sacedon, Diez et al. 2005). Considering the connections between inflammation with both fibrosis and muscle fiber degeneration we next tested whether Hh stimulation decreased the inflammatory response in the diaphragm since this may represent a potential mechanism to explain the observed reduction in Collagen 1 deposition and central nuclei. To quantify inflammation we performed CD45 immunostaining and quantified the proportion of immunoreactive area. Immunostaining revealed no significant reduction in CD45 immunoreactive area in treated mice (15.4% +/- .4) compared to vehicle control (16.2% +/- 4.1) (Figure 4.8 A). As an additional measure of inflammation, we next compared gene expression of immune cytokines including TNFα, IL-1β, and TGFβ1 in the diaphragm muscle. Expression levels of TNFα, IL-1β, and TGFβ1 were not significantly changed in diaphragms of purmorphamine treated mice compared to controls (Figure 4.8 B). These findings suggested that purmorphamine treatment does not significantly reduce the number of inflammatory cells or inflammatory
gene expression in the diaphragm muscle. However, this analysis was conducted on mice at the conclusion of treatment at 12 weeks; therefore this did not rule out the possibility that Hh may attenuate muscle inflammation at an earlier time point in disease. In order to test the effect of Hh stimulation earlier in the disease, we treated $mdx^{5cv}$ mice with purmorphamine beginning when mice reach eight weeks of age and extending for one week. In this case we utilized flow cytometry to quantify the proportion of CD45+ cells isolated from limb muscles from BL6, $mdx^{5cv}$ controls, and treated $mdx^{5cv}$ mice. The major advantage to using this method is that it allowed us to obtain precise quantifications of the numbers of CD45+ cells and it allowed us to perform this quantification over multiple whole muscles. Our analysis revealed significantly reduced numbers of immune cells among the muscle mononuclear compartment compared to control $mdx^{5cv}$ mice (Figure 4.8 C). While treatment was not able to reduce the proportion of CD45 cells in $mdx^{5cv}$ muscles to uninjured BL6 levels, there was no statistically significant difference found between treated $mdx^{5cv}$ mice and BL6 mice (Figure 4.8 C). This finding suggests that Hh stimulation reduces the number of immune cells in the limb muscles of $mdx^{5cv}$ mice between the ages of eight and nine weeks.

4.4 Discussion

As previously mentioned Hh signaling has only recently been acknowledged as an active signaling pathway in adult regeneration programs (Lavine, Long et al. 2008; Mak, Bi et al. 2008; Ochoa, Syn et al. 2010; Bond,
Angeloni et al. 2013). Recent studies in skeletal muscles have shown activation of the pathway in response to injury in both muscle ischemia and acute damage models (Pola, Ling et al. 2003; Straface, Aprahamian et al. 2009). Importantly, it has been shown in these studies that Hh signaling elicits pleiotropic effects on the regenerative program serving to enhance number of satellite cells during regeneration, prevent fibrosis, and stimulate angiogenesis (Straface, Aprahamian et al. 2009). Because of these multiple functions carried out by Hh signaling, we decided to determine if Hh signaling is up-regulated in dystrophic muscle as a failure to stimulate the pathway may play a significant role in the progression of disease pathology. While Hh signaling is normally induced upon acute regeneration, our data clearly shows that Hh signaling is not induced in the \textit{mdx}\textsubscript{5cv} quadriceps muscle and is decreased in the \textit{mdx}\textsubscript{5cv} diaphragm (Straface, Aprahamian et al. 2009). Due to this finding we next utilized the small molecule purmorphamine to up-regulate Hh signaling in \textit{mdx}\textsubscript{5cv} mice in order to determine whether Hh signaling may be effective at ameliorating multiple disease parameters characteristic of dystrophic mice.

**Stimulation of hedgehog signaling reduces fibrosis**

Our results show a trend towards reduced Collagen 1 deposition in the diaphragm. This reduction in the diaphragm may account for the observed increase in post-exercise activity and reduced fatigue. However, the mechanism responsible for reduction in Collagen 1 is unclear. TGFβ is regarded as a major
driver of the fibrotic response in DMD and *mdx* mice by promoting the expression of collagen gene expression (Inagaki, Truter et al. 1994; Jimenez, Varga et al. 1994; Bernasconi, Torchiana et al. 1995). For example, multiple studies have shown through direct inhibition of TGFβ or through inhibition of downstream Smad signaling, a significant decrease in *mdx* diaphragm fibrosis (Gosselin, Williams et al. 2004; Andreetta, Bernasconi et al. 2006; Turgeman, Hagai et al. 2008). Our results presented here demonstrate that while Hh stimulation reduces the amount of Collagen 1 deposition in the diaphragm, TGFβ mRNA levels are unchanged. Additionally, while purmorphamine and SAG treatments induce Gli1 expression *in vivo*, the mRNA expression of Collagen 1 and Collagen 3 in freshly isolated fibroblasts are also unaffected. In combination these results suggest that the observed decrease in Collagen 1 deposition is not due to inhibition of TGFβ or the inhibition of collagen gene expression. These results suggest that the reduction in Collagen 1 protein deposition may be a result of an alternative mechanism not utilized by current anti-fibrotic strategies. Considering there is currently no method capable of completely reducing fibrosis the identification of such mechanism may provide a new anti-fibrotic mechanism or a strategy that may be useful in complementing current methods of TGFβ inhibition for a synergistic effect that may be superior to current strategies. To better understand this process it will be necessary to investigate multiple mechanisms that may be responsible for the reduction in Collagen 1. First, we have only analyzed Collagen 1 at the gene expression level; changes in collagen protein expression or secretion may be a potential mechanism affected by Hh signaling. In order to
address these possibilities we will culture primary fibroblasts with purmorphamine and assay for the number of cells expressing Collagen 1 and the number of cells presenting Collagen 1 on their surface. Secondly, Hh signaling may indirectly regulate collagen by regulating the proliferation of FAPs and/or fibroblasts. Since there are no available markers for cell sorting pure fibroblasts we will isolate primary fibroblasts by preplating and assay proliferation in response to Hh stimulation by immunostaining and quantification of fibroblasts expressing the Ki67 proliferation marker. For FAPs we can assay the effects of Hh in vivo by quantifying the proportion of Pdgfra cells positive for BrdU by flow cytometry. A third potential mechanism may be that Hh signaling is increasing the rate of ECM degradation and therefore slowing the accumulation of Collagen 1 in the diaphragm. In this case Hh may be mediating this process by directly or indirectly influences expression of enzymes responsible for collagen/ECM degradation such as matrix metalloproteinases (MMPs) (Backstrom and Tokes 1995; Saito, Katoh et al. 1998). Since expression of multiple MMPs including MMP2 and MMP9 (Li, Mittal et al. 2009; Miyazaki, Nakamura et al. 2011), have been shown to elicit dramatic effects on mdx pathology, we will measure expression of these enzymes to determine if Hh stimulation influences their expression in dystrophic muscle. Lastly, Hh may be reducing Collagen 1 deposition by reducing immune cell infiltration or modulating immune cell subtypes that make up the inflammatory response. Hh signaling albeit not characterized in regards to immune regulation in skeletal muscle, has been shown to regulate multiple aspects of the immune system. Moreover, since multiple studies have
demonstrated a strong contribution of the immune system to fibrosis development in the \textit{mdx} mouse (Spencer, Montecino-Rodriguez et al. 2001; Villalta, Nguyen et al. 2009), we have performed multiple studies to determine if Hh stimulation elicits effects on the immune cell infiltrate or immune cytokines.

