Development of approaches to improve the regenerative potential of muscle stem cells

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Development of Approaches to Improve the Regenerative Potential of Muscle Stem Cells

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Stem cell therapy is a promising treatment for diseases such as Duchenne muscular dystrophy (DMD) and ischemic heart failure. However, low survival and differentiation of transplanted cells hinders therapy. In this study, we examined ways to enhance the effectiveness of muscle cells for cardiomyoplasty by increasing antioxidant levels, explored the role of vascular endothelial growth factor (VEGF) in mechanical stimulation pre-treatment and characterized muscle derived stem cells (MDSCs) from normal and dystrophic mice.

First we demonstrated that increasing antioxidant levels positively correlated with the early survival of myoblasts after implantation into infarcted hearts, but did not result in long term functional benefits, indicating that early survival does not necessarily correlate with long term regeneration and repair.

Next we aimed to determine the effect of VEGF on mechanically stimulated MDSCs transplanted into dystrophic muscle. MDSCs were transduced with vectors carrying the *LacZ* reporter gene (lacZ-MDSCs), the soluble VEGF receptor *Flt1* (sFlt1-MDSCs) or short hairpin RNA targeting VEGF messenger RNA (shRNA_VEGF MDSCs). They were subjected to 24 hours of cyclic strain and injected into the gastrocnemius muscles of dystrophic *mdx*/SCID mice. After 2 weeks, there was an increase in angiogenesis in muscles transplanted with mechanically stimulated lacZ-MDSCs compared to non-stimulated lacZ-MDSCs and sFlt1-MDSCs. Dystrophin positive myofiber regeneration and in vitro myotube differentiation were significantly lower in the shRNA_VEGF-MDSC group compared to the lacZ-MDSC and sFlt1-MDSC groups. Thus, the beneficial effects of mechanical stimulation on MDSC mediated muscle repair were lost by inhibiting VEGF.

Finally, we aimed to compare wild-type (wt) MDSCs with MDSCs obtained from *mdx* and dystrophin/utrophin double knock out (DKO) mice, which are models of muscular

dystrophy. We demonstrated that wt and *mdx* MDSCs did not have differences in proliferation, differentiation, or VEGF secretion. We compared DKO homozygous MDSCs and DKO heterozygous MDSCs and found that DKO homo MDSCs had decreased proliferation, differentiation, and cell survival capabilities compared to DKO het MDSCs. Finally, we pre-treated DKO MDSCs with mechanical stimulation and increased their proliferation rates. In conclusion, efforts to optimize cell therapy are necessary to improve transplantation outcomes for both ischemic cardiac repair and muscular diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NON-STANDARD ABBREVIATION AND ACRONYMS

ANOVA, analysis of variance

CHF, Congestive Heart Failure

DEM, Diethyl Maleate

DMD, Duchenne Muscular Dystrophy

DMEM, Dulbecco's Modified Eagle's Medium

FS, Fractional Shortening

FAC, fractional area change

FsMHC, Fast Skeletal Myosin Heavy Chain

GSH, Glutathione

H3, Anti-Phospho-s10 Histone

HPF, high power fields

LV, Left Ventricle

MDSCs, Muscle-Derived Stem Cells

nLacZ, Nuclear-Localized LacZ

MI, Myocardial Infarction

NAC, N-acetylcysteine

PI, Propidium Iodide

PBS, phosphate-buffered saline

PM, Proliferation Medium

SE, standard error

SOD, Superoxide Dismutase

VEGF, Vascular Endothelial Growth Factor

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1.0 INTRODUCTION

1.1 SIGNIFICANCE

Despite many years of hard work, stem cell therapy is as of yet insufficient to completely regenerate damaged tissue. Un-modified allogenic or autologous stem cells have not yet met the overwhelming clinical need. Methods to modify stem cells in order to increase their potency, proliferation, paracrine profile, survival, and ability to recruit endogenous cells to the site of injury and disease are necessary to achieve the objective of complete regeneration and repair of damaged tissue and organs, and especially to improve the quality of life of the people who need these therapies.

1.2 DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is an inherited neuromuscular disorder which affects 1:3500 live male births (Towbin et al. 2002; Deconinck et al. 2007). In 1987, it was discovered that DMD patients are missing the muscular structural protein dystrophin, which attaches the sarcolemma to the extracellular matrix (Hoffman et al. 1987). This causes a lack of stabilization which results in rips in the cellular membrane, and an influx of calcium and

proteases which leads to muscle fiber necrosis. Initially, there is a balance between cell death and repair, however after approximately 5 years muscle regeneration is replaced by fibrosis and fatty tissue infiltration. The end result of DMD is muscle wasting in both skeletal and cardiac tissue (Deconinck et al. 2007; Wallace et al. 2009). Patients with DMD develop dilated cardiomyopathy as early as 9 years of age and the incidence is almost 100% by the time they reach their 30's; cause of death is usually respiratory or cardiac failure in the third decade (McNally 2007).

The *mdx* mouse is the most well characterized and well established murine model of DMD. The mouse model and human disease share many similarities, including initially normal cardiac function which later evolves to dilated cardiomyopathy with hypertrophy and necrosis (Quinlan et al. 2004). However, the *mdx* mice do not develop cardiomyopathy until well into their second year of life, which does not mimic the human condition in which patients begin to develop heart failure as early as their first decade. Other limitations of the model include diffuse (rather than regional) dyskinesia and fibrosis and a lower degree of fibrotic tissue overall in the *mdx* mouse (Quinlan et al. 2004). Therefore, it is necessary to make use of other models such as a more recent mouse model of DMD in which the utrophin protein is knocked out of the *mdx* mouse by targeted mutagenesis (Grady et al. 1997; Grady et al. 1997), and as such both dystrophin and utrophin are absent (DKO). Utrophin is a homologue of dystrophin which is distributed throughout the sarcolemma in fetal and regenerating muscle, but is down-regulated at birth and restricted to the myotendinous and neuromuscular junctions in normal adult muscle (Pons et al. 1991). Dystrophin on the other hand, is distributed throughout the sarcolemma (Ervasti 2007). Utrophin and dystrophin bind the same complement of proteins and have significant homology, therefore utrophin may be capable of compensating for dystrophin

deficiency in the *mdx* mouse model and in fact DKO mice have more severe symptoms and die within three months unlike *mdx* mice which have near normal life spans (Janssen et al. 2005). As dystrophin is also up-regulated in humans, (Mizuno et al. 1993), it is unclear why there is such a difference between *mdx* mice and human patients, but it is possible that the mouse has better regenerative capacities than humans (Deconinck et al. 2007; Sacco et al. 2010), or that due to the small fiber size of *mdx* mice compared to humans, less utrophin is needed to stabilize membrane integrity (Grady et al. 1997).

There are other murine models of DMD which exhibit symptoms closer to the human disease, all of which are achieved by knocking out one other protein besides dystrophin on an *mdx* background. They include *mdx*:MyoD-/- (Megeney et al. 1999), *mdx*:B1integrin (Elsherif et al. 2008), and *mdx* mice lacking telomerase activity (Sacco et al. 2010). Furthermore, the genetic background of *mdx* mice affects their phenotype. *Mdx* mice on a DBA/2 background had fewer myofibers, increased fat and fibrosis accumulation, and insufficient regeneration of myofibers compared to their BL/10 counterparts (Fukada et al. 2010).

An interesting commonality these models share is an effect on the stem cell compartment. Sacco et al. elegantly elucidated that when the telomeres of satellite cells are shortened and these progenitor cells are exhausted at earlier time points, the murine disease progression follows the human condition much more closely (Sacco et al. 2010). The *mdx*:MyoD-/- mice displayed an increased severity of skeletal myopathy, leading to premature death at 1 year of age. Interestingly, the muscle of these mice contained a 2 fold increase in satellite cells undergoing self-renewal rather than proliferation and differentiation into *de-novo* muscle fibers (Megeney et al. 1999). *Mdx* mice on a DBA/2 background displayed a more severe pathology than their BL/10 counterparts as well as lower self-renewal efficiency of satellite cells (Fukada et al. 2010).

The most common treatment for DMD is steroids, which prolongs ambulation and improves upper limb function as well as increases muscle mass without replacing dystrophin (Balaban et al. 2005). Interestingly, steroid therapy is associated with greater numbers of satellite cells due to increased proliferation (Hussein et al. 2010). Taken together, these data all point to a role for stem cell dysfunction in DMD.

Current research into DMD treatment focuses largely on gene therapy with the aim of replacing dystrophin. However, gene replacement may not be the best option to treat the heart owing to the limited turnover of cells and thus very slow replacement of deficient cells with those containing the dystrophin protein. New research has shown that from birth to death only about half of the heart is regenerated with new cells (Bergmann et al. 2009). In addition, even with gene therapy in the muscle there has been no evidence of diffuse, persistent, non-toxic production of dystrophin (Deconinck et al. 2007). Other treatments for muscular dystrophy are cell therapy and drug therapy. Options for drug therapy include a chemical drug that up-regulates utrophin (Tinsley et al. 1998) or nitric oxide (NO) releasing anti-inflammatory agents, which have been shown to slow down the phenotype in *mdx* mice (Cossu et al. 2007). These studies demonstrate that it is feasible to treat muscular dystrophy without replacing dystrophin and that targeting the stem cell compartment may be an efficient and effective treatment.

1.3 STEM CELLS FOR CARDIAC AND SKELETAL REPAIR

Despite major advances in our understanding of the pathology of the disease and improved therapeutic options, heart disease is a serious problem. DMD patients die of cardiac or respiratory failure in their 3rd decade (Deconinck et al. 2007), and it is the major cause of death

in both industrialized and emerging countries. In fact, in America alone, nearly 2300 people die of cardiovascular disease each day, an average of 1 death every 38 seconds. Cardiovascular disease claims more lives each year than cancer, chronic lower respiratory disease, and accidents combined (Lloyd-Jones et al. 2010). Ischemic heart disease is characterized by reduced blood supply to the heart caused by blockage of the coronary arteries, otherwise known as myocardial infarction (MI). This leads to cardiomyocyte death, high levels of fibrosis, inflammation, and a resulting decrease in contractile strength. Characteristics of untreated MI include left ventricular wall thinning and dilation which are associated with reduced pumping of the heart (Towbin et al. 2002). Currently, the only therapeutic option for end stage ischemic MI is heart transplantation. Heart transplantation, however, has several hurdles, including limited availability of organs and the need for life-long immunosuppressive therapies.

Damage to the heart is such a critical problem because native heart muscle does not restore damaged tissue on its own to a level that will alleviate major damage. Instead, ischemic areas are replaced by non-contractile fibrotic tissue. Recent studies indicate the existence of a small pool of resident cardiac progenitor cells but they are not sufficient to re-grow a significant amount of human cardiac muscle (Bergmann et al. 2009). Therefore, tissue engineering methods are necessary to re-build damaged tissue. Two potential ways to accomplish this are either through recruitment of endogenous progenitor cells or delivery of stem cells to the injured area.

Stem cells are characterized by their ability to self-renew and their capacity for long term proliferation. They can be found in most vascularized tissue including muscle, adipose, bone marrow and placenta (Crisan et al. 2008). Options for cell therapy include skeletal myoblasts, bone marrow derived stem cells, endothelial progenitor cells, adipose stromal tissue cells, mesenchymal cells, embryonic stem cells, cardiac stem cells, umbilical cord blood cells, smooth

muscle cells, hematopoietic stem cells, induced pluripotent stem cells, and muscle derived stem cells (Caplice et al. 2005; Laflamme et al. 2005; Zheng et al. 2007; Segers et al. 2008). Cell therapy aids repair through a variety of mechanisms including decreasing fibrosis, improving angiogenesis, activating endogenous stem cells, preventing adverse remodeling, increasing survival, decreasing apoptosis and necrosis of host cells, and reducing inflammation. However, although there is much speculation, the mechanism of stem cell mediated repair is largely unknown. To date, however, there is increasing evidence for a paracrine mechanism due to the low percentage of cells that engraft into damaged tissue (Zhang et al. 2001; Laflamme et al. 2005).

One important effect of stem cell therapy is the facility to increase vascularization to ischemic areas of the heart. Angiogenic factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin like growth factor 1 (IGF-1) and nitric oxide (NO). These factors influence the survival, proliferation and migration of endothelial cells and vascular support cells such as smooth muscle cells (Lamalice et al. 2007).

Another paracrine effect observed following cell therapy is a decrease in harmful remodeling (Jain et al. 2001). This may be due to pro-survival effects on the remaining tissue as well as the secretion of anti-fibrotic factors such as matrix metallo-proteases (MMPs) and the inhibition of fibroblast proliferation (Rota et al. 2008).

A final consideration to take into account regarding cellular therapy is survival of the transplanted cells. Stem cells have high resistance to oxidative stress (Ogasawara et al. 2009). This resistance to stress may help them survive in the harsh environment following ischemic injury.

Currently, many different types of stem cells have been used for cell therapy after MI with only moderate success. Hurdles that remain include minimal engraftment into tissue, cells that do not couple with the surrounding cardiac tissue, limited survival of transplanted cells, and insufficient delivery methods. For these reasons cell enhancement strategies need to be employed in order to realize the full restorative potential of stem cells for cardiomyoplasty. Stem cells have great potential to regenerate cardiac tissue, decrease harmful remodeling, and increase angiogenesis to the affected area; this potential, however, still needs to be properly harnessed and optimized to yield the best clinical results. We have started to look at ways to do this using a population of cells called muscle derived stem cells.

1.4 MUSCLE DERIVED STEM CELLS

Muscle derived stem cells (MDSCs) are progenitor cells isolated from skeletal muscle. Muscle is physically and enzymatically digested and then plated onto collagen-coated flasks. Two hours later the floating cells are transferred to a fresh collagen coated flask. This is then repeated every 24 hours for four days (Shown in Figure 1). This process results in cell fractions isolated according to their adhesion characteristics (Qu-Petersen et al. 2002; Gharaibeh et al. 2008).

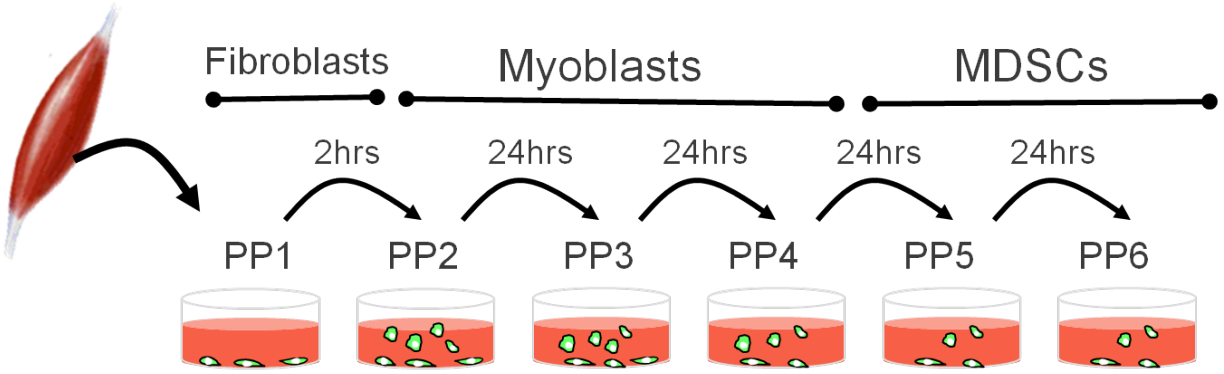

Figure 1: Pre-plate technique

This technique was originally developed to separate myoblasts from non-myogenic cells such as fibroblasts (Rando et al. 1994). However, our group has taken it a few steps further to separate early plating (EP) cells from late plating (LP) cells. The late plating fractions contains cells that have stem cell characteristics including long term proliferation in vivo, self-renewal capabilities, and multi-lineage differentiation (Qu-Petersen et al. 2002; Peng et al. 2004; Oshima et al. 2005; Kuroda et al. 2006; Lavasani et al. 2006; Matsumoto et al. 2008; Matsumoto et al. 2009) MDSCs also have potential in a variety of in vivo applications including muscle repair (both skeletal and cardiac), bone, cartilage, and peripheral nerve (Lee et al. 2000; Qu-Petersen et al. 2002). This cell population is separate from that of myoblasts in that myoblasts are more committed to a muscle cell lineage than MDSCs and they express late myogenic cell markers such as paired box protein 7 (pax7) while MDSCs express stem cell markers such as CD34 and stem cell antigen 1 (sca-1). However, it is important to note that while MDSCs have been characterized for their marker profiles they are a heterogeneous population derived by their adhesion characteristics. This population of cells is not isolated or defined by surface protein expression. The ontogeny of MDSCs is unknown but it is postulated that they may derive from vascular associated cells

(Zheng et al. 2007; Crisan et al. 2008). In the following chapters we will explore previous research with MDSCs for cardiac repair, which will lead up to the aims of this thesis.