Our studies have produced differing results regarding the reduction of immune cell infiltrate in response to purmorphamine. On one hand, our analysis of limb muscles by FACS following a single week of treatment resulted in a significant reduction in the proportion of CD45 cells. However, analysis of our long-term purmorphamine study, lasting 8 weeks, revealed no significant difference in CD45+ immunoreactive area in the diaphragm. There are multiple possible explanations for these differing observations. First it is possible that purmorphamine reduces CD45 cell numbers in the diaphragm early during treatment but it unable to maintain the effect as pathology progresses as the mice age to 12 weeks of age. Secondly, there may be compensatory mechanisms occurring in the \textit{mdx}^{5cv} mouse limiting Hh induction when stimulated for an extended period of time. Additionally, the discrepancy in our results may result from differences in methods used to measure CD45 immune cells. It is also possible that Hh stimulation is effective at reducing immune cell infiltration in the limb muscles but not in the diaphragm. While CD45 immunostaining is not significantly reduced in the diaphragm, it remains possible that immune modulation is still present and may contribute to reduced collagen1 deposition. For example, multiple studies have demonstrated reduced diaphragm fibrosis
resulting from alterations to the immune cell populations (Farini, Meregalli et al. 2007; Vidal, Serrano et al. 2008). For instance, immune modulation via loss of osteopontin in the \textit{mdx} mouse has been shown to significantly reduce fibrosis deposition in the \textit{mdx} diaphragm (Vetrone, Montecino-Rodriguez et al. 2009). Additionally, \textit{mdx} mice lacking T cells display significantly reduced fibrosis (Farini, Meregalli et al. 2007). In order to better understand the effects of Hh on the \textit{mdx} muscle immune infiltrate, future studies will use flow cytometry analysis to investigate how Hh signaling may regulate the abundance of immune cell populations in dystrophic muscle with a focus on the diaphragm muscle.

\textbf{Hh signaling and muscle regeneration/degeneration}

In the present study we show multiple data supporting a positive role for Hh signaling in the expression of multiple myogenic transcription factors in myogenic cells. These results in combination with previous reports in muscle regeneration studies suggest that stimulation of Hh signaling may be effective at enhancing regeneration in \textit{mdx} muscle towards the goal of alleviating pathology (Straface, Aprahamian et al. 2009; Piccioni, Gaetani et al. 2013). In fact there appears to be an effect of Hh stimulation on either of the regenerative or degenerative parameters in \textit{mdx}^{5cv} muscle based on reductions in central nuclei quantifications, but the specific process modulated by Hh signaling remains unclear. Interpretation of central nuclei quantification is difficult due to the fact that once cell fuses into a myofiber, the nucleus migrates to the center of the myofiber and remains there indefinitely. Because of this process the assay can
only infer that changes in regeneration/degeneration occurred at some point in
time during life of the subject. We have performed further studies investigating
the processes of degeneration and regeneration on diaphragm tissues collected
at the endpoint of treatment. Both embryonic myosin heavy chain staining (a
measure for regeneration) and immunoglobulin G staining (a measure for
degeneration) are unchanged in the diaphragm at twelve weeks of age.
Considering this data it appears that Hh stimulation does not affect the processes
of regeneration or degeneration at the time point of twelve weeks in the
diaphragm muscle. Interestingly, while we do not observe a significant difference
in the proportion of centrally nucleated fibers in our eight to twelve week
treatment group, we did observe a trend towards decreased numbers of centrally
nucleated fibers in mice from the four to twelve week treatment. These findings
suggest that either the process of regeneration or degeneration was altered
during the 4 to 8 week time frame, but further studies will be required in order to
determine which parameter.

In particular, Hh has been shown in multiple studies to induce the
proliferation of satellite cells in culture models (Koleva, Kappler et al. 2005; Elia,
Madhala et al. 2007). Therefore future studies will be aimed at evaluating in
greater detail specific parameters of satellite cell biology including proliferation,
activation and differentiation. These studies may utilize the Pax7-ZsGreen
reporter mouse in order to directly analyze satellite cells from purmorphamine
treated mice and determine if Hh stimulation significantly increases the number
of proliferating cells by measuring the number of BrdU+ Pax7+ cells by FACS (Bosnakovski, Xu et al. 2008). While Hh signaling has been shown to positively influence the expression of myogenic regulatory factors MyoD and Myf5 (Gustafsson, Pan et al. 2002; Voronova, Coyne et al. 2013), there has been no connection with Pax7 expression. This analysis will provide an additional measure to determine if Hh stimulation increases the number of Pax7 satellite cells and determine if Hh induction increases the expression of Pax7 on a cell by cell basis. In addition, future studies may utilize MyoD and Myogenin immunostaining to quantify the number of activated satellite cells and differentiating myoblasts at multiple stages in the \( \text{mdx}^{5cv} \) diaphragm. While number of satellite cells has been quantified in \( \text{mdx} \) limb and DMD, quantifications in the mouse diaphragm are currently lacking (Ishimoto, Goto et al. 1983; Kottlors and Kirschner 2010). Moreover, in the diaphragm there has yet to be a comprehensive analysis analyzing the effects of the disease on multiple stages of myogenesis, from the number of total satellite cells to the number of activated satellite cells and differentiating myoblasts. By incorporating the combination of these assays our studies will not only evaluate the effects of Hh signaling on satellite cells and myoblasts but also fill in gaps in our knowledge involving the effect of pathology in the diaphragm on multiple stages of muscle regeneration.

A possible explanation for not observing a stronger improvement in muscle regeneration/degeneration parameters may be that Hh is not induced at a
sufficient level to elicit all of the pathway’s effects. This may be the case due to
the morphogen nature of Hh in which case cellular responses are proportional to
the intensity of pathway activation (Su, Jones et al. 2007). Also, while Hh
signaling positively influences Myf5 expression and MyoD function, it is currently
unclear how different levels of Hh induction may influence these functions
(Gustafsson, Pan et al. 2002; Voronova, Coyne et al. 2013). Our data in Figure 4.7 showing that SAG is capable of increasing the expression of multiple
myogenic transcription factors is promising and encourages further study
investigating whether SAG treatment of mdx<sup>5cv</sup> mice results in a more dramatic
improvement in pathology. Additionally, future studies should be aimed towards
better understanding the effects of the intensity of Hh induction on myogenic
transcription factor expression by comparing induction mediated by both
purmorphamine and SAG in myogenic cells.

**4.5 Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations in the
Guide for the Care and Use of Laboratory Animals of the National Institutes of
Health. All animal protocols were approved by the Animal Care and Use
Committee of Nationwide Children’s Hospital.
**Animals**

Mdx^{5cv} mice (a gift of Louis Kunkel, Harvard Medical School, Boston, MA) were used in all experiments. Mdx^{5cv} mice have a mutation in exon 10 of the DMD gene that disrupts dystrophin expression. C57BL/6J mice were used as background matched wild type controls, originally purchased from Jackson Laboratories. All mice were bred in house.

**Pre-plating Preparation**

Limb muscles were dissected and digested producing a mononuclear cell suspension as mentioned previously in the SP cell isolation protocol in chapter III. Afterwards, cell suspensions are incubated on 0.2% Gelatin coated tissue culture dishes for 1 hour at 37°C. After the hour incubation, fibroblasts are adherent to the plate while non-adherent cells are transferred to a second Gelatin coated dish and incubated for an additional hour at 37°C. Afterwards, non-adherent cells are collected containing myogenic cells.

**RNA Isolation**

For whole tissue, muscles were excised and stored in Trizol (Invitrogen). Tissues were homogenized using a TissueLyser (Qiagen). Once homogenized, samples were spun in a micro centrifuge at top speed for 5 min to remove debris and un-homogenized material. Supernatant was removed and transferred to a new tube where the protocol was continued as previously described beginning with the addition of chloroform. After the addition of an equal volume of 70%
EtOH, samples were purified using an RNeasy Mini Column (Qiagen) using the manufacturer protocol. RNA was eluted in 50ul of nuclease free water.

For RNA isolation of fibroblasts, Trizol is added to adherent cells contained in the first preplate and incubated at RT for 10 min while rocking. Afterwards, Trizol is collected and RNA is extracted as previously mentioned using an RNeasy MinElute column. For RNA isolation of myogenic cells, non-adherent myogenic cells are collected after the second preplate and spun for 10 min at 1500RPM, Trizol is added to the cell pellet, incubated at RT for 10 min, and collected. Afterwards, the extraction proceeds as similarly mentioned using an RNeasy MinElute column.

**CDNA Synthesis and Real Time PCR**

First strand synthesis was performed by using the Superscript Vilo Kit (Life Technologies) according to the manufacturer’s protocol. Primers used for real-time RT-PCR include: Gli1: F 5’-ACTTGCCAACCATCATATCCAG-3’, R 5’-GTGTACCACATGACTCTACTCG-3’, Probe FAM 5’-TGCAACCTGCCAGCTGAAGTCA-3’, TBP: F 5’-ACCCCAACAACCTCTTCACATTC-3’, R 5’-AGCCAAGATTTCCAGGTAGATAC-3’, Probe Cy5 5’-CCTATCACTCCTGCCACACCAGC-3’, HPRT: F 5’-CCTCATGGACTGATTGGACAG-3’ R 5’-TCAGCAGAAGAATTATAGCCCG-3’, Probe MAX 5’-AGATGTCATGAGGAGATGGGAGGC-3’, IL-1B primer probe set was supplied as a complete assay containing primers and probes (Life
Technologies, Mm00434228_m1). TNFα F 5’-
CCCTCACACTCAGATCTTCT-3’, R 5’- GCTACGACGTGGGCTACAG-3’,
TGFβ1: F 5’- TGCCCCTATATTTGGAGCCTGGAC-3’, R 5’-
GCCCCGGTTGTGTTGGTTTAGAG-3’, Collagen1a1: F 5’-
CCCGAACCCCCAAGGAAAAGAAGC-3’, R 5’-
CTGGGAGCCTCGGTGGACATTA-3’, Collagen3a1: F 5’-
TGGAAAGAGTGGTGACAGAGGAGA-3’, R 5’-
TTAGAGCCACGTTCACCAGTTTCA-3’. For probe based reactions Real Time
PCR was performed using Taqman Fast Advanced Master Mix (Life
Technologies) using 10ul reaction volumes in an ABI 7500 (Life Technologies).
For SYBR green reactions (those not including probe sequences) Real Time
PCR reactions was performed using SYBR Select Master Mix (Life
Technologies) using 10ul reaction volumes in an ABI 7500 (Life Technologies).
All reactions were conducted using TBP or TBP and HPRT in combination as
housekeeping genes. Reactions utilizing probes were performed in duplex or
triplex using TBP or both TBP and HPRT as housekeeping genes respectively.
Efficiency of each PCR primer set was validated to amplify with 90% or greater
efficiency. Validation experiments were conducted using probe based reactions
confirming that dynamic range, efficiency, and limit of detection are unaffected in
multiplexed reactions compared to singleplex. Fold changes were calculated
using the delta-delta Ct method using Data Assist software (Life Technologies).
**Purmorphamine Injections**

Mice were injected with purmorphamine (Toronto Research Chemicals # P840300) at a dosage of 10mg/kg twice a day for 4 to 8 weeks for animal studies. Stock purmorphamine solution was prepared at a concentration of 30mg/mL in DMSO. Working solutions were made at a concentration of 1mg/mL in vehicle solution composed of 10% DMSO, 18% Cremaphore, and 20% glucose in distilled water.

**Exercise-Induced Fatigue Assay**

Activity measurements were taken on a Photobeam Activity System (San Diego Instruments, San Diego, CA) under dim light in the early morning before and after a single bout of treadmill exercise. Activity was recorded for 1 hour at 5-minute interval. The DMD_M.2.1.002 TREAT-NMD standard operating procedure was followed, and the first 5 minutes of recorded activity were discarded from analysis. Treadmill (Columbus Instruments) settings were 15° decline, 10 m/min speed, with a 10-minute run. For analysis, percentage decline in activity is calculated as the difference between post-exercise and pre-exercise levels.

**Quantification of Centrally Nucleated Fibers and Fiber Diameter**

10μm diaphragm sections were fixed with 4% paraformaldehyde for 15 minutes and immunostained with antibody directed against Laminin (Sigma). Sections were stained with secondary antibodies (Jackson Immuno). For each
section 20x montages were photographed, the numbers of total myofibers and myofibers with a centrally located nucleus were counted and used to calculate the percentage of fibers containing a central nucleus. Minimal fiber diameter was quantified using Laminin stained sections and measuring minimal feret diameter on approximately 1000 fibers per section with Image J software (NIH).