1.5 ENGRAFTMENT: MDSCS REGENERATE DYSTROPHIC HEART MUSCLE

To explore the regenerative potential of MDSCs in the heart a model of muscular dystrophy was employed. The *mdx* mouse is a naturally occurring mutant which lacks the structural protein dystrophin and is widely used as a model for DMD. However, unlike human patients the murine model develops a very mild cadiomyopathy. Therefore this study was primarily designed to examine cell fate and engraftment; it was found that MDSCs isolated from a wild type mouse engrafted into the dystrophic heart of an *mdx*/SCID mouse and formed dystrophin positive fibers. These were identified up to 12 weeks post-implantation. There were two noteworthy findings, one is that a small number of MDSCs will contribute to the vasculature of the surrounding tissue and the second is that a small number on the edge of the engraftment area will fuse with resident cardiac cells. However, most of the MDSCs injected into the *mdx*/SCID heart formed myotubes and fused with each other (Payne et al. 2005). One reason that the number of MDSCs fusing with resident tissue is so small may be that scar tissue forms at the border of the graft-host myocardium interface. This barrier of scar tissue may have blocked the migration of transplanted cells and decrease their effectiveness as fibrosis is known to be a major impediment to cellular transplantation (Wynn 2008). Methods have been employed to reduce fibrosis by degrading collagen and therefore increasing cellular migration (Li et al. 2005). It is also possible that some of the fibrosis comes from the cell delivery method of injection. For this reason, other cell delivery methods are being investigated that are less-invasive, such as cell sheets, patches, or

delivery through the circulatory system, in which it is necessary that the cells hone to the site of injury.

1.6 MDSCS IMPROVE CARDIAC FUNCTION AFTER ISCHEMIC INJURY

Numerous groups have shown that skeletal muscle derived cells (satellite cells and myoblasts) can engraft into cardiac tissue and improve cardiac function (Taylor et al. 1998; Menasche 2008). Skeletal muscle derived cells are particularly attractive as muscle can be harvested from patients, expanded, and transplanted back where needed. However, after myoblast transplantation most cells die rapidly and only a small sub-population plays a role in muscle regeneration. This suggests a heterogeneous population of cells, some that are able to survive at a higher rate and in harsher conditions than others. For this reason alternative methods of isolating stem-like cells from muscle tissue have been examined, including the pre-plate technique to isolate MDSCs (Qu-Petersen et al. 2002; Gharaibeh et al. 2008).

MDSCs and myoblasts were compared as a therapy after acute coronary ligation, a murine model of myocardial infarction. MDSCs were found to generate larger grafts compared to myoblasts after implantation into the heart for up to 12 weeks following injection. This larger engraftment correlated with decreased remodeling and superior cardiac function. After 12 weeks the end diastolic diameter (EDD) of the left ventricle was significantly higher in the myoblast treated group compared to the MDSC treated group, indicating that treatment with MDSCs delayed dilation of the ventricular cavity typically associated with ischemic injury. Fractional area change (FAC) was also compared between the groups and the MDSC treated group had a higher FAC, indicating that treatment with MDSCs resulted in better heart function than

treatment with myoblasts. In this study MDSCs prevented harmful remodeling and improved cardiac function more effectively than myoblasts (Oshima et al. 2005).

Most of the MDSCs and myoblasts differentiated toward a skeletal muscle lineage. However, the overall amount of engraftment was small in both cell types. Similar to an earlier study, a few of the cells at the border of the injection area expressed cardiac markers. There was a significantly lower number of myoblasts that acquired a cardiac phenotype compared to MDSCs. However, 50% of myoblasts with a cardiac phenotype also expressed connexin 43, indicating the possibility for electrically coupling between the graft and the host.

One of the largest differences seen between the two groups was the number of *lacZ* positive cells found 2, 6 and 12 weeks after treatment. There were significantly more MDSCs than myoblasts surviving after therapy. This indicated that cell survival may be a key mediator to success in cell therapy.

1.7 CELL SURVIVAL: RESISTANCE TO OXIDATIVE STRESS

Survival is an integral component of cell therapy mediated repair, whether it is a reduction in death of the native tissue or endurance of injected cells. There are numerous methods of studying survival, such as differences in survival between cell types, induction of survival signals in the cells themselves as well as the environment, the cell delivery method, or resistance to stress (Penn et al. 2008). It has been determined that MDSCs repair the heart after ischemic injury in a more effective manner than myoblasts, with a greater number of MDSCs surviving after 12 weeks (Oshima et al. 2005). However, the mechanism for this has not been completely elucidated. It may have to do with the fact that MDSCs secrete different paracrine factors than

myoblasts, that they can induce angiogenesis in a more effective manner, or that they simply survive the unfavorable environment after an infarction more effectively than myoblasts. The environment after ischemic injury is harsh and not conductive to cell survival. This may be a main reason why there is an universally low survival rate among implanted cells following this type of injury (Segers et al. 2008).

In order to explore the mechanism behind which MDSCs repair the heart, apoptosis was assayed under oxidative stress conditions in vitro, and at 250 and 500 μM of H_2O_2 exposure there were fewer apoptotic MDSCs than myoblasts (Oshima et al. 2005). This indicates that MDSCs are more resistant to oxidative stress induced apoptosis than myoblasts. Inflammatory stress-induced cell death was also examined, and after TNF-alpha stimulation for 18 hours there was significantly more death in myoblasts compared to MDSCs, highlighting their unique survival advantage over myoblasts (Urish et al. 2009). This may be accounted for by the observation that MDSCs displayed increased levels of the antioxidant glutathione (GSH) and super-oxide dismutase (SOD) as well as decreased levels of reactive oxygen species (ROS) after exposure to H_2O_2 (Urish et al. 2009).

To further asses the role of antioxidant capacity in cell transplantation, MDSCs were treated with diethyl maleate (DEM) a thiol-depleting agent that decreases GSH levels. This resulted in decreased engraftment in skeletal muscle, similar to that of myoblast transplantation, indicating that in vivo, as well as in vitro, antioxidant levels are critical to survival and transplantation (Urish et al. 2009).

This leads to the logical next questions, if decreasing antioxidants is detrimental to MDSC survival, what will be the result if antioxidants are increased? In order to explore this, MDSCs were treated with the glutathione precursor N-acetylcysteine (NAC). NAC is currently

being used clinically to treat diseases with glutathione deficiency or liver toxicity (Dodd et al. 2008). In vitro, NAC treatment of MDSCs increased survival under oxidative and inflammatory stress compared to untreated MDSCs while, conversely, treatment with DEM decreased their survival (Drowley et al. 2010). NAC treatment does not influence proliferation of MDSCs, however, it does increase myogenic differentiation compared to untreated and DEM treated cells (Drowley et al.).

Transplantation with MDSCS pre-treated with NAC increased cardiac fractional shortening compared to therapy with untreated MDSCs after six weeks in an acute murine MI injury model. This correlated with increased angiogenesis and decreased fibrosis; the NAC treated MDSC group showed a significant increase in CD31+ structures after 6 weeks compared to untreated MDSCs, and there was a decrease in fibrotic scar tissue in the NAC treated group compared to untreated MDSCs (Drowley et al. 2010). This leads to the conclusion that survival is a critical aspect of cellular therapy for cardiac repair and that methods to increase survival of transplanted cells, as well as native tissue, may aid in the repair process. In this example, the cells were pre-treated with an antioxidant, and thus their capacity to neutralize oxidative species was increased, which may explain their increased survival. Other studies have also demonstrated that survival is important for cell therapy in the heart, mesenchymal stem cells had functional and histological improvements after myocardial infarction, but when the cells were transduced with the survival factor Akt this improvement was significantly increased (Mangi et al. 2003). However, one drawback to that study was the use of gene therapy to modify the cells. Although chemical pre-treatment of cells requires optimizing timing and dose, the use of NAC has been shown to be safe and is already in clinical use, making it an attractive option for enhancing cell therapy after human myocardial infarction. Whereas using stem cell gene therapy has been

shown to pose some rare risks linked to insertional mutagenesis of transplanted hematopoietic stem cells leading to leukemia in patients with severe combined immunodeficiency (Hacein-Bey-Abina et al. 2003; Wu et al. 2011).

NAC is not the only way to increase cell survival. For example, VEGF is certainly a potent angiogenic factor, but there is evidence that it also has a protective effect on the surrounding cardiomyocytes as a survival factor (Jiang et al. 2006). Certainly, cell survival and the paracrine factors that are secreted are two inter-related areas of importance to cell therapy. One beneficial paracrine effect is angiogenesis.

1.8 ANGIOGENESIS

In studies examining MDSC engraftment into cardiac muscle it was found that small numbers of MDSCs would co-localize with CD31 (an endothelial cell marker). This indicated that MDSCs might have some role in angiogenesis. However, since the number of CD31+ MDSCs was so small, this role is most likely due to secreted paracrine factors which induce survival in neighboring cells and potentially recruit endothelial cells or resident/circulating stem cells.

Studies have shown that angiogenic paracrine factors such as vascular endothelial growth factor (VEGF) secreted by transplanted cells are an integral part of the benefits seen after cell therapy (Seeger et al. 2007). In the study comparing myoblast and MDSC transplantation into infarcted murine hearts it was shown that MDSCs secrete VEGF after transplantation (Oshima et al. 2005). To examine this further MDSCs were transduced with the VEGF specific antagonist soluble Flt1 (sFlt1) and injected into a murine model of acute myocardial infarction five minutes after injury. The MDSCs transduced with sFlt1 showed significantly less angiogenesis in the

infarct area compared to MDSCs transduced with LacZ or VEGF. In fact the levels of angiogenesis were similar to those seen in hearts treated with the PBS control. The benefits usually seen by wt-MDSCs with regard to scar tissue area and infarct size were lost when VEGF was blocked (Payne et al. 2007). Furthermore, antagonism of VEGF diminished the therapeutic effect of MDSC cell therapy on cardiac function. The sFlt1-MDSC treated hearts had fractional shortening and fractional area change values similar to PBS treated controls.

However, it is interesting to note that too much VEGF can actually be detrimental to the reparative process. High levels of VEGF induced dis-organized vasculature and actually resulted in decreased engraftment. These same phenomena have also been observed in myoblast populations transduced with VEGF (Suzuki et al. 2001). This emphasizes the importance of the "cell" in cell therapy as not only a source of growth factors, but also a regulator of these factors. Also, it highlights the importance of an optimal level of VEGF, as when it is blocked the effect of MDSC cell therapy are severely diminished. In this particular study increasing VEGF levels was accomplished through genetically modifying MDSCs. However, there are other ways to stimulate cells to produce VEGF, such as mechanically stimulating the cells with cyclic stretch or culturing the cells under hypoxic conditions (Payne et al. 2007) . These may represent options to safely and simply condition cells in order to increase their potential for effectiveness for cellular therapy.

1.9 ANTI-FIBROSIS AND CARDIOMYOGENESIS

The mechanism of increased or decreased fibrosis after MDSC therapy has not been studied directly; however, a high correlation was observed between an increase in angiogenesis and a

correlating decrease in fibrosis. The replacement of healthy, contractile tissue with fibrotic tissue is one of the hallmarks of ischemic injury. When this occurs in other tissues, such as skeletal muscle, resident progenitor cells may be able to repair the damage. However, since the heart has such a low percentage of these cells it is unable to repair the damage on its own (Bergmann et al. 2009). Also, in diseases where skeletal progenitor cells may be compromised, such as DMD, fibrotic replacement of muscle cells is a main cause of muscle weakness (Mann et al. 2011). Cell therapy may delay fibrosis by the secretion of survival factors or by the recruitment of building block cells such as endothelial or progenitor cells to the injured area. In MDSCs mediated therapy, VEGF may be a particularly potent survival/angiogenic factor and methods to increase the survival of transplanted cells have significantly beneficial effects.

As mentioned earlier, a fraction of engrafted murine MDSCs differentiate into and/or fuse with cardiomyocytes (Oshima et al. 2005) at a ratio significantly higher than myoblasts. In order to enhance their cardiomyogenic potential, non-canonical wnt11, which is highly involved in cardiogenesis and cardiac tissue formation and capable of promoting cadiomyogenic differentiation in stem/progenitor cells, was transduced into MDSCs (Xiang et al. 2011). Wnt11-MDSCs had increased expression of the early cardiac markers NK2 transcription factor related 5 (NKx2.5) and GATA-binding protein-4 (GATA4), as well as more mature cardiac markers: Connexin43 (Cx43), α, β-myosin heavy chain (MHC), brain natriuretic protein (BNP), and Troponin I (TnI). Although some of the cells beat spontaneously, most Wnt11-transduced MDSCs are not fully differentiated cardiomyocytes in culture. Upon transplantation into acutely infarcted myocardium, Wnt11- MDSCs showed greater survival and cardiac differentiation, suggesting the feasibility of enhancing the cardiomyogenic capacity of MDSCs for therapeutic purposes (Xiang et al. 2011).

1.10 CONCLUSION

MDSCs represent a promising skeletal muscle-derived stem cell population for cardiac repair and regeneration. They induce increases in neovascularization to the ischemic myocardium mediated through angiogenic paracrine factors, and have an increased resistance to stress compared to conventional myoblasts. It is possible to further improve their beneficial effects by treating the cells with antioxidants to increase their ability to survive in the harsh environment following post-infarction intra-myocardial transplantation. Moreover, other areas of interest to improve therapeutic efficacy of MDSCs include new methods of cell delivery, measures to increase expression of angiogenic growth factors, and other modalities to improve cell survival, such as applying different antioxidants or using antioxidants to treat other cell types to improve survival and regenerative capacity. Furthermore, human skeletal muscle derived stem cells have similar potential to repair the ischemic heart as murine MDSCs, indicating the feasibility of translating the promising outcomes from animal models to the clinical setting. In fact, we have initiated a phase I clinical trial in Canada for myocardial infarction patients to be treated using MDSCs.

2.0 SPECIFIC AIMS OF DISSERTATION

2.1 AIM 1: THE ROLE OF ANTIOXIDANT MEDIATED CELL SURVIVAL ON CARDIAC REPAIR

Hypothesis: Cell survival is an integral aspect of cell mediated repair. Treating myoblasts with the antioxidant N-acetylcysteine (NAC) will increase cell survival and engraftment and increase their capacity for cardiac repair compared to untreated myoblasts and myoblasts treated with diethyl-maleate (DEM).

2.2 AIM 2: THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN MECHANICALLY STIMULATED MDSC MEDIATED TISSUE REGENERATION AND ANGIOGENESIS

Hypothesis: Mechanically stimulated MDSCs (MS-MDSCs) secrete significantly more VEGF than non-stimulated MDSCs (NS-MDSCs). These MS-MDSCs have a superior capacity for cardiac repair compared to NS-MDSCs. Conversely, when VEGF signaling is blocked by transducing MDSCs with the soluble VEGF receptor sFlt1, their repair capacity diminishes. Therefore, we hypothesize that increased VEGF secretion is the mechanism behind the improved repair observed with mechanically stimulated MDSCs; we hypothesize that when sFlt1-MDSCs

or shRNA_VEGF MDSCs are mechanically stimulated, their capacity for inducing angiogenesis and muscle regeneration will be eliminated.

2.3 AIM 3: CHARACTERIZING MDSCS FROM MURINE MUSCULAR DYSTROPHY MODELS

Hypothesis: Duchenne muscular dystrophy is a stem cell disease, and as such supplementing the stem cell compartment may help to delay disease progression. To this aim we examined the characteristics of MDSCs from two different murine muscular dystrophy models, *mdx* and *mdx*/utr-/- (DKO) mice, in order to assess their stem cell characteristics to better understand the feasibility of using dystrophic MDSCs as a possible therapy for DMD.

3.0 THE ROLE OF ANTIOXIDANTS IN MYOBLAST MEDIATED CARDIAC REPAIR

3.1 INTRODUCTION

Cell therapy is a promising approach to treat heart disease, which includes both myocardial infarction (MI) and congestive heart failure (CHF). In the United States alone 1 in every 2.8 deaths is caused by cardiovascular disease (Lloyd-Jones et al. 2010). A wide variety of progenitor cell types have shown modest improvement in animal models of ischemic heart disease and early clinical trials (Mummery et al. 2010; Wollert et al. 2010). Benefits of stem/progenitor cell therapy result largely from increased angiogenesis (Payne et al. 2007), decreased apoptosis of native cardiomyocytes, decreased fibrosis (Li et al. 2009) and increased contractile properties of the left ventricle (LV). However, low engraftment (Robey et al. 2008) which may be due to low cell survival (Penn et al. 2008; Drowley et al. 2010), and/or low cell retention, remains a universal obstacle to cell therapy, with only 1-10% of implanted cells surviving in the heart (Zhang et al. 2001; Laflamme et al. 2005). The unfavorable microenvironment after myocardial infarction accumulates high levels of inflammation and oxidative stress, leading to irreversible damage to donor cells (Segers et al. 2008). Therefore, methods to relieve adverse effects inflicted by environmental stress may improve donor cell survival and consequently be beneficial to cell therapy.

Muscle-derived stem cells (MDSCs) belong to a subpopulation of muscle stem cells that can be isolated from skeletal muscle based upon their low adhesion to collagen coated flasks during isolation (Qu-Petersen et al. 2002; Oshima et al. 2005; Gharaibeh et al. 2008). Previously, we have shown that MDSCs have promoted cardiac repair and demonstrated superior cell survival when compared to myoblasts, largely attributable to their higher levels of antioxidants (Oshima et al. 2005; Payne et al. 2005). Similar results have been observed in MDSC-mediated skeletal muscle repair through greater cell survival under oxidative stress due to increased levels of the antioxidant glutathione (GSH) and enzyme superoxide dismutase (SOD) (Urish et al. 2009). Altering the cellular redox state by depleting GSH using diethyl-maleate (DEM) decreased the engraftment of MDSCs into skeletal muscle (Urish et al. 2009). Pre-treating MDSCs with the free radical scavenger N-acetylcysteine (NAC), a precursor to the antioxidant glutathione (GSH),(Dodd et al. 2008) increased fractional area change, decreased fibrosis and increased angiogenesis following cell delivery in an acute murine myocardial infarction model (Drowley et al. 2010). On the contrary, pre-treating MDSCs with DEM (a non-toxic chemical which binds to and de-activates GSH) decreased function and angiogenesis and increased fibrosis to levels similar to PBS injection (Urish et al. 2009; Drowley et al. 2010).

Myoblasts, one of the most abundant cell sources for tissue repair, have been relatively restricted in regenerative applications by poor cell survival following cell therapy, a deficiency presumably due to their lower levels of antioxidants compared to MDSCs (Menasche 2008). We hypothesize that survival is an integral part of muscle cell-mediated cardiac repair, and that by up-regulating the antioxidant level in myoblasts prior to implantation, it will increase their survival and consequently improve regenerative potential. In the present study, we demonstrate that increasing antioxidant levels positively correlates with the early survival of myoblasts after

implantation in infarcted hearts. NAC was chosen because it is already being used clinically as an antioxidant (Dodd et al. 2008). NAC pre-treatment further increases proliferation and decreases apoptosis in the engraftment area. At 6 weeks post-infarction, myoblasts pre-treated with DEM prior to implantation resulted in reduced functional repair, close to that of PBS injections, as well as increased fibrosis and decreased angiogenesis, suggesting the importance of antioxidant level in myoblast-mediated cardiac repair.

3.2 METHODS

Animal studies: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol #0901823). As such, all animal studies conform to the Guide for the Care and Use of Laborataory Animals as published by the National Institute of Health.

Myoblast isolation: Mice were sacrificed by isoflurane overdose followed by cervical dislocation and the skeletal muscles were removed. Myoblasts were then isolated from the skeletal muscle of three week old normal C57BL mice (Jackson, Bar Harbor, ME) using the modified preplate technique as previously described (Gharaibeh et al. 2008). Myoblasts were cultured in proliferation media (PM) containing Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen,), 10% horse serum (HS), 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical, Westbury, NY).

Differentiation: Myoblasts were plated at 50 cells/mm^2 on a 24-well collagen type-I coated plates. Twenty-four hours later, the media was changed to DMEM + 2% FBS, DMEM +

2% FBS + 50 µmol/L DEM (Sigma, St Louis, MO), or DMEM + 2% FBS + 10 mM NAC (Sigma). At five days, the plates were stained with mouse anti-fast skeletal myosin heavy chain antibody (fsMHC, 1:400; Sigma,) and 4',6-diamidino-2-phenylindole (DAPI, for nuclei), and the percentage of nuclei in fsMHC+ myotubes compared to total DAPI+ nuclei was quantified to assess myotube formation.

VEGF secretion: Myoblasts were plated in PM at 105 cells/mm^2 in 6-well collagen type-I coated plates and treated with PM, PM + 50 µmol/L DEM, or PM + 10 mmol/L NAC. Twenty-four hours prior to media collection, the media was switched to DMEM with 2% FBS and 1% penicillin/streptomycin. ELISAs specific for mouse VEGF (R&D Systems, Minneapolis, MN) was performed according to manufacturer's instructions and as previously described (Payne et al. 2007). The VEGF levels were normalized to total cell number at the end of the experiment.

Glutathione levels: Myoblasts were plated at a density of 30 cells/mm^2 in a 96 well plate (Perkin Elmer, Waltham, MA) and treated with PM + 100 µmol/L H_2O_2, PM + 100 µM H2O2 + 50 µmol/L DEM or PM + 100 umol/L H_2O_2 + 10mmol/L NAC for 2.5 hours. Cells were then treated with PM + 2.5mmol/L Probenecid (Sigma) + 50uM Monochlorobimane (MCB) (Invitrogen) for 20 min at room temperature. Cells were then rinsed twice with PBS (Lonza, Allendale, NJ) and analyzed using a plate reader (excitation 380 nm and emission 380nm).

Cell survival under oxidative stress: Myoblasts were plated in PM at 10 cells/mm^2 in a 24-well collagen type-I coated plate. Twenty-four hours later, the media was switched to PM with propidium iodide (unstressed) (PI, 1:500, Sigma, St. Louis, MO), or PM containing 375 µmol/L hydrogen peroxide (H_2O_2) with PI (oxidative stress). Each group was then broken down into one of three treatment groups: PM, PM + 50 µmol/L DEM, or PM + 10 mmol/L NAC. The plates were next placed onto a previously described live cell imaging system (LCI, Kairos

Instruments, LLC, Pittsburgh, PA), and fluorescent and bright field images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageJ software (NIH, Bethesda, MD). Cell proliferation was determined by counting the number of cells present in the bright field images at 12 hour intervals. Cell survival was determined by counting the number of PI-positive cells (dead cells) in the fluorescent images at 12 hour intervals and subtracting the dead cells from total cell number.

Myoblasts transduced with a retrovirus carrying the LacZ reporter gene: The myoblasts were retrovirally transduced with a *LacZ* gene prior to their transplantation. The construct used was a retroviral vector containing a modified *LacZ* gene with a nuclear localization sequence which has been used previously in our laboratory (Payne et al. 2005).

Cell transplantation to infarcted myocardium: Twenty eight male immunodeficient, NOD-SCID mice at 14-18 weeks of age were used in this study (Jackson, Bar Harbor, ME). Mice were anesthetized during the surgical procedure with 1-1.5% isoflurane in 100% O_2. Mice that underwent permanent left descending coronary artery ligation using a 7-0 prolene suture to create myocardial infarction(Oshima et al. 2005; Payne et al. 2007; Drowley et al. 2009) were randomly allocated between the treatment groups (7 mice per group: PBS, untreated myoblasts, and myoblasts treated with either 10mM NAC or 50uM DEM). Five minutes after the creation of the infarction, the mice received a 30 µl injection of one of the following: 30 µl PBS, $3x10^5$ NAC-treated myoblasts in PBS, $3x10^5$ DEM-treated myoblasts in PBS, or $3x10^5$ untreated control cells in PBS. The cells or PBS were injected into the left ventricular free wall five minutes after the permanent ligation of the left coronary artery as previously described (Oshima et al. 2005; Drowley et al. 2010). Echocardiography was performed in each mouse at 2 and 6 weeks after cell transplantation. Two-dimensional images were obtained at the mid-papillary

level. The end-diastolic area (EDA) and end-systolic area (ESA) were measured from short-axis images of the LV, as previously described (Oshima et al. 2005; Payne et al. 2007). At 3 days and 6 weeks the mice were sacrificed by isoflurane overdose followed by cervical dislocation. Subsequently, the hearts were harvested, flash-frozen in 2-methylbutane, and cryo-sectioned into 7 μm thick sections.

Histology and Immunohistochemistry: Previously described techniques were used to stain section for both *nLacZ* and eosin (Qu-Petersen et al. 2002). Terminal dUPT nick end-labeling (TUNEL) staining was carried out according to the manufactures protocol (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Temecula, California) and counter-stained with hematoxylin. Apoptotic cells were normalized to total cell number. Phospho-Histone3 staining was used to quantify proliferating cells. Mitotic phase cells were identified by anti-phospho-S10 Histone H3 (1:300 Milipore, Billerica, Massachusetts) with donkey anti-rabbit Alexa Flour 594 (1:200; Sigma)(Clause et al. 2010) and DAPI (Vecta-Shield; Vector Labs). Masson Modified IMEB Trichrome (IMEB, San Marcos, CA), which stains both collagen (blue) and muscle (red), was performed according to the manufacturer's guidelines following a protocol previously described by our research group (Oshima et al. 2005; Payne et al. 2007; Drowley et al. 2009). The sections were assessed for the percentage area of collagen in 5 sections per heart which were normalized to total muscle area within the section using CellProfiler image analysis software (http://www.cellprofiler.org). The number of CD31 positive cells in the infarct area was determined by staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma, St. Louis, MO) and donkey anti-rat Alexafluor 594 secondary antibody (1:300, Sigma, St. Louis, MO). Endothelial cells within the cell-injected areas were

determined by counting the number of CD31 positive cells per 400X high powered field in 3 fields per heart.

Microscopy: Fluorescence and bright field microscopy were performed using either a Nikon Eclipse E800 microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix Imaging, Cheektowaga, NY) or a Leica DMIRB inverted microscope with a Retiga digital camera and Northern Eclipse software.

Statistical analysis: The means and standard errors were calculated for all measured values, and statistical significance between the groups was determined by a 1-way or 2-way ANOVA (for cardiac function) (SPSS). In the event of a significant ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (S-N-K).

3.3 RESULTS

Modulation of antioxidants does not affect muscle cell differentiation: N-acetylcysteine (NAC) is a synthetic precursor to intracellular cysteine which is the rate limiting peptide for synthesis of the endogenous antioxidant GSH as well as a direct reactive oxygen species scavenger. It is used clinically as both an antioxidant and a mucolytic agent (Dodd et al. 2008). The antioxidant Glutathione (GSH) is increased in NAC treated myoblasts and decreased in diethyl-maleate (DEM) treated myoblasts. There is a significant difference in GSH expression in the NAC treated group compared to the DEM treated group (*$P < 0.05$, n=6, Figure 2a). To examine their myogenic potential after drug treatment, myoblasts were first treated with NAC or DEM for 24 hours then changed to low-serum differentiation media, with untreated cells serving

as controls. After 5 days the number of nuclei in myotubes (as shown by fast skeletal myosin heavy chain staining) was quantified. As shown by Figure 2b there is no difference in differentiation after treatment of myoblasts with either NAC or DEM.

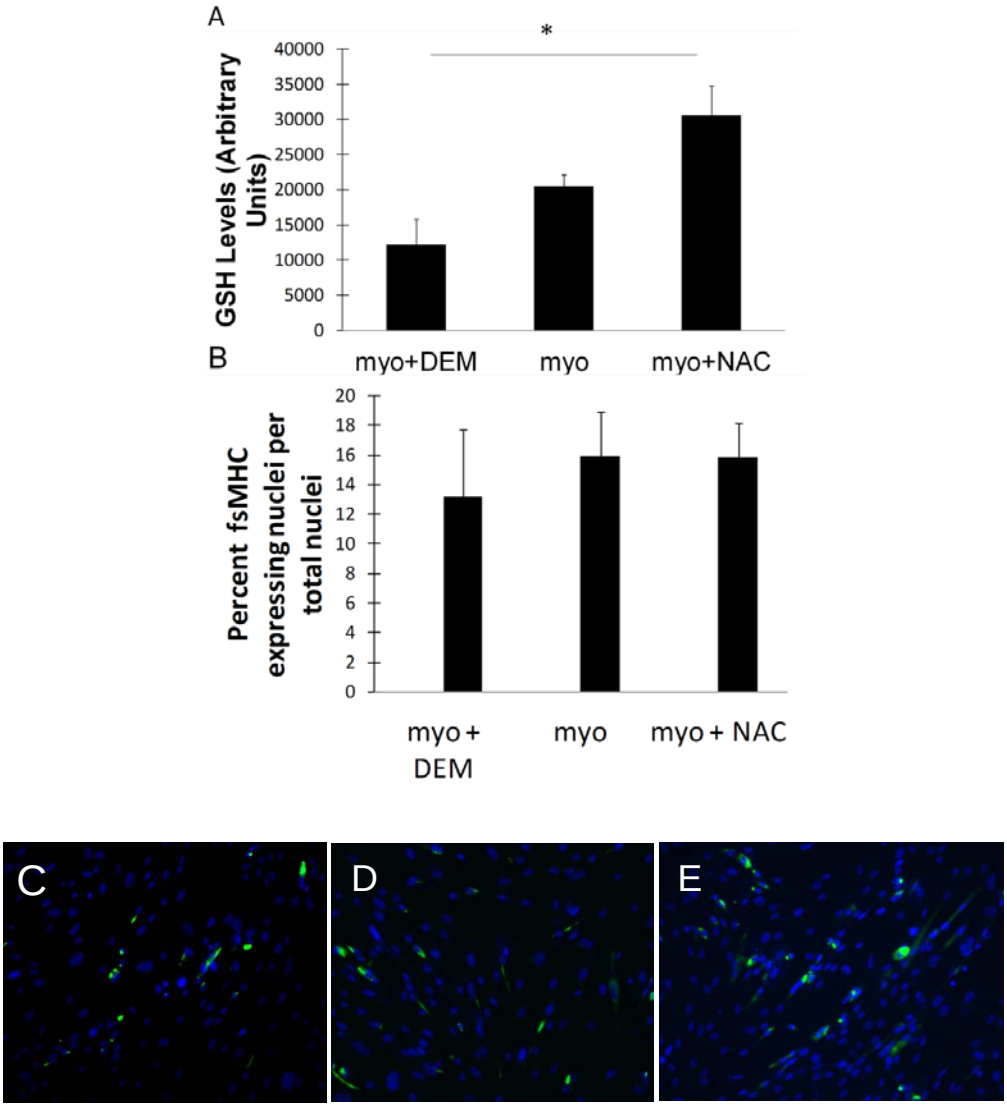

Figure 2: Modulation of antioxidant levels does not affect myogenic differentiation.

(A) Glutathione expression is increased in myoblasts treated with NAC compared to myoblasts treated with DEM (n=4, *P < 0.05,). Fluorescence is shown as measured by Monochlorobimane (MCB) staining. (B) Differentiation of myoblasts is unchanged after treatment with NAC or DEM as determined by the percent of nuclei in fast-skeletal myosin heavy chain (fsMHC) stained myotubes n=6. (C-E) Representative images of fsMHC staining, Myo+DEM, myo, and myo+NAC respectively.