**Immunohistochemistry**

10um sections were fixed with either acetone or 4% paraformaldehyde. Sections were blocked and permeabilized with 10% horse serum (Gibco)/0.2% Triton-X (Fisher) in PBS for 1 hour at room temperature. Primary antibodies include Collagen 1 (Cedarlane), embryonic myosin heavy chain (DSHB), and CD45 (Ebiosciences). Primary antibodies were incubated overnight at 4°C in blocking solution. Sections were washed in PBS and stained with secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature. For IgG quantification, donkey anti-mouse antibodies (Jackson Immuno) were incubated for 1 hour at RT. Slides were mounted with n-propyl gallate (Sigma) mounting media. 6 to 8 non-overlapping images were captured with an Olympus microscope (Olympus, Center Valley, PA). The immunoreactive area was determined for Collagen 1, CD45, and IgG staining after manual outlining with the use of ImageJ software (NIH) and was expressed as a percentage of positive staining of the outlined section area. For measuring regeneration, eMHC staining was quantified as a proportion of positively stained eMHC fibers compared to total number of fibers.
C2C12 Culture

C2C12 myoblasts were grown for three days in growth medium (GM) consisting of Dulbecco Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). After 24 hours in culture, purmorphamine (Toronto Research Chemicals # P840300) or cyclopamine (Toronto Research Chemicals # C988400) were added to the growth medium at concentrations of 2um and 20um, respectively. Fresh growth medium was added every 24 hours.

CD45 Flow Cytometry Analysis

Limb mononuclear cells were isolated using the protocol mentioned previously in Chapter 3 (SP Isolation) using both Collagenase IV (Worthington Biochemicals) and Dispase (Worthington Biochemicals) enzymatic digestion. Following digestion and cell counting, cells were pre-incubated with Fc Block (BD Biosciences) followed by CD45 antibody (BD Biosciences clone#30F11) for 15 min on ice. Afterwards cells were rinsed and staining was visualized on a FACS Vantage DIVA (BD Biosciences). Prior to analysis, cells were counterstained with Propidium Iodide (Fisher Scientific) for exclusion of dead cells.

Statistical Analysis

Statistical tests for pairwise comparisons were performed using the non-parametric Mann-Whitney test. For multiple comparisons ANOVA was utilized
followed with the Kruskal-Wallis post-hoc test for non-parametric analysis. Calculations were made using Graphpad Prism software.
Figure 4.1: Real time RT-PCR comparing whole muscle Gli1 expression in diaphragm and quad from \(mdx^{scv}\) and BL6 mice. Gli1 is significantly down-regulated in the diaphragm muscle of \(mdx^{scv}\) mice compared to BL6 (A), while not significantly changed in the quadriceps (B). Values in (A, B) represent means generated from biological replicates, N= 4-6 biological replicates. Error bars represent SD. Significance was evaluated by performing the non-parametric Mann-Whitney test.* indicates Pval<.05. (C, D) Individual fold changes of biological replicates for Diaphragm and Quad muscles.
Figure 4.2: Schematic displaying *mdx* disease pathology and the timing of our purmorphamine treatment groups.
Figure 4.3: Eight week purmorphamine treatment significantly reduces post-exercise fatigue in mdx\textsuperscript{5cv} mice. Percent decrease in vertical activity measured in both vehicle control and mdx\textsuperscript{5cv} mice following both 1 month and 2 months of treatment with purmorphamine. **P<.01, *P<.05. N= 3 mdx\textsuperscript{5cv} mice treated with purmorphamine, 10-11 mdx\textsuperscript{5cv} controls including vehicle injected controls and uninjected controls. Values represent means +/- SD. Significance was evaluated by performing the non-parametric Mann-Whitney test.
Figure 4.4: Purmorphamine treatment reduces collagen deposition in the diaphragm. A,B Immunohistochemistry highlighting reducing in Collagen 1 staining in Purmorphamine treated diaphragm (A) compared to Vehicle (B). Quantification of Collagen 1 immunoreactive area in the diaphragm muscle at after four weeks (C) and eight weeks (D) of treatment. Purmorphamine reduced Collagen 1 immunoreactive area, Values represent means generated from biological replicates, error bars represent SD. Significance was evaluated by performing the non-parametric Mann-Whitney test. The observed decrease in Collagen1 was not found to be statistically significant.
Figure 4.5: Hh induction does not significantly reduce collagen gene expression. (A) QRT-PCR comparing expression of Collagen 1 and Collagen 3 in diaphragms of purmorphamine treated mice. N= 2 BL6, 5 Mdx5cv controls including vehicle injected and uninjected, and 3 Purmorphamine treated Mdx5cv mice. (B, C) QRT-PCR comparing gene expression of Gli1, collagen1, and Collagen 3, in fibroblasts freshly isolated from mdx5cv mice following 1 week of purmorphamine treatment, mdx5cv controls, and BL6 mice. (B) 1 week of purmorphamine treatment up-regulates Gli1 expression in mdx5cv mice, but does not restore expression to BL6 levels. (C) Expression of Collagen 1 and Collagen 3 are not significantly changed in response to purmorphamine treatment. N= 2 biological replicates for BL6, Mdx5cv control, and Purmorphamine treated Mdx5cv. (D, F) QRT-PCR comparing gene expression of Gli1, collagen1, and Collagen 3, in fibroblasts freshly isolated from mdx5cv mice following 1 week of SAG treatment, and mdx5cv controls. (E) SAG treatment increases Gli1 expression in treated fibroblasts. (F) Hh up-regulation via SAG does not significantly affect Collagen 1 or Collagen 3 gene expression in fibroblasts. N= 2 biological replicates for Mdx5cv control, and Purmorphamine treated Mdx5cv. Significance determined by non-parametric Mann Whitney test for pairwise comparisons, multiple comparisons were conducted using one-way ANOVA, pairwise comparisons were performed using Kruskal-Wallis posthoc. Lack of asterisk indicates lack of statistical significance.
Figure 4.5