NAC pre-treatment increases early survival of myoblasts: Myoblasts were treated with NAC or DEM and cultivated under oxidative stress conditions (375 μmol/L H_2O_2). Treatment of myoblasts with NAC increases cell survival under *in vitro* oxidative stress at all time points for up to 48 hours following treatment. The *in vitro* cell survival is significantly greater in myoblasts treated with NAC than myoblasts treated with DEM (*P < 0.05, n=36, Table 1).

Table 1: Percent survival under oxidative stress.

		Table 1: Percent cell survival under oxidative stress				
		Time (hours)				
Treatment	n	0	12	24	36	48
NAC	36	100%	93% ± 2% p = 0.12 to Control, p = 0.003 to DEM	88% ± 3% p = 0.30 to Control, p = 0.009 to DEM	84% ± 3% p = 0.26 to Control, p = 0.003 to DEM	79% ± 3% p = 0.23 to Control, p = 0.006 to DEM
Control	36	100%	85% ± 3% p = 0.12 to NAC, p = 0.38 to DEM	81% ± 4% p = 0.302 to NAC, p = 0.29 to DEM	76% ± 4% p = 0.26 to NAC, p = 0.17 to DEM	70% ± 3% p = 0.28 to NAC, p = 0.24 to DEM
DEM	36	100%	79% ± 4%	73% ± 4%	67% ± 4%	62% ± 4%

NAC = N-acetylcysteine; DEM = diethyl-maleate. Data are mean ± SEM

To test their *in vivo* survival treated and un-treated myoblasts transduced with *LacZ* were transplanted into an acutely infarcted heart and survival was examined after 3 days. This was quantified by counting the total number of *LacZ*+ cells normalized to tissue area in the injected hearts. Three days after transplantation, a significantly greater number of *LacZ*+ NAC treated

myoblasts was observed when compared to untreated and DEM treated myoblasts in the infarct area (*P < 0.05, n=6, Figure 3).

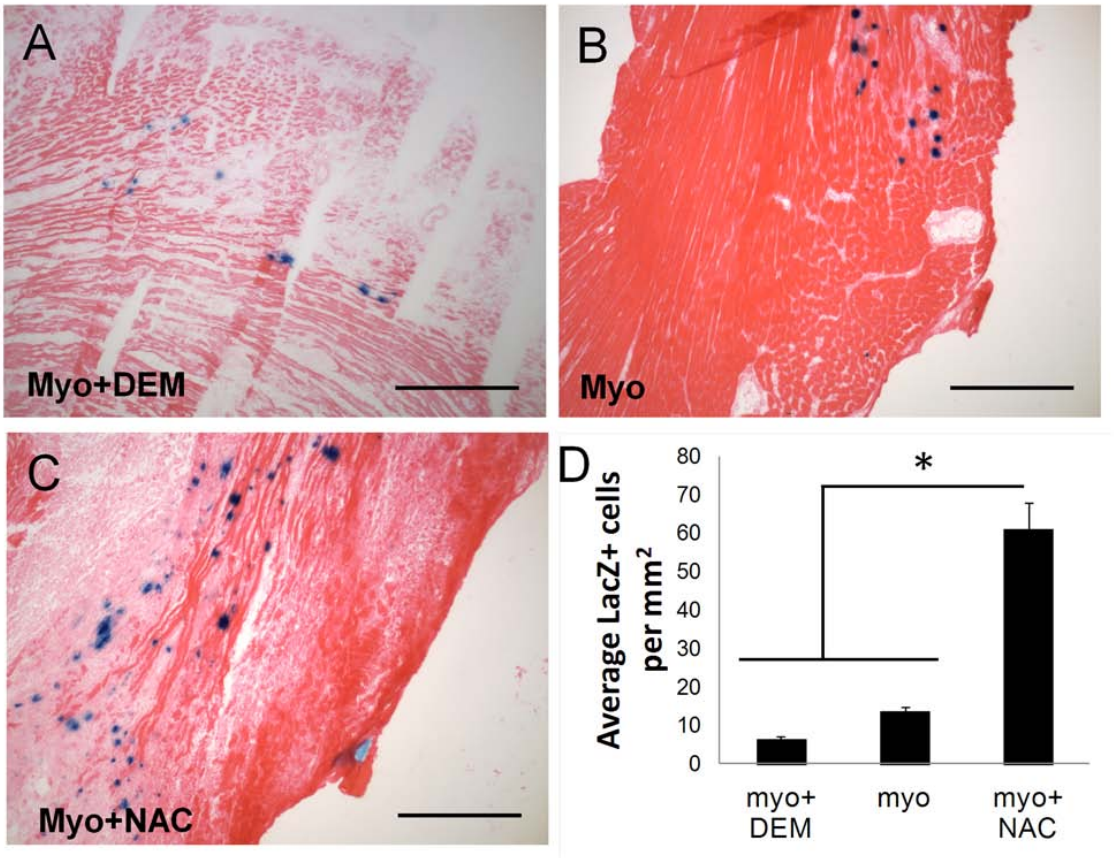

Figure 3: NAC treated myoblasts show superior survival

Cardiac injections of treated cells were performed 3 days5 minutes post MI, 3 days later hearts were harvested and stained for LacZ to monitor transplanted cells. (A)(B)(C) Representative images of sections stained with LacZ positive myoblasts that are treated with DEM, untreated, or treated with NAC, respectively. (D) There was a significantly greater amount of LacZ positive transplanted cells in the hearts injected with myoblasts+NAC compared to myoblasts treated with DEM or untreated myoblasts (n=6, *P < 0.05). (Scale bar = 0.3 mm)

This suggests that treatment with NAC has a positive effect on cell survival at early time points.

Cellular apoptosis was examined with TUNEL staining in the engraftment area (Figure 4).

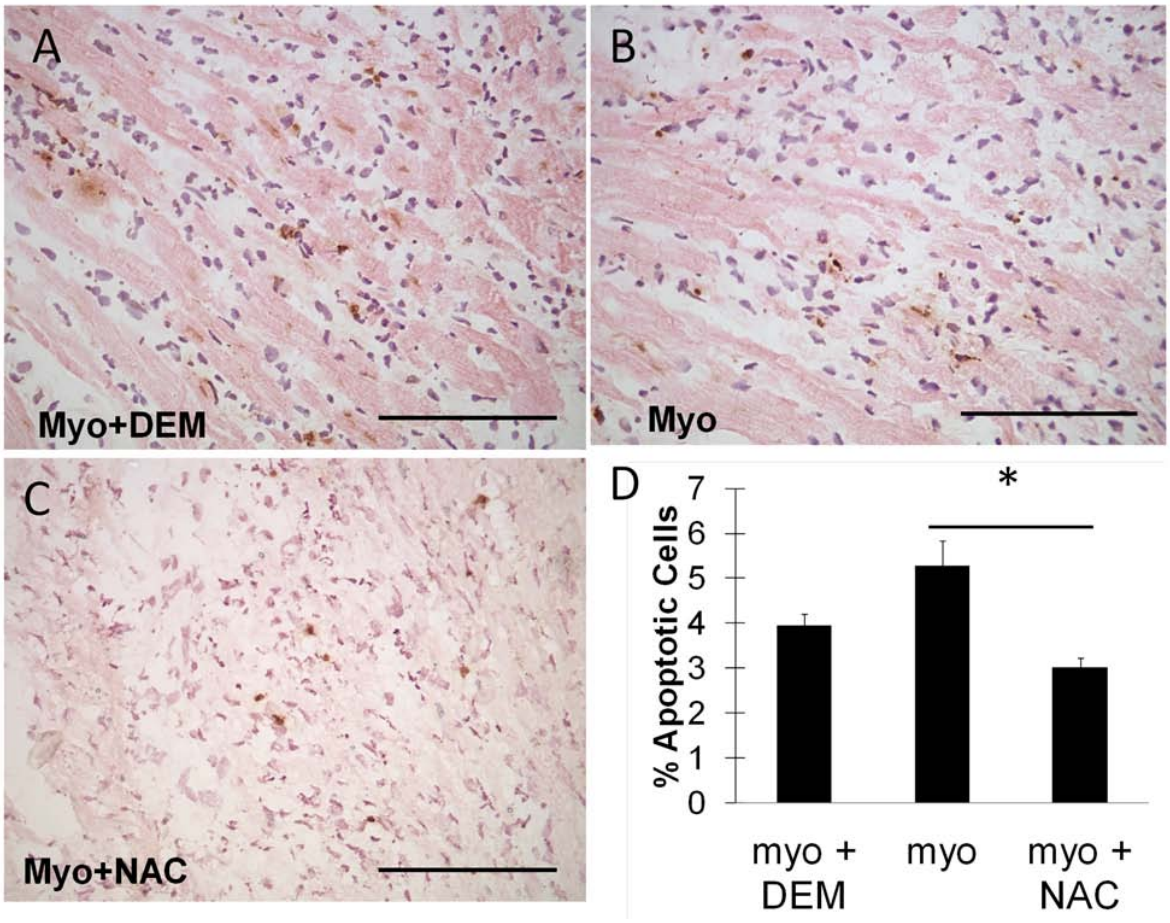

Figure 4: NAC pretreatment decreases apoptosis

Terminal dUPT nick end-labeling (TUNEL) staining (brown) demarks apoptotic nuclei 3 days after transplantation. (A)(B)(C) Representative images of TUNEL stained sections treated with, myoblasts treated with DEM, untreated myoblasts or myoblasts treated with NAC, respectively. Scale bar = 0.1 mm. (D) There was significantly less apoptosis in the samples treated with myo+NAC as compared to myo (n=6, *P < 0.05).

Three days after injection, there was less apoptosis in the groups treated with myoblasts+NAC compared to those injected with untreated myoblasts (*P < 0.05, n=6, Figure 4). Proliferation was examined in the engraftment area using Histone-3 staining. There is twice as much proliferation in the NAC treated group compared to the other groups but this is not statistically significant (Figure 5).

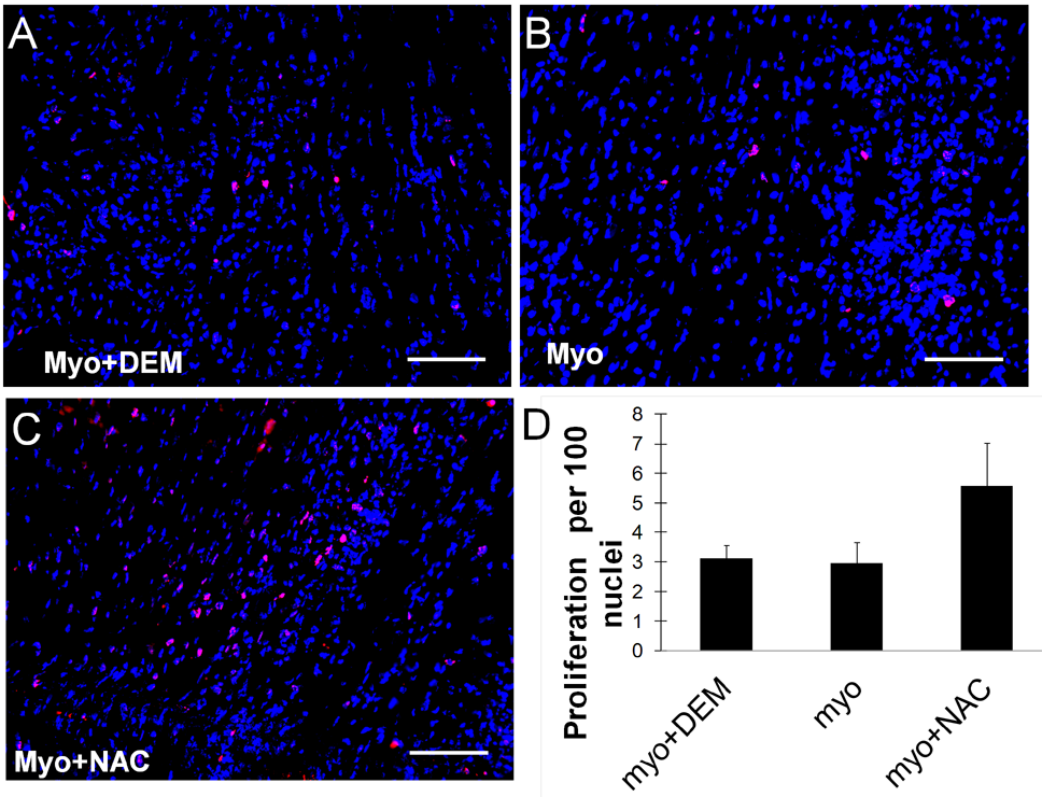

Figure 5: NAC pretreatment increases myoblast proliferation.

Mitotic phase cells were identified by anti-phospho-S10 Histone H3 Phospho-Histone3 (H3) staining (purple) 3 days after transplantation. The number of H3 positive cells was normalized to total number of nuclei (DAPI, blue). (A)(B)(C) show representative images of: myo+DEM, myo, and myo+NAC, respectively (D) The hearts treated with myoblasts+NAC have had twice as many proliferating cells as those treated with myo+DEM or untreated myoblasts, however this was not statistically significant. N=6, Scale bar = 0.1 mm.

Modulation of Antioxidants Affects Cardiac Function: The cardiac functional parameters fractional shortening (FS) and fractional area change (FAC) were analyzed by echocardiography at 2 and 6 weeks. FS in the myo+NAC groups was significantly increased compared to PBS (*$P < 0.05$, n=6, Figure 6a) at 6 weeks. FAC in the myo+NAC group was significantly greater than the myo+DEM and PBS groups while the untreated myoblast group was significantly greater than PBS at 6 weeks (*$P < 0.05$, Figure 6b). Collectively, there was a trend toward positive functional improvement of NAC treated myoblasts over the other groups

and this was statistically significant when compared to myo+DEM and PBS at 6 weeks (*P < 0.05, Figure 6b). There was no significant difference in the parameters tested at 2 weeks.

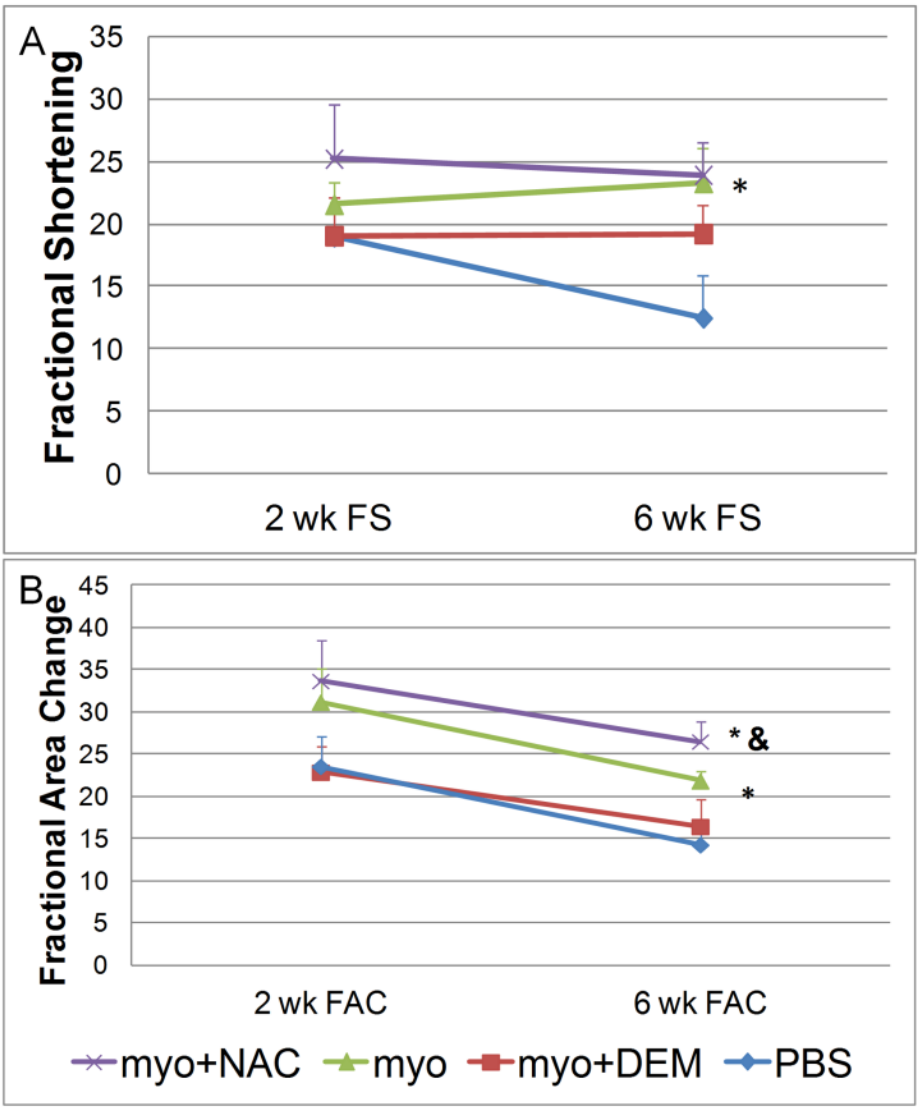

Figure 6: Cardiac Function at 2 and 6 weeks post transplantation

(A) There was no difference in fractional shortening between the groups at 2 weeks. However, at six weeks the myo+NAC treatment group had significantly increased FS compared to PBS. (* P<0.05 compared with PBS injection) (B) There is no difference in fractional area change at 2 weeks. At 6 weeks the myo+NAC treatment group had significantly increased FAC compared to PBS and the myo+DEM group and the myo group are significantly greater than the PBS control. (n=6, *P < 0.05 compared with PBS, &P< 0.05 compared with myo+DEM injection)

Depleting Antioxidants Increases Fibrosis: Total fibrotic area was determined by analysis of Masson's trichrome staining by calculating percent fibrosis (blue stain) compared to total tissue area. The myo+NAC and untreated myoblast groups contained significantly less fibrosis in comparison to the myo+DEM and PBS treated hearts (*$P < 0.05$, n=6, Figure 7). There was no difference between the groups treated with PBS and myoblasts+DEM. There was no significant difference between the NAC treated and untreated myoblast groups.