A

B

D

E

F
Figure 4.6: Analysis of purmorphamine treatment on muscle regeneration and degeneration. (A, B) Minimal Feret fiber diameter quantification of the diaphragm muscle. (A) Comparison of fiber diameter of mice treated for 4 weeks, group B, between \( mdx^{5cv} \) controls, purmorphamine treated \( mdx^{5cv} \), and BL6 mice. (B) Comparison of fiber diameter of mice treated for 8 weeks, group A, between \( mdx^{5cv} \) control, and purmorphamine treated \( mdx^{5cv} \). (C) Quantification of number of myofibers containing a central nucleus. Proportion of central nuclei is not significantly changed in group B, but there is a trend towards reduced central nuclei in group A. (D) Quantification of percentage of myofibers staining positive for embryonic myosin heavy chain. The number of regenerating myofibers is not significantly changed in response to purmorphamine. (E) Quantification of IgG reactive area to detect muscle degeneration. Degeneration is not significantly changed in response to purmorphamine. Values are means +/- SD. *P<.05. N= 2 BL6 mice, 3 \( mdx^{5cv} \) mice treated with purmorphamine, 2-5 \( mdx^{5cv} \) controls including vehicle injected controls and uninjected controls. Significance determined by one-way ANOVA, pairwise comparisons were performed using Kruskal-Wallis posthoc. None of the analyses yielded statistically significant differences.
Figure 4.6

A

B

C

D

E

% IgG Positive Area

0 5 10 15 20 25 30

Mdx Cntrl  Pur

% Positive EMHC Fibers

0 1 2 3 4 5 6 7

Mdx Cntrl  Pur

Percent Fibers Containing Central Nuclei

0 10 20 30 40 50 60

Mdx control  Group A  Group B

Relative Frequency

0 5 10 15 20 25 30 35

Mdx Cntrl  Pur  BL6

Min Feret Diameter μM

5 10 15 20 25 30 35 40 45 50 55 60

Min Feret Diameter μM

19 15 20 25 30 35 40 45 50 55 60
Figure 4.7: Hh signaling is down-regulated in dystrophic myogenic cells: preliminary data on potential functions of Hh signaling in myogenic cell function. (A) QRT-PCR comparing Gli1 expression in preplated myogenic cells isolated from \textit{mdx}^{5cv} and BL6 mice. Gli1 expression is down-regulated in preplated myogenic cells isolated from \textit{mdx}^{5cv} compared to BL6. N= 1, Values represent means generated from technical triplicate reactions. (B) QRT-PCR comparing expression of Gli1 and myogenic regulatory factors (Myf5, MyoD, Pax7, and Myogenin) in main population cells (MP) isolated from SAG treated \textit{mdx}^{5cv} mice (1 week), \textit{mdx}^{5cv} controls, and BL6 mice. Data obtained from a single experiment. Gli1 is induced in response to SAG treatment. Myf5, Pax7, and Myogenin expression is increased in response to SAG. (C) QRT-PCR comparing expression of myogenic regulatory factors in proliferating C2C12 cells treated with purmorphamine or cyclopamine. N= 3 wells per group, QRT-PCR for each group performed in triplicate. Error bars represent SD. Significance determined by one-way ANOVA, pairwise comparisons were performed using Kruskal-Wallis posthoc. * indicates Pval < .05. Lack of asterisk indicates lack of significance.
Figure 4.8: Analysis of purmorphamine treatment on inflammation and inflammatory gene expression. (A) Quantification of CD45 immunoreactive area reveals no significant reduction in inflammation in purmorphamine treated diaphragms compared to control. N= 3 mdx<sup>5cv</sup> controls including vehicle injected and uninjected controls and 3 purmorphamine injected mdx<sup>5cv</sup> mice. (B) Expression of cytokines IL-1β, TNFα, and TGFβ are not significantly reduced in the mdx<sup>5cv</sup> diaphragm in response to purmorphamine treatment. N= 4 mdx<sup>5cv</sup> controls including vehicle injected and uninjected mdx<sup>5cv</sup> controls, 3 treated mdx<sup>5cv</sup>, and 5 BL6. (C) Following 1 week of purmorphamine treatment, limb muscle mononuclear cells were isolated from treated mdx<sup>5cv</sup> mice; vehicle control injected mdx<sup>5cv</sup> mice, and BL6 wild type mice, and stained for CD45 expression. Cells were analyzed by FACS and the percentage of CD45+ cells is quantified. Purmorphamine treatment significantly reduced the abundance of CD45+ cells. N=5 mdx<sup>5cv</sup> control, 2 purmorphamine injected mdx<sup>5cv</sup>, and 5 BL6. Values are means +/- SD. Significance determined by one-way ANOVA, pairwise comparisons were performed using Kruskal-Wallis posthoc. * indicates Pval < .05. Lack of asterisk indicates lack of significance.
DMD is a devastating disease that despite intense research efforts currently has no treatment capable of significantly improving patient outcome. This is likely due to the fact that general mechanisms responsible for DMD have been identified but our understanding of the disease still remains incomplete. One of the remaining mysteries is that we still do not understand why muscle regeneration is insufficient in maintaining muscle mass in response to chronic damage associated with focal myofiber degeneration. In DMD myogenesis progressively fails resulting in a gradual replacement of muscle with fibrosis and fat tissue (Stephens, Duance et al. 1982; Hantai, Labat-Robert et al. 1985). Intensive research has been conducted since the late 1980s aimed at utilizing myogenic cells in cell based therapies in order to enhance the regenerative response and inhibit the loss of muscle tissue (Partridge, Morgan et al. 1989; Law, Bertorini et al. 1990; Law, Goodwin et al. 1992; Mendell, Kissel et al. 1995). However despite over two decades of research, cell based therapies remain clinically ineffective. These negative results may infer that the number of myogenic cells may not be the limiting factor responsible for the gradual decline
in muscle regeneration; alternatively, the disease may create a microenvironment that opposes the inherent regenerative response and/or the activity of transplanted cells in cell therapies.