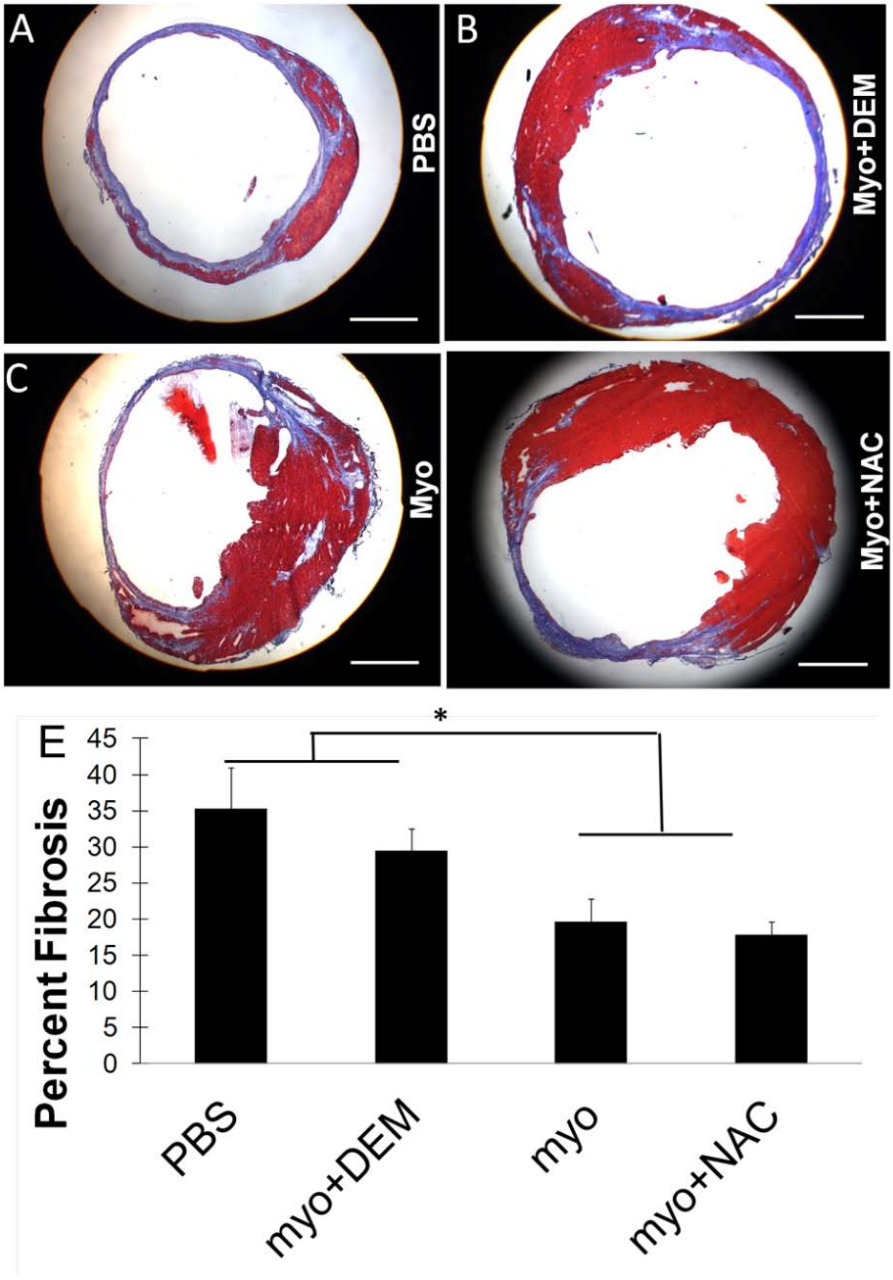

Figure 7: Fibrosis in injected hearts at 6 weeks post transplantation

Masson trichrome stains collagen (blue) and muscle (red). Representative images of hearts injected with (A) PBS, (B) myo+DEM, (C) myo or (D) myo+NAC are shown. Scale bar = 1.0mm. (E) Percent fibrosis was measured by quantifying the collagen staining compared to the total area. The PBS and myo+DEM treated hearts had more fibrosis compared to the myo+NAC and myo hearts (n=6, *$P < 0.05$).

Depleting Antioxidants Decreases Angiogenesis: Angiogenesis is an integral component of cell-mediated repair following cardiac injury. In-vivo angiogenesis was determined by counting the number of CD31 positive cells per square millimeter of tissue area. The myo and myo+NAC groups had significantly more CD31 positive cells than both the PBS and myo+DEM groups (*$P < 0.05$, n=6, Figure 8). There was no significant difference between the NAC treated and untreated myoblast groups. There was no difference between the PBS group and the myo+DEM group.

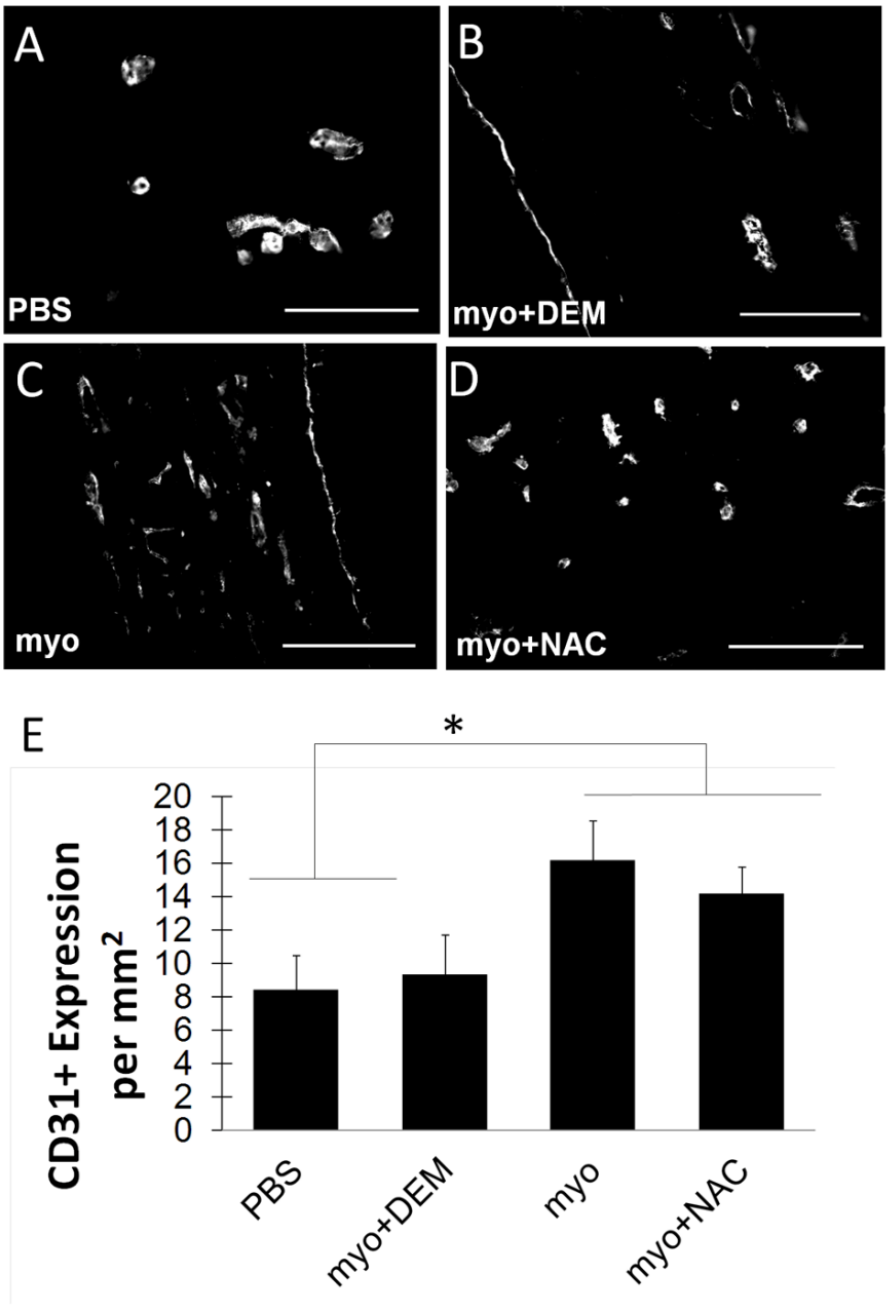

Figure 8: CD31 positive cells at 6 weeks post implantation

Angiogenesis was measured in the peri-infarct area at 6 weeks by counting the number of CD31 positive cells and normalizing to the tissue area. Representative images of the groups are shown: (A) PBS, (B) myo+DEM, (C), myo, (D) myo+NAC. Scale bar = 0.1mm. (E) The groups treated with myo and myo+NAC had significantly more CD31 positive cells than those treated with PBS or myo+DEM (n=6, *P < 0.05).

3.4 DISCUSSION

The environment after infarction is unfavorable for cell survival, with inflammation, oxidative stress, and ischemia all playing a role. Cells that are transplanted into this environment often do not adhere to damaged tissue, survive in significant numbers, or differentiate into cell types native to the resident tissue (Mummery et al. 2010; Mohsin et al. 2011). However, host cells do impart some benefits, which are likely caused by paracrine factors (Gnecchi et al. 2008). Even if transplanted cells do not become cardiomyocytes they still have positive effects on the tissue, which may include increased blood supply (Payne et al. 2007), less overall tissue death, decreased remodeling and less harmful LV dilation. One way to increase these effects is to increase cell survival.

Antioxidants are one of the first lines of cellular defense against free radicals and toxins. Antioxidants reduce ROS levels and increase survival in many cell types including: cardiomyoblasts (Rodriguez-Porcel et al. 2010), endothelial cells (Voghel et al. 2008), mesenchymal stem cells (Song et al.), chondrocytes (Ueno et al.), and muscle derived stem cells (Urish et al. 2009; Drowley et al. 2010). Oxidative stress can be caused either by improper reactive oxygen species (ROS) elimination or an increase in ROS themselves; antioxidants help to remove excess ROS from the cell. Dis-regulated free radicals have been implicated in muscle diseases such as Muscular Dystrophy and are thought to lead to death of proliferating myoblasts and differentiated myotubes (Pervaiz et al. 2009). In addition, progenitor cells have been shown to have high levels of antioxidants, indicating that resistance to stress may be a stem cell characteristic (Dernbach et al. 2004).

Another benefit provided by antioxidants may be an influence on their integrin levels. Anoikis, cell death caused by detachment of cells from their extracellular matrix, appears to be

an overlooked aspect of cellular therapy (Thomas et al. 1999). Cell adhesion may be an integral aspect of their function. When cells are digested prior to transplantation this will disrupt their integrin signaling. Also, reactive oxygen species may inhibit adhesion of transplanted cells but NAC has been shown to rescue integrin expression in mesenchymal stem cells (Song et al. 2010). In addition, recent studies indicate that the rigidity of the substrate upon which cells are grown can affect cell survival and increase engraftment into skeletal muscle (Gilbert et al. 2010). Muscle stem cells grown on soft (12kPa) substrates, which mimic the elasticity of muscle, have reduced cell death compared to those grown on plastic (Gilbert et al. 2010).

Survival of transplanted cells is a critical hurdle to overcome in order to improve cell therapy for cardiac repair following infarction. One way to achieve a more sustained survival of the transplanted cells is through gene therapy (Mohsin et al. 2011). Previous work has shown that MSCs transduced with the survival factor Akt improve heart repair after MI compared to untransduced MSCs (Mangi et al. 2003). In another study, MSCs were genetically engineered to express the anti-apoptotic Bcl-2 gene and then used therapeutically in an animal model of myocardial infarction. The modified MSCs were protected against apoptosis; treated hearts had increased capillary density and resulted in smaller infarct sizes (Li et al. 2007). Furthermore, when MSCs were transduced with a hypoxia-regulated heme oxygenase-1 (HO-1) vector, this therapy improved the survival of stem cells in a model of myocardial ischemia and improved functional recovery of infarcted hearts 2 weeks after MI, HO-1 is an anti-apoptotic and antioxidant enzyme, and the hypoxia-inducible system turned on the enzyme when it was needed (Tang et al. 2005).

This indicates that increasing the survival of transplanted cells can improve functional cardiac outcomes. Pre-treatment with antioxidants is one way to achieve this. Previous papers

published by our group indicate that treating MDSCs with the antioxidant NAC increased functional cardiac repair and that depleting the cells of glutathione with DEM decreased antioxidant levels in the cell and the functional benefit of these antioxidant-depleted cells was similar to PBS (Urish et al. 2009; Drowley et al. 2010). However, we did not see this same increase in cardiac function when we treated myoblasts with NAC.Myoblasts are an abundant source of cells for cardiac and skeletal repair but are limited by their poor survivability (Qu-Petersen et al. 2002; Oshima et al. 2005). We hypothesized that pre-treating myoblasts with NAC would increase their cell survival and regenerative capacity, making them an equally-effective therapeutic cell source to the rare MDSCs. The results showed that hearts injected with NAC treated myoblasts had significantly increased cardiac function when compared to DEM treated myoblasts or PBS injection, but not control myoblasts. Moreover, the levels of CD31 positive cells and fibrosis were similar between NAC treated and un-treated myoblasts. Early data showed that NAC pre-treatment increased survival of transplanted cells compared to untreated and DEM treated myoblasts as well as increased proliferation and decreased apoptosis in the myo+NAC group compared to the untreated myo group. However, by the 2 and 6 week time points these early benefits diminished. The differences we observed between treatment of MDSCs and treatment of myoblasts could be explained a few different ways. First of all, the transient effect of NAC may not be sufficient to increase the survival to a level necessary to make a significant difference to function in the myoblasts. This suggests a more sustained survival factor may be necessary to increase the cardiac regenerative potential. The effects of NAC were not sufficient for long-term survival benefits in myoblasts. It is also evidence that an early increase in survival may not translate to functional repair at later time points. Future studies would include transducing myoblasts with antioxidant or survival genes such as HO-1,

Akt, or super oxide dismutase (SOD) to garner a sustained increase in antioxidant capacity, and consequently, the beneficial effect of treated stem cells on cardiac repair may be more pronounced.

Another possible reason for the differences observed may be the cells themselves. We have shown in multiple studies that MDSCs are more effective than myoblasts at regenerating tissue including the heart (Oshima et al. 2005; Payne et al. 2005) and skeletal muscle (Qu et al. 1998). This may be due to differences in the paracrine factors secreted by myoblasts and MDSCs. Paracrine factors are critical for cardiac repair (Gnecchi et al. 2008). Also, MDSCs display stem cell characteristics while myoblasts are more myogenic, indicating the MDSCs are likely more able to proliferate and differentiate into different cell types (albeit at low levels), which makes them better prepared to regenerate injured tissue (Qu-Petersen et al. 2002). Increasing myoblast cell survival may not be enough to improve upon repair if the cells themselves are not capable of efficiently regenerating tissue. It may be better to switch to another cell source, or try and modify a different facet of myoblasts, such as improving secretion of survival signals to the tissue, factors to induce angiogenesis, or prevent remodeling.

Finally, it is interesting to note that treating myoblasts with DEM resulted in less fibrosis and angiogenesis compared with the untreated control group and imparted a functional benefit similar to PBS. This suggests that some level of antioxidants are important for myoblast cell survival in a cardiac MI model.

In summary, pre-treatment of myoblasts with NAC increases early cell survival following cardiac transplantation and decreases cellular apoptosis. However, this therapy fails to impart a functional benefit over non-treated myoblasts at 6 weeks and future work is necessary to

determine if sustained myoblast survival could impart functional benefits on damaged tissue or if cell functionality is more important to modulate than cell survival in this cell type.

4.0 THE BENEFICIAL EFFECT OF MECHANICAL STIMULATION ON THE REGENERATIVE POTENTIAL OF MDSCS IS LOST BY INHIBITING VEGF

4.1 INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited neuromuscular disorder which affects 1:3500 live male births, and is caused by lack of the membrane-stabilizing dystrophin protein (Deconinck et al. 2007) (Towbin et al. 2002). Without dystrophin, muscle fibers experience periods of pathological degeneration and regeneration and subsequent loss of membrane integrity (Deconinck et al. 2007). DMD is the most severe form of muscular dystrophy, exhibiting progressive weakness leading to respiratory or cardiac failure and premature death (Deconinck et al. 2007).

Stem cell therapy is a promising treatment for numerous disorders including degenerative muscle diseases such as DMD, heart failure, liver failure, bone degeneration etc (Usas et al. 2007; Mohsin et al. 2011; Vilquin et al. 2011). However, the low survival of transplanted cells and their decreased differentiation capacity into desired cell types hinder positive therapeutic outcomes (Mohsin et al. 2011). Many benefits of stem cell transplantation are attributed to paracrine effects, including increased angiogenesis, decreased fibrosis, immunomodulation, and secretion of survival and stem cell recruitment factors (Rafii et al. 2003; Gnecchi et al. 2008; Schlosser et al. 2012). A critical paracrine factor is vascular endothelial growth factor (VEGF),

whose main function is to promote angiogenesis by improving survival, inducing proliferation, and enhancing migration and invasion of endothelial cells (Byrne et al. 2005). However, recent evidence suggests that VEGF has effects on other cell types as well, such as regulating cardiac (Dai et al. 2007), myoblast (Germani et al. 2003), podocyte (Goldman et al. 1998), and hematopoietic stem cell (Gerber et al. 2002) survival, bone differentiation (Arfin et al. 1995), neurogenesis (Kendall et al. 1996) and the stimulation of skeletal muscle regeneration (Arsic et al. 2004; Bouchentouf et al. 2008). Muscle derived stem cells (MDSCs) are a stem/progenitor cell population obtained using a modified pre-plate technique which have long term proliferation, self-renewal, and multi-lineage differentiation capabilities (Qu-Petersen et al. 2002; Gharaibeh et al. 2008). Our previous work showed that murine MDSCs were superior to myoblasts at generating dystrophin positive myofibers after transplantation into hearts and skeletal muscles of dystrophic mice (Qu-Petersen et al. 2002; Payne et al. 2005). MDSCs repaired the heart after myocardial infarction to a greater extent than myoblasts and demonstrated a higher level of VEGF expression in the MDSC graft (Payne et al. 2005). In dystrophic skeletal muscle, the transplantation of MDSCs expressing VEGF resulted in increased angiogenesis in the engraftment area; conversely, the transplantation of MDSCs expressing the soluble receptor for VEGF, sFlt1 (sFlt1-MDSCs) demonstrated decreased angiogenesis in the engraftment area (Deasy et al. 2009). Likewise, in ischemic cardiac muscle, treatment with sFlt1 abrogated the regenerative capacity of these cells. Increasing VEGF levels in muscle cells prior to transplantation by ex-vivo gene therapy increased levels of angiogenesis in the recipient muscle, reduced death of donor cells, and improved engraftment of donor cells; (Germani et al. 2003; Payne et al. 2007; Bouchentouf et al. 2008; Deasy et al. 2009) however, excessive levels of VEGF can cause dis-organized vascularization and have deleterious effects, such as the

formation of angiomas or edema (Dvorak et al. 1995; Franco et al. 2011). Thus, there is a critical level of VEGF required for optimal tissue regeneration.

An alternative method to increase VEGF secretion of cells is through mechanical stimulation (MS) (Payne et al. 2007). In experiments comparing MS-MDSCs and non-stimulated (NS) MDSCs, VEGF secretion of MS-MDSCs was significantly greater than NS controls (Byrne et al. 2005; Payne et al. 2007). Mechanical stimulation improved transplantation outcomes in the injured heart compared to non-stimulated stem cells, (Byrne et al. 2005; Pijnappels et al. 2008) and has been shown to be a potent stimulus for angiogenesis (Byrne et al. 2005; Kasper et al. 2007; Schad et al. 2011) and cell fate decisions (Park et al. 2004; Pijnappels et al. 2008; Li et al. 2011). Mechanical stimulation may represent an effective method to pre-condition stem cells in order to increase VEGF secretion, and improve regeneration capacity, without the deleterious effects of VEGF over-expression.

We hypothesized that the augmented angiogenesis following transplantation of MS-MDSCs was due to increased VEGF secretion. Therefore, in the current study, we examined the importance of VEGF secretion on MS-MDSCs by blocking VEGF through two different approaches: 1) transducing MDSCs with sFlt1, which binds to and blocks VEGF in the extracellular space from both the donor and the host or 2) transducing MDSCs with specific shRNA targeting VEGF (shRNA_VEGF MDSCs), which degrades the mRNA of VEGF, and effectively reduces VEGF secretion from only the donor cells while not affecting host cell VEGF secretion. This allowed us to compare the overall reduction of VEGF within the host tissue (sFlt1-MDSCs) with the reduction of VEGF secretion only from donor cells (shRNA_VEGF MDSCs). We found that mechanical stimulation of lacZ-MDSCs significantly increased angiogenesis in the engraftment area, which was not observed with mechanically stimulated

sFlt1-MDSC or shRNA_VEGF MDSCs. Transplantation of shRNA_VEGF MDSCs resulted in fewer dystrophin positive myofibers. There was also a decrease in the in vitro myogenic differentiation and adhesion capacity of shRNA_VEGF MDSCs. Taken together, our results support the hypothesis that VEGF is integral and critical to the increased angiogenesis observed following transplantation of MS-MDSCs into dystrophic skeletal muscle and that inhibiting VEGF secretion in the transplanted MDSCs resulted in loss of the beneficial effects of mechanical stimulation on the regenerative potential of MDSCs.

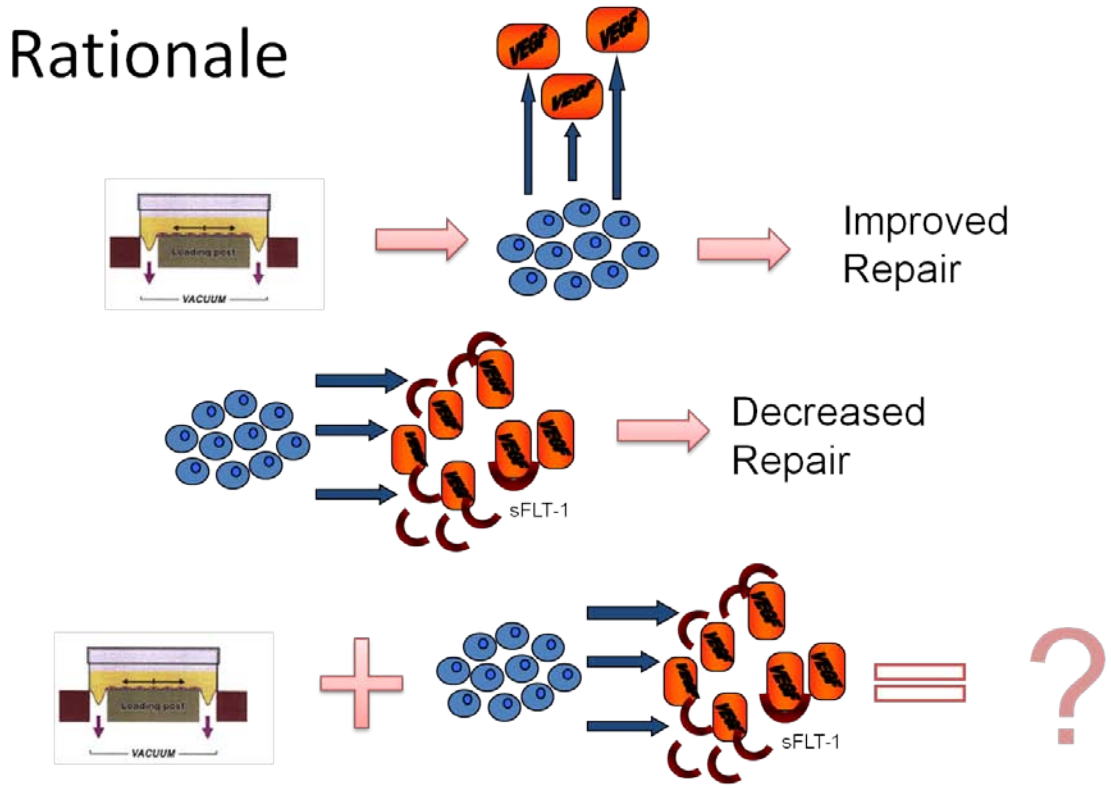

Figure 9: Rationale for VEGF-Stretch Study

4.2 METHODS

Animal studies: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. As such, all animal studies conformed to the Guide for the Care and Use of Laboratory Animals as published by the National Institute of Health.

MDSC isolation: As previously described**,** the pre-plate technique was used to isolate MDSCs from the skeletal muscle of three week old normal C57BL mice (Jackson, Bar Harbor, ME) (Oshima et al. 2005). MDSCs were cultured in proliferation media (PM) containing Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 10% horse serum (HS), 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical, Westbury, NY).

MDSCs transduced with a retrovirus carrying the *LacZ* reporter gene or *sFLT or shRNA to VEGF*: MDSCs were transduced with a retrovirus encoding the *LacZ* or *sFlt1* gene. A retroviral vector containing a modified *LacZ* gene with a nuclear localization sequence has been used previously in our laboratory (Peng et al. 2005; Payne et al. 2007; Deasy et al. 2009). The retroviral vector encoding human *sFlt1* (Invitrogen) containing a retro-backbone of pCLX driven by the hCMV/LTR promoter was derived from pLXSN (Clontech) as described previously (Peng et al. 2002). Based on a retro-CL vector named retro-CLB4G (Peng et al. 2004), we removed the *BMP4* gene and IRES-GFP cassettes by digesting with BgI *II* and Not *I* enzymes, and the purified retro-backbone was filled by klenow for ligation with an insert with a size of 1.7 kb from the recombinant adeno-associated viral vector digested by the same enzymes and also filled by klenow. This insert comprises of the not only the human U6 driving shRNA cassette that targets to the 198 – 216 region of mouse VEGF mRNA (Ambion #240535), but

also contains the CMV promoter controlling the ZsGreen reporter gene to monitor virus transduction efficiency. The detailed sequence of mVEGF/shRNA includes 19 bp sense (5'-CGAGATAGAGTACATCTTC), 9 bp loop (5'-TTCAAGAGA) 19 bp antisense (5'-GAAGATGTACTCTATCTCG).

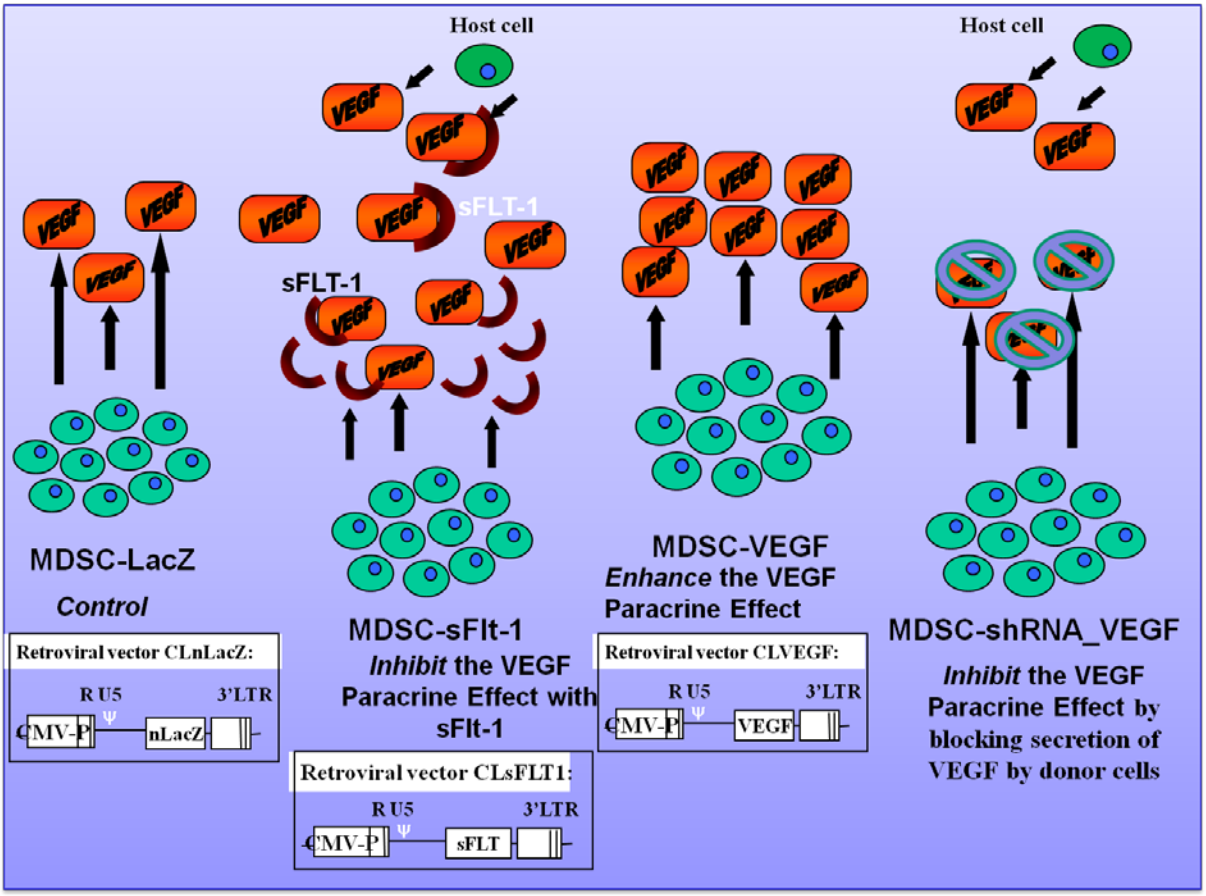

Figure 10: Schematic of vectors used in this study.

Mechanical Stimulation: MDSCs were cultured on bioflex plates-- flexible 6-well culture plates coated with collagen type I (105 cells/mm^2, Flexcell Intl. Corp.). After 12 hours of culture, an FX-4000T strain unit subjected the cells to 10% equibiaxial strain with a 0.5 Hz sine wave for 24 hours (Byrne et al. 2005; Payne et al. 2007). Control MDSCs were cultured on the

same plates without strain. The mechanical stimulation parameters were chosen based on previous studies which indicated increased VEGF secretion under these conditions in vitro (Gruden et al. 1999; Seko et al. 1999; Payne et al. 2007).