**Beyond the satellite cell and into the side population**

It has been over 50 years since the discovery of the satellite cell, the cell that has been credited as the major cellular effector of muscle regeneration (Mauro 1961). Until recently, the satellite cell has been regarded as the only muscle precursor cell responsible for muscle regeneration. However, reports have accumulated over the past 15 years documenting alternative myogenic cell populations with the capacity to undergo myogenesis in culture and *in vivo* engraftment experiments (Gussoni, Soneoka et al. 1999; Qu-Petersen, Deasy et al. 2002; Dellavalle, Sampaolesi et al. 2007). Interestingly, in contrast to satellite cells which are localized in close apposition to an associated myofiber, these alternative myogenic cells have been identified in other locations including the interstitium, the endothelium, and the bone marrow (Ferrari, Cusella-De Angelis et al. 1998; Asakura, Seale et al. 2002; Dellavalle, Sampaolesi et al. 2007; Liadaki, Casar et al. 2012). Research has rapidly expanded in efforts to establish these alternative cell types for therapeutic purposes in muscular disorders such as DMD. Many of these cell types are attractive for therapeutics due to distinct advantages over satellite cells. One major advantage is the ability of non-satellite cells to migrate to multiple muscles following systemic delivery (Gussoni, Soneoka et al. 1999; Dellavalle, Sampaolesi et al. 2007). Additionally, many of
these cells maintain their myogenic activity for brief periods in vitro allowing for expansion (Dellavalle, Sampaolesi et al. 2007; Wei, Li et al. 2011; Pacak, Eddy et al. 2013); unlike satellite cells which rapidly lose their engraftment potential when cultured in vitro (Montarras, Morgan et al. 2005). Despite these advantages, however, therapeutic use of these cell types remains limited likely due to our limited understanding of the biology of these new cell types. For example, it is currently unclear how the regenerating muscle environment regulates their activity or how muscle pathology may alter their behavior. The research presented in this dissertation serves to investigate how the muscle environment may affect the myogenic potential of the one these alternative populations, the muscle SP cells. We have gathered interesting data showing that the myogenic activity of the SP cells is strongly influenced by the state of the tissue of origin. Significantly these findings provide further evidence for the dystrophic environment negatively affecting myogenesis. For example, the dystrophic environment is thought to negatively affect myogenic differentiation due to muscle fiber abnormalities (Morris and Raybould 1971; Schmalbruch 1984), and altered in vitro muscle differentiation of muscle satellite cells isolated from dystrophic muscle (Cheng, Chuang et al. 1996; Schuierer, Mann et al. 2005; Yablonka-Reuveni and Anderson 2006).

**Muscle SP cells: does the donor environment matter?**

Initial muscle SP cell experiments provided exciting results documenting that transplanted SP cells successfully engraft into myofibers following systemic
delivery into regenerating muscle (Gussoni, Soneoka et al. 1999). These initial results suggested that muscle SP cells may be a promising candidate for cell therapy in DMD by serving as a vector to reintroduce dystrophin expression. Autologous cell therapy using a patient’s own cells would be advantageous by avoiding potential rejection by the immune system and not requiring immunosuppressive medications. However, follow up studies transplanting \( mdx^{5cv} \) SP cells back into \( mdx^{5cv} \) recipient mice resulted in a low level of engraftment in dystrophic muscle (Bachrach, Li et al. 2004). These negative results shed doubt on the potential use of autologous derived SP cells for therapy. Additionally, SP cells were found to be dependent on C2C12 co-culture in order to undergo myogenesis \textit{in vitro}, making the study of SP cell biology difficult and preventing their clinical development (Asakura, Seale et al. 2002).

We present here in chapter II a novel culture system capable of supporting SP cells in isolation without the requirement for co-culture. This system provides a tool to analyze SP cells in isolation and determine their spontaneous differentiation and lineage choices without the influence of other cell types. We have utilized the newly developed \textit{in vitro} system to culture SP cells and directly assay the myogenic capacity of cells isolated from different muscle environments. One major finding in chapter II is the observation that muscle SP cells isolated from both acutely damaged and dystrophic muscle fail to undergo myogenisis, differentiating into both fibroblasts and adipocytes. Taken together these results caution against the use of dystrophic SP cells for autologous cell therapies due to the risk of introducing fibroblasts that may potentially exacerbate
or accelerate fibrosis in dystrophic muscles. While many of the new non-satellite cell populations such as MDSC’s, pericytes, and PIC cells are myogenic in vivo, they are not restricted to the myogenic lineage and therefore contain the inherent risk of fibroblast formation in dystrophic muscle (Li and Huard 2002; Besson, Smeriglio et al. 2011). For example studies transplanting MDSC’s into lacerated muscle result in myofibroblast differentiation warranting future investigation to confirm that dystrophic MDSC’s do not undergo fibroblast differentiation in vivo (Li and Huard 2002). These results in combination with our findings presented here encourage investigation into the effects of the dystrophic environment on other non-satellite myogenic cells and validation that cells transplanted into dystrophic mice do not give rise to fibroblast or adipocyte cell types in vivo.

The Pdgfra SP cell

The over activity of the receptor tyrosine kinase, Pdgfra, has the ability to induce fibrosis in multiple organ systems including the skeletal muscle (Olson and Soriano 2009). DMD and mdx muscles contain significantly elevated levels of Pdgf ligands, suggesting this receptor may play a mechanistic role in fibrosis development (Tidball, Spencer et al. 1992; Zhao, Haginoya et al. 2003). Cells within skeletal muscle that express the Pdgfra receptor and respond to Pdgf ligands have recently been characterized as a non-myogenic population of cells serving as progenitors of fibroblasts and adipocytes (Joe, Yi et al. 2010; Uezumi, Ito et al. 2011). Surprisingly, our studies in chapter II reveal a population of cells expressing Pdgfra within the SP compartment capable of undergoing
myogenesis. Previous studies involving Pdgfrα cells likely did not include these cells in their analysis due to their method of eliminating cellular debris by excluding events exhibiting low Hoechst fluorescence, a characteristic of the SP cells (Joe, Yi et al. 2010). Significantly these data demonstrate for the first time that Pdgfrα may not be a unanimously fibrotic marker thus opening the door to the possibility that there may be populations of cells expressing Pdgfrα that may contribute to myogenesis. While these myogenic Pdgfrα SP cells make up a very small proportion of cells within the mouse skeletal muscle mononuclear cell pool it should not be assumed that they are not functionally important for muscle regeneration. For example, a very rare population of satellite SP cells expressing syndecan 4 has recently been reported to significantly contribute to the satellite cell pool, in fact superior to traditionally characterized satellite cells, and participate significantly in regenerating muscle fibers following acute injury (Tanaka, Hall et al. 2009). To address whether Pdgfrα cells contribute significantly to myogenesis future studies may utilize a lineage tracing mouse model using a conditionally activated Pdgfrα promoter driven Cre recombinase reporter mouse model (Tripathi, Rivers et al. 2010). The use of a conditional model like this would allow us to unambiguously assess the contribution of Pdgfrα cells to myogenesis in both the embryonic and adult regeneration programs. Additionally it will be useful to utilize this model in both regenerating wild type and dystrophic models in order to determine if the dystrophic environment potentially reduces the myogenesis of Pdgfrα cells at multiple stages of the disease. Recently, imatinib has been tested as an anti-fibrotic in
*mdx* mice acting by inhibiting PdgfRα function (Huang, Zhao et al. 2009). This genetic model will provide an additional tool to determine whether PdgfRα inhibition may antagonize muscle regeneration by negatively influencing PdgfRα myogenesis thus limiting the clinical efficacy of drugs inhibiting PdgfRα.

**Hedgehog signaling restores SP myogenesis**

Our results in chapter III are significant because they illustrate that myogenesis of dystrophic SP cells is not irreversibly lost and in fact may be rescued by modulation of signaling pathways. Additionally these findings open the door to new research efforts aimed at investigating the function of these pathways in SP cell biology but also encourage further study into additional biological mechanisms and pathways that may regulate SP cell behavior. These findings in chapter IV have implications for future therapeutic development of SP cells in DMD. For example, a potential reason for *mdx<sup>5cv</sup>* SP cells engrafting into dystrophic muscle at low efficiency may be due to negative effects mediated by the dystrophic microenvironment (Bachrach, Li et al. 2004). Because we have shown that *in vivo* Hh stimulation partially restores downstream *in vitro* myogenesis of the SP cells, Hh stimulation *in vivo* may be also be able to improve the efficiency of transplanted *mdx<sup>5cv</sup>* SP cells contributing to muscle regeneration in dystrophic muscle. Additionally, Hh stimulation *in vitro* may be a method to maintain or increase myogenesis of isolated *mdx<sup>5cv</sup>* SP cells; thus allowing for *ex vivo* manipulation and the potential use of SP cells as a gene
A recent study has reported a novel culture system utilizing bioreactor culture of SP cells capable of expanding and maintaining a significant fraction of undifferentiated SP cells (Pacak, Eddy et al. 2013). Stimulating Hh signaling in this system would allow us to test the downstream gene expression changes occurring in SP cells responding to Hh activation while remaining in an undifferentiated state. Additionally this new culture system may allow for increased expansion of SP cells for future SP studies in vivo; in which case it would be interesting to test whether Hh stimulation of SP cells during expansion improves downstream engraftment into recipient muscle.

**Hh signaling in muscle regeneration: implications for DMD**

Until recently it has been assumed that Hh signaling primarily functions during development while remaining relatively inactive in the adult. While Hh has not previously been implicated in muscle disorders, recent evidence shows that Hh signaling functions in adult muscle biology by impacting both proliferation of myogenic cells and fibrosis during muscle regeneration (Straface, Aprahamian et al. 2009). Additionally, inhibition of Hh in these experiments reveal significant secondary effects resulting in reduced expression of IGF-1 and VEGF, both factors that function significantly during muscle regeneration and have shown benefits in mdx pathology when overexpressed (Shavlakadze, White et al. 2004; Messina, Mazzeo et al. 2007; Straface, Aprahamian et al. 2009). Additionally, studies comparing the Hh response during acute injury over multiple ages have
shown that aged mice do not exhibit significant Hh induction compared to young mice suggesting that dampened Hh signaling may play a role in the decreased regenerative potential of aged muscle (Piccioni, Gaetani et al. 2013). In fact, overexpression of Hh signaling using a plasmid significantly increased the number of activated satellite cells at the site of injury, decreased the fibrotic response, and significantly increased levels of IGF-1 and VEGF in aged mice (Piccioni, Gaetani et al. 2013). Interestingly due to the pleiotropic functions of Hh signaling in muscle regeneration, these studies suggest that a potential defect in Hh signaling may have a significant impact on muscle regeneration by affecting multiple processes including satellite cell proliferation and fibrosis. Considering these results in combination with the fact that dystrophic muscle undergoes a chronic level of regeneration, it is reasonable to hypothesize that Hh signaling is induced in dystrophic muscle. However, our studies presented in chapter III show the contrary; in the limb muscle Hh signaling is not significantly changed and in the diaphragm muscle Hh is significantly down-regulated. These results suggest that this failure to induce Hh signaling may represent a new disease mechanism in DMD pathology and may be doing so by negatively influencing multiple aspects of the muscle regeneration program. By the same token Hh stimulation may represent a new treatment strategy that may be useful in lessening disease pathology by improving satellite cell activity and reducing fibrosis.

Hh signaling clearly induces the proliferation of satellite cells in multiple studies but there remains significant controversy regarding the effect of Hh
signaling on muscle cell differentiation. In order for any potential Hh therapy to be successful, Hh signaling must not significantly inhibit the differentiation process. Interestingly there have been conflicting reports, including pro-differentiation effects via stimulation of the MAPK/AKT pathway in myogenic cells (Elia, Madhala et al. 2007) but anti-differentiation effects observed by an inhibitory effect of Hh signaling on myotube formation in vitro (Koleva, Kappler et al. 2005). Our results in chapter IV support a pro-proliferation/pro-differentiation model of Hh activity in myogenic cells. To review, stimulation in vivo with SAG up regulated Pax7 and Myf5 normally associated with satellite cells, and up regulated myogenin associated with differentiating myoblasts. Conversely, inhibition of Hh signaling via cyclopamine resulted in dramatic down-regulation of these same factors. These results are in agreement with a recent study in P19 cells where myogenin expression was found to be positively regulated by Hh signaling (Voronova, Coyne et al. 2013). Due to the fact that Hh signaling positively influences myogenin expression, which normally functions to promote terminal differentiation, these results suggest that Hh signaling supports myogenic differentiation. Additional evidence generated through significant overexpression of Hh in aged regenerating muscle shows a significant increase in the number of muscle fibers containing a central nucleus suggesting that Hh promotes the differentiation process (Piccioni, Gaetani et al. 2013). In combination these findings support Hh stimulation as a method to increase expansion of myogenic progenitor cells and enhance regeneration in the mdx mouse.
While previous in vitro studies have shown significant effects of Hh stimulation on myogenic cell proliferation and differentiation, our studies currently do not show an effect on these parameters in the \( mdx^{5cv} \) diaphragm. An important consideration is that Hh is a morphogen factor meaning that instead of functioning like an on/off switch, it elicits unique responses relating to the degree of activation (Gurdon and Bourillot 2001; Casali and Struhl 2004; Su, Jones et al. 2007). The most classic example of this is the role that Hh plays in patterning the somite in which case differential responses regulate cell fate determination (Borycki, Mendham et al. 1998). In skeletal muscle there has been no study comparing the outcomes resulting from multiple levels of Hh stimulation during post-natal regeneration. Considering this, an explanation for our findings may be that Hh signaling induction provided by purmorphamine is simply not strong enough to result in an obvious effect on muscle regeneration parameters \textit{in vivo}. Recent studies involving overexpression via Shh plasmid in aged muscle resulted in up-regulation of Gli1 expression of about 4 fold over control uninjected muscles (Piccioni, Gaetani et al. 2013). This level of overexpression of Hh showed significant benefits with regard to number of activated satellite cells and significantly boosted levels of IGF-1 and VEGF. Considering that purmorphamine is only capable of restoring Hh signaling to wild type levels, we are likely not obtaining the maximal effect of Hh stimulation and it is possible that this level of induction is not significantly increasing IGF-1 and VEGF levels. Therefore it will be very important to perform additional treatments with SAG in order to address whether higher levels of Hh induction result in a more obvious benefit in the
$mdx^{5cv}$ diaphragm and potentially increase expression of IGF-1 and VEGF. Overexpression of both of these factors has previously been shown to improve disease pathology in the mdx mouse by reducing myofiber necrosis and enhancing muscle regeneration, respectively (Shavlakadze, White et al. 2004; Messina, Mazzeo et al. 2007; Verma, Asakura et al. 2010). By determining the optimal induction of Hh signaling to increase IGF-1 and VEGF levels Hh stimulation may be able to improve pathology from multiple angles resulting in a synergistic effect that may be greater than studies overexpressing either IGF-1 or VEGF alone. Completion of these studies will not only determine whether increased levels of Hh induction convey superior benefits in pathology but also have the potential to enhance our understanding of the functions of Hh signaling in muscle biology.