VEGF and sFlt1 secretion: MDSCs were plated in PM at 105 cells/mm^2 in a 6-well collagen type I coated plates. Prior to collection, the media was switched to DMEM with 1% penicillin/streptomyocin for 24 hours. Enzyme-linked immunosorbent assays (ELISAs) for VEGF or sFlt1 (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's instructions and as previously described (Payne et al. 2007). VEGF levels were normalized to cell number. The mechanical stimulation parameters were chosen based on previous studies which indicated increased VEGF secretion under these conditions in vitro (Gruden et al. 1999; Seko et al. 1999; Payne et al. 2007).

Western Blot: Cell lysates were prepared in RIPA buffer (#9806, CellSignaling Technology, Inc., Danvers, MA) supplemented with protease (P8340) and phosphatase inhibitors (P5726 and P0044, 1:100, Sigma-Aldrich, St. Louis, MO) and quantified using the Bio-rad Protein Assay Kit 2 (#500-0002, Bio-Rad, Hercules, CA). Membranes were incubated with polyclonal rabbit anti-VEGF (Ab46154, Abcam, Cambridge, MA, USA, 1:1000) at 4°C overnight in 5% milk or BSA in TBST. Following washing in TBST, Membranes were incubated with the secondary antibody, HRP-conjugated polyclonal goat anti-rabbit (#31460, Thermo Fisher Scientific, Rockford, IL). In order to ensure equal loading, membranes were probed with mouse HRP-conjugated rabbit anti-GAPDH (ab9482, Abcam) or stained with Ponceau S (P7170, Sigma-Aldrich).

Cell transplantation: For each replicate, 300,000 MDSCs in 20µl PBS were transplanted into each gastrocnemius muscle of 4-6 week old male *mdx/scid* mice (Jackson, Bar Harbor, ME).

Before transplantation the mice were anesthetized with 2-4% isoflurane in O_2. After 2 weeks the muscles were collected, flash frozen in liquid-nitrogen cooled methyl-2-butane and cryosectioned to 10µm thickness, as previously described (Deasy et al. 2009).

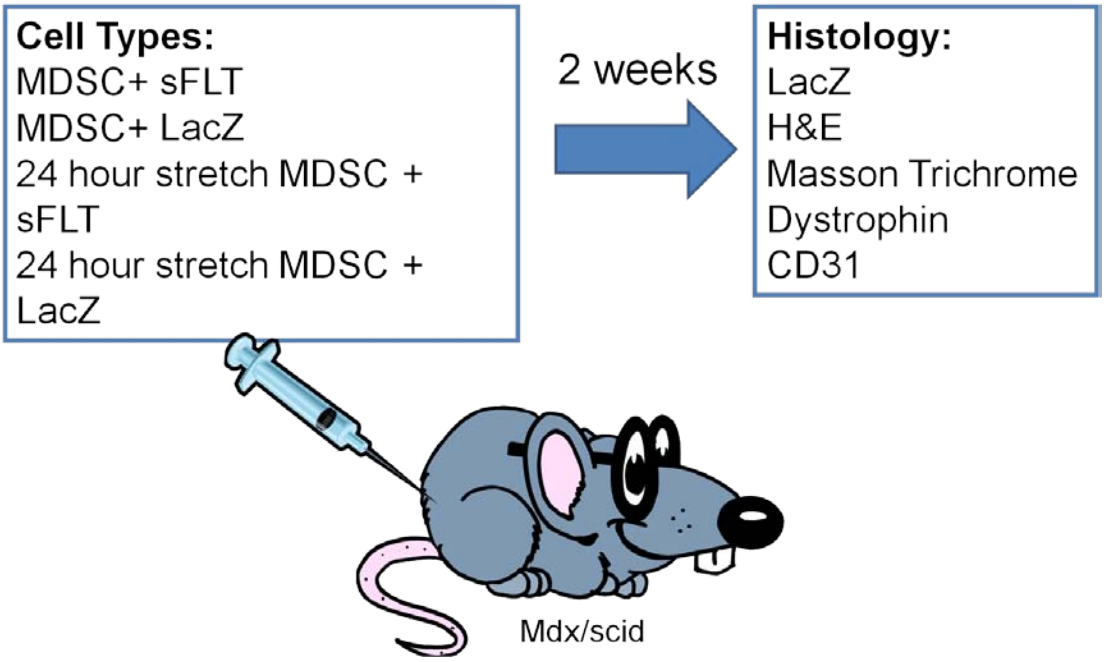

Figure 11: Injection schematic

Histology and Immunohistochemistry: Masson Modified IMEB Trichrome (IMEB, San Marcos, CA), which stains both collagen (blue) and muscle (red), was performed according to the manufacturer's guidelines following a protocol previously described by our research group (Oshima et al. 2005; Payne et al. 2007; Drowley et al. 2009). The sections were assessed for the percent area of collagen in 3 sections per muscle which were normalized to total muscle area using CellProfiler image analysis software (www.cellprofiler.com). Briefly, each image was unmixed to generate a "blue" and "red" grayscale image. These images were then thresholded to identify the appropriate areas and the identified area was measured (Lamprecht et al. 2007;

Luciana Barros Sant'Anna 2011). Percent center-nucleation was determined by H&E staining which was performed according to a previously described protocol (Deasy et al. 2009). The number of CD31 positive cells in the engraftment area was determined by staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma, St. Louis, MO) and goat anti-rat Alexafluor 594 secondary antibody (1:300, Invitrogen). Dystrophin was stained with rabbit anti-dystrophin (1:300; Abcam, Cambridge, MA) and donkey anti rabbit Alexafluor 488 (1:300, Invitrogen). Endothelial cells within the dystrophin positive and distal areas and were determined by counting the number of CD31 positive cells per high powered field in 3 fields per muscle using image J software (NIH, Bethesda, MD).

Proliferation and motility: MDSCs were plated in PM at a density of 10 cells/mm^2 in a 24-well collagen type-I coated plate. Twenty-four hours later, the media was switched to PM, or PM containing 250μM hydrogen peroxide (H_2O_2). The plates were placed onto a live cell imaging system (LCI, Kairos Instruments, LLC, Pittsburgh, PA), and bright field images were taken every 10 minutes over a 60 hour period in 3 locations per well (Schmidt et al. 2008). Images were analyzed with ImageJ software. Cell proliferation was determined by counting the number of cells present in the bright field images at 12 hour intervals, and cell numbers were normalized. Cell motility was analyzed using the ImageJ plug-in Manual Tracking, and 3 cells were analyzed per well for total distance traveled.

Wound Healing (Migration) Assay: MDSCs were plated in PM at a density of 10^5 cells/mm^2 on 12 well collagen type-I coated plates until confluent. A single straight wound was created in the center of each well by scratching the cell monolayer with the tip of a sterile 5 ml serologic pipette. Plates were washed twice with PBS and subsequently placed on the LCI system described above in PM. Each population was cultured in duplicate wells and

photographed (3 pictures per well) every 10 minutes for 18 hours. The entire assay was repeated 3 times independently. LCI images were analyzed using Image J. The distance between cells on either side of the wound at wound creation (D_0) and end point (D_1) were randomly measured 3 times per picture. Migration rate (%) was calculated according to the following equation: ((D_0-D_1)/ D_0)*100.

Differentiation: MDSCs were plated at 50 cells/mm^2 on a 24-well collagen type-I coated plates. Twenty-four hours later, the media was changed to DMEM + 2% FBS. At five days, the plates were stained with mouse anti-fast skeletal myosin heavy chain antibody (fsMHC, 1:400; Sigma,) and 4',6-diamidino-2-phenylindole (DAPI, for nuclei). The percentage of nuclei in fsMHC+ myotubes compared to total DAPI positive nuclei was quantified to assess myotube formation.

Adhesion: MDSCs were plated at a density of 316 cells/mm^2 on 96-well collagen type-I coated plates. After 30 minutes cells were washed with ice cold PBS then fixed in cold methanol for 10 minutes. Adherent cells were stained with 0.5% Crystal Violet for 20 minutes and then rinsed in tap water and let to air dry. Cells were solubilized in methanol and absorbance at 540 nm was determined on a spectrophotometer. The absorbance obtained from wells in the absence of cells was subtracted from all data points.

Microscopy: Fluorescence and bright field microscopy were performed using either a Nikon Eclipse E800 microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix Imaging, Cheektowaga, NY) or a Leica DMIRB inverted microscope with a Retiga digital camera and Northern Eclipse software.

Statistical analysis: The means and standard errors were calculated for all measured values, and statistical significance between the groups was determined by a 1-way ANOVA

(SPSS). In the event of a significant ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (S-N-K or Games-Howell).

4.3 RESULTS

In vitro VEGF secretion and expression of mechanically stimulated MDSCs

To examine the effect of sFlt1 and shRNA to VEGF on the secretion of VEGF after mechanical stimulation of MDSCs, cells were genetically modified to express either the soluble VEGF receptor (sFlt1-MDSCs) (Payne et al. 2007; Deasy et al. 2009) or shRNA to VEGF (shRNA_VEGF-MDSCs) in order to block VEGF secretion. Control MDSCs were transduced to express the *lacZ* reporter gene (lacZ-MDSCs). VEGF secretion from transduced cells was evaluated in vitro after 24 hours of mechanical stimulation. VEGF secretion of lacZ-MDSCs, measured by ELISA, was significantly increased after mechanical stimulation (Figure 12A, n=4 *P<0.05). There was no statistical difference in VEGF secretion among the sFlt1-MDSC and shRNA_VEGF MDSC groups, regardless of mechanical stimulation (Figure 12A); however, their VEGF secretion levels were lower than the lacZ-MDSCs (Figure 12A). Cellular VEGF expression was also examined by western blot (Figure 12 B-E). We found an increase in cellular VEGF levels from mechanically stimulated lacZ-MDSCs compared to NS lacZ-MDSCs (Figure 12B) and this was significant when normalized to GAPDH (Figure 12C, n=3, *P<0.05). As expected, we found that under normal conditions, cellular VEGF levels were decreased by sFLT1 or shRNA-VEGF expression (Figure 12D). Furthermore, following stretch, we found that the sFLT1 or shRNA-VEGF expression abrogated the stretch-induced VEGF increase (Figure 12E). This suggests that the increased VEGF secretion observed by ELISA is at least in part due

to increased VEGF expression, although we cannot totally rule out the effect of release of membrane associated heparin-binding VEGF (Ashikari-Hada et al. 2005; Krilleke et al. 2009) that could be released upon mechanical stimulation.

We also assayed the increased sFlt expression from sFlt1-transduced MDSCs and found a 6x increase in sFlt1 secretion from transduced MDSCs (Figure 13).

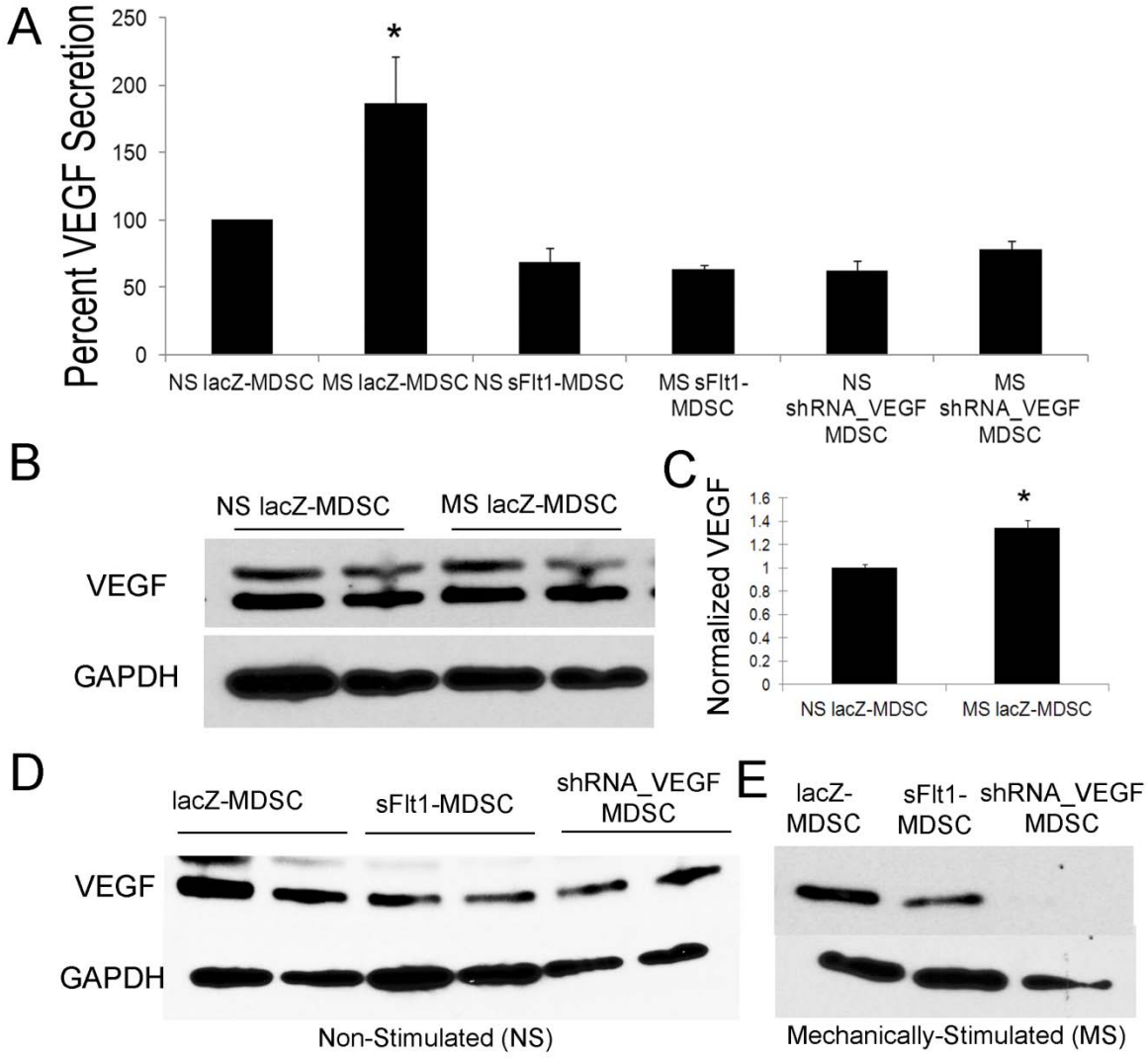

Figure 12: Secreted and cellular VEGF

(A) VEGF secretion was analyzed with ELISA and normalized to percent NS lacZ-MDSC. VEGF secretion was significantly increased after mechanical stimulation (n=12, *P<0.05). (B) Cellular VEGF in the NS and MS lacZ-MDSC groups is shown. (C) Cellular VEGF levels in lacZ-MDSCs were significantly increased after mechanical stimulation (n=3, *P<0.05) (D) Cellular VEGF in NS lacZ-MDSC, sFlt1-MDSC and shRNA-VEGF

MDSC groups was analyzed with western blot. GAPDH is shown as loading control. (E) Cellular VEGF in MS lacZ-MDSC, sFlt1-MDSC and shRNA_VEGF MDSC groups is shown.