While Hh clearly impacts myogenesis in *in vitro* systems, the influence of the pathway on myogenesis may be more complex when considered in the context of dystrophic muscle. Our results presented here clearly show that dystrophic muscle fails to induce Hh signaling and encourage future study to determine the mechanism responsible for this finding. This may be due to multiple possibilities including alterations in Hh expression, changes in the post-translational modifications of Hh ligand which may affect ligand range of action, or other signaling pathways activated in the dystrophic environment that may cross-talk and inhibit Hh action. First, while studies in regenerating muscle (Straface, Aprahamian et al. 2009) have shown that Hh is expressed from
muscle fibers it will be important to characterize the location and amount of Hh ligand in dystrophic muscle to determine if a change in ligand expression or localization is responsible for the decrease in Hh signaling. Secondly, cholesterol modification is a mechanism that functions to regulate the range of activity of Hh ligand (Lewis, Dunn et al. 2001; Jeong and McMahon 2002), a process which may be altered in response to dystrophic disease. Thirdly, multiple pathways including TGFβ/BMP and Wnt pathways have been shown to crosstalk with Hh signaling which may in turn inhibit the pathway’s activation (Mullor, Dahmane et al. 2001; Watt 2004; Katoh and Katoh 2009; Javelaud, Alexaki et al. 2011). While Hh inhibition clearly impacts myogenesis parameters during acute injury, future studies should investigate whether increasing Hh signaling during acute muscle injury enhances muscle regeneration either by boosting satellite cell numbers or accelerating the formation of new myofibers. These experiments provide an opportunity to determine if the lack of a clear regenerative effect in our purmorphamine treatments is due to Hh lacking the ability to enhance muscle regeneration in vivo or if the dystrophic environment is counteracting such potentially positive effects; this may in turn reveal new mechanisms in dystrophic pathology. Overall our findings encourage further study of Hh signaling in adult muscle regeneration and dystrophic pathology that in turn has the potential to enhance our understanding of basic Hh biology but also help us to gain insight into alterations in Hh pathway activity that may apply not only to dystrophic muscle but also to other disorders where Hh signaling may be affected.
Multiple attempts have been made to inhibit fibrosis via inhibitors to fibrotic pathways TGFβ and PDGF (Andreetta, Bernasconi et al. 2006; Huang, Zhao et al. 2009), but no treatment to date has been capable of completely preventing fibrosis accumulation in mdx diaphragm. While blocking TGFβ early in mdx pathology significantly reduces fibrosis deposition near wild type levels in young mice, long term studies in older mdx mice using the angiotensin type II antagonist Losartan previously shown to inhibit TGFβ signaling, show that TGFβ inhibition is only partially effective as the disease progresses resulting in a significant amount of remaining fibrosis (Andreetta, Bernasconi et al. 2006; Cohn, van Erp et al. 2007). Because of this there is a critical need to identify further mechanisms and pathways that may contribute to fibrotic pathogenesis. In chapter IV we show that stimulation of the Hh pathway in mdx5cv mice is effective at reducing Collagen 1 deposition in the dystrophic diaphragm muscle. In order to determine whether Hh stimulation in our studies restores Collagen 1 deposition to wild state levels we have begun evaluating collagen I deposition in wild type diaphragms. Our preliminary studies show however that there still remains a significant amount of collagen I in mdx5cv diaphragms compared to wild type. Hh stimulation therefore does not appear to be as effective as immunomodulation of TGFβ using a blocking antibody in which case fibrosis is reduced to a level observed in wild type mice (Andreetta, Bernasconi et al. 2006). It must be considered that our results show a trend towards reduced Collagen 1 deposition with a relatively weak level of Hh induction compared to other in vivo studies. Furthermore, our findings encourage future studies with higher levels of Hh
stimulation via SAG that may be able to elicit a more dramatic anti-fibrotic response. Additionally, while studies blocking TGFβ result in down-regulation of collagen gene expression, our studies show that Hh stimulation reduces collagen protein deposition while not impacting mRNA expression of either TGFβ or Collagen 1. These findings are significant because they suggest that Hh signaling may be reducing collagen deposition by an alternative mechanism not targeted by current anti-fibrotic strategies. Further investigation of this process may reveal novel mechanisms and methods for inhibiting fibrosis that may be exploited and utilized to complement TGFβ antagonism in order to develop superior anti-fibrotic strategies.

In summary, our studies utilizing purmorphamine are promising revealing that Hh stimulation in the \textit{mdx}^{5cv} mouse is capable of reducing Collagen 1 deposition, improving post-exercise fatigue, and reducing the number of centrally nucleated fibers. These findings reveal for the first time that Hh signaling impacts the disease process in the \textit{mdx} mouse and have provided potential new mechanisms in disease pathology. However, these studies show a relatively mild improvement in \textit{mdx} pathology as there is a significant amount of remaining Collagen 1 remaining in the diaphragm muscle, fiber size distribution is largely unchanged, and muscle fiber degeneration and regeneration are not significantly improved. While these results show that purmorphamine is likely not suitable for future therapeutic development, taking into account our studies show improvements in pathology with only a weak induction of Hh signaling via
purmorphamine these studies strongly encourage future studies of Hh stimulation using a stronger method of Hh activation in the $mdx^{5cv}$ mouse.

**Conclusion**

The studies presented in this dissertation have increased our understanding in muscle SP biology and in turn have enhanced our understanding of disease processes involved in DMD. Our studies reveal that the dystrophic microenvironment negatively influences the myogenesis of the muscle side population cells in part due to a reduction in Hh signaling. This finding led to the discovery that dystrophic muscle fails to significantly induce Hh signaling and motivated the evaluation of Hh stimulation as a potential therapeutic in the $mdx^{5cv}$ mouse. The results from these studies are exciting and provide the groundwork supporting future investigation for Hh stimulation as a potential future therapeutic in DMD.
References