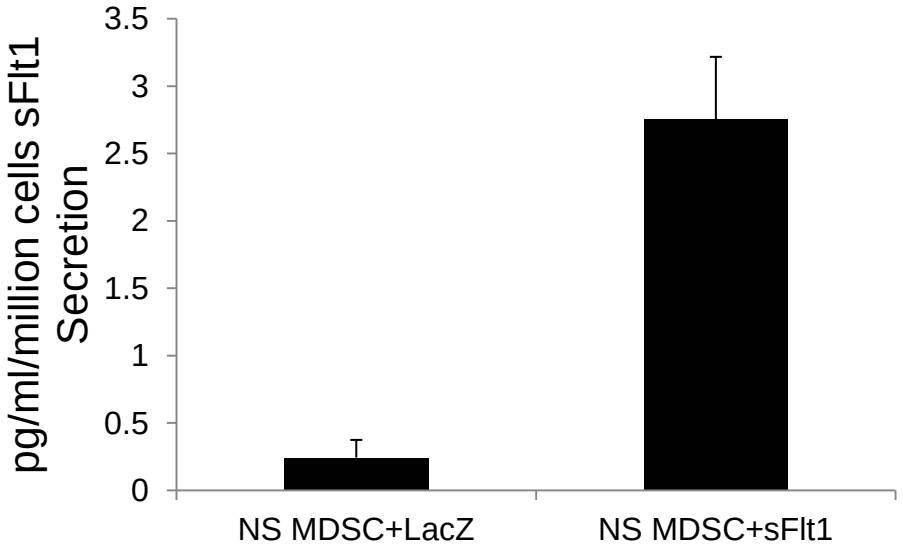

Figure 13: sFlt1 secretion from NS MSDC and sFlt1-MDSC

Inhibition of VEGF secretion increases tissue fibrosis in dystrophic muscle

We next aimed to determine the effect of blocking VEGF on MDSC transplantation in dystrophic muscle. Three hundred thousand cells from each of the six treatment groups were injected into gastrocnemius muscle of *mdx/scid* mice. These mice are a model of muscular dystrophy that are both dystrophin deficient and are immune-compromised, and a useful model for cell transplantation. Muscle regeneration is often hindered by the formation of fibrotic tissue (Serrano et al. 2010). To address how VEGF secretion and mechanical stimulation of MDSCs might affect fibrosis levels and muscle regeneration after cellular transplantation into dystrophic tissue, fibrosis levels were determined by Masson's trichrome staining and regeneration was examined by H&E staining. Fibrosis levels were higher in muscles transplanted with sFlt1-MDSCs or shRNA_VEGF-MDSCs as compared to lacZ-MDSCs, independent of mechanical stimulation (Figure 14G, n=6 *$P<0.05$). The percent centrally nucleated fibers were analyzed and

there was significant reduction in fiber regeneration in the muscles transplanted with MDSCs where VEGF was blocked (Figure 14H, n=6 *P<0.05). These results are consistent with our previous finding that blocking VEGF in dystrophic tissue by transplanting sFlt1-MDSCs led to increased levels of fibrosis (Deasy et al. 2009), and further suggests that VEGF produced by the transplanted MDSCs reduced fibrotic scarring in the recipient mice and increased muscle regeneration.

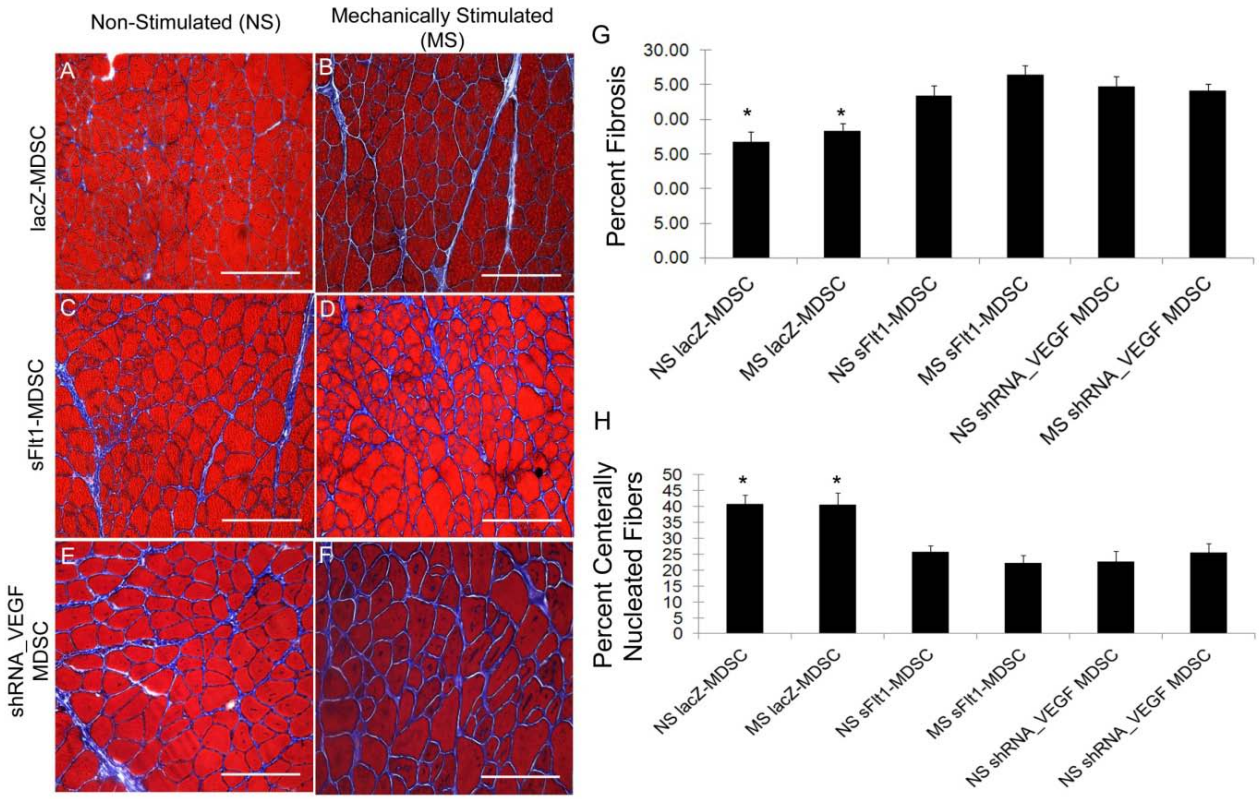

Figure 14: Fibrosis and muscle regeneration

(A-F) Masson's trichrome staining of representative images of NS-MDSC, MS-MDSCs, NS sFlt1-MDSCs , MS sFlt1-MDSCs, NS shRNA_VEGF MDSCs, and MS shRNA_VEGF MDSCs, respectively. Red represents muscle and blue represents collagen formation (fibrosis). Scale bar = 0.2mm. The percent blue staining was quantified. (G) There was significantly increased fibrosis in muscles transplanted with sFlt1-MDSCs and shRNA_VEGF-MDSCs compared to lacZ-MDSCs (n=6, *P<0.05). (H) The percentage of centrally nucleated fibers was quantified and there was significantly greater regeneration in the lacZ-MDSC groups compared to the other groups (n=6, *P<0.05).

The pro-angiogenic effect of mechanical stimulation is abolished when VEGF is blocked

Two weeks following transplantation, dystrophin positive myofibers (green) and CD31 positive capillaries (red) were immunohistochemically detected (Figure 15 A-F). Dystrophin is a marker for donor-derived regenerating myofibers and CD31 (or platelet endothelial cell adhesion molecule) is a marker for endothelial cells. Within the engraftment area, the MS lacZ-MDSC group had significantly greater CD31 structures compared to all other groups (Figure 15G, n=6 *P<0.05). In addition, all four groups with VEGF blockage had significantly less CD31 structures when compared to control lacZ-MDSCs (Figure 15G, n=6 $^{\&}$P<0.05). To further examine the paracrine effects of the implanted cells, angiogenesis distal to the engraftment area was analyzed. Similarly, all four groups with VEGF blockage exhibited significantly less CD31 positive structures in areas distal to the engraftment site when compared to the NS and MS lacZ-MDSC groups (Figure 15H, n=3 *P<0.05). To take into account variability in the level of skeletal muscle regeneration, we also quantified the number of CD31 positive structures adjacent to dystrophin positive muscle fibers. This ratio was significantly greater in the MS-MDSCs transplantation group as compared to NS-MDSCs and both MS and NS sFlt1-MDSCs (Figure 15I n=6 *P<0.05). There was no difference in the CD31/Dystrophin ratio between MS and NS sFlt1-MDSCs (Figure 15I). There was no difference in the CD31/Dystrophin ratio between NS and MS shRNA_VEGF MDSCs, however this ratio was significantly greater than the ratios of the other groups (Figure 15I, $^{\#}$P<0.05). This is likely due to the fact that, as we will discuss in the next section, the dystrophin positive fiber engraftment is significantly reduced in the shRNA_VEGF transduced MDSC group. Overall, this suggests that the pro-angiogenic effect of

mechanical stimulation on MDSCs is abrogated when VEGF is blocked, as there was no difference in angiogenesis between the stimulated and non-stimulated groups, but there was an increase in angiogenesis after MS in the control group.

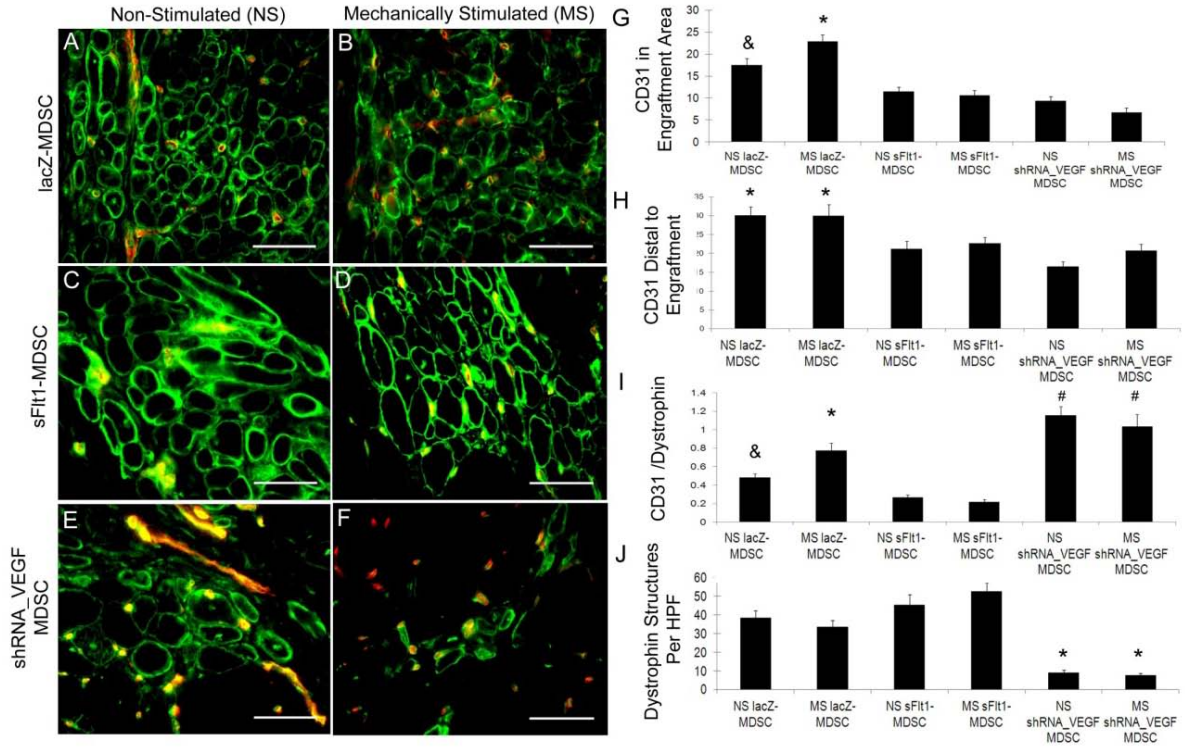

Figure 15: Angiogenesis and dystrophin positive myofiber engraftment

MDSCs were transplanted into the gastrocnemius muscles of *mdx/SCID* mice. Two weeks later CD31 and dystrophin expression were quantified. Green staining represents dystrophin and red staining represents CD31. (A-F) Representative pictures of transplantation of NS-MDSC, MS-MDSCs, NS sFlt1-MDSCs, MS sFlt1-MDSCs, NS shRNA_VEGF MDSCs, and MS shRNA_VEGF MDSCs, respectively. Scale bar = 0.05 mm. (G) CD31 was quantified in the engraftment area and there was increased CD31 expression after mechanical stimulation of lacZ-MDSCs (n=6, *P<0.05 to all other groups), as well as increased CD31 expression in the NS lacZ-MDSC group compared to the MS and NS sFlt1-MDSC and shRNA_VEGF MDSC groups ($^{\&}$P<0.05). (H) CD31 distal to the engraftment area was also quantified and their was increased CD31 expression in the lacZ-MDSC groups compared the other groups (n=3, *P<0.05). (I) The CD31/Dystrophin ratio was quantified and this was increased in the MS lacZ-MDSC group compared to the NS lacZ-MDSC group and the sFlt1-MDSC groups (n=6, *P<0.05). There was also an increased ratio in the NS lacZ-MDSC group compared to the sFlt1-MDSC groups ($^{\&}$P<0.05). The CD31/Dystrophin ratio was increased in the shRNA_VEGF MDSC groups compared to all other groups ($^{\#}$P<0.05). (J) Quantification of dystrophin positive myofiber engraftment. The NS and MS shRNA_VEGF MDSC transplantation groups had significantly less dystrophin positive engraftment than all other groups. (n=6, *P<0.05 to NS/MS MDSC, NS/MS sFlt1-MDSC.)

Dystrophin positive fiber engraftment is reduced after transplantation of shRNA_VEGF-MDSCs, but not sFlt1-MDSCs

To further investigate whether the innate myogenicity of MDSCs was influenced by the inhibition of VEGF secretion, we quantified the regenerating dystrophin-positive myofibers within the engraftment area. Dystrophic muscles implanted with shRNA_VEGF-MDSCs had significantly reduced dystrophin positive myofiber regeneration (NS: 9±1, MS: 7±1) compared with lacZ-MDSCs (NS: 38±4, MS: 34±3) and sFlt1-MDSCs (NS: 45±6, MS: 52±5); this was independent of mechanical stimulation (Figure 15J, *$P<0.05$). Moreover, dystrophin positive fiber engraftment was significantly reduced after transplantation of shRNA_VEGF-MDSCs, but not sFlt1-MDSCs, suggesting that internal autocrine VEGF signaling may play a role in MDSC engraftment into dystrophic muscle.

shRNA_VEGF transduction does not decrease MDSC proliferation or motility

The decrease in the number of dystrophin-positive myofibers observed after transplantation of MSDCs lacking VEGF could possibly be due to either a failure of proliferation, an impaired adhesion/migration, or defective myogenicity of engrafted cells. To further investigate the underlining mechanism(s), we examined the in vitro proliferative, migratory, and myogenic capacities of these cells.

Previous reports showed increased oxidative stress in dystrophic muscle compared to wild-type controls (Ragusa et al. 1997; Disatnik et al. 1998). Consequently, we examined the proliferation and motility of MDSCs in normal and oxidative stress culture conditions. Proliferation medium supplemented with H_2O_2 was used to model oxidative stress. Cells were monitored for 60 hours on a LCI system, and cell number was quantified every 12 hours. The proliferation of shRNA_VEGF MDSCs in normal media was significantly greater than sFlt1-

MDSCs but not significantly different from the lacZ-MDSCs (Figure 16A, n=12 *P<0.05). The proliferation of MDSCs under oxidative stress was not affected by shRNA_VEGF or sFlt1 transduction (Figure 15B). Cellular motility was assessed based on the total distance traveled of individual cells. The motility of MDSCs was not affected by blocking VEGF, however in the MS sFlt1-MDSC group and MS shRNA_VEGF group motility was decreased after exposure to oxidative stress conditions (Figure 16C, *P<0.05, n=12 per group). This suggests that the shRNA_VEGF MDSCs do not have a defect in their in vitro proliferative abilities but that the combination of blocking VEGF, mechanical stimulation and oxidative stress reduces MDSC motility.

Blocking VEGF decreases MDSC migration

Next we examined the effect of mechanical stimulation and blocking VEGF on the migration and adhesion of MDSCs. A wound healing assay was performed where MDSCs were grow until confluent and then scratch-wounded with a pipette. We analyzed percent wound closure after 18 hours of incubation and found that all MS groups tended to have slower wound healing compared to NS groups, particularly in the shRNA_VEGF MDSC group (Figure 65D, n=6, $^{\&}$P<0.05 between NS and MS shRNA_VEGF MDSCs). Blocking VEGF secretion in general also decreased wound healing as all groups had significantly slower migration compared to the NS lacZ-MDSC group (Figure 16D *P<0.05). This suggests that blocking VEGF decreases MDSC migration time.

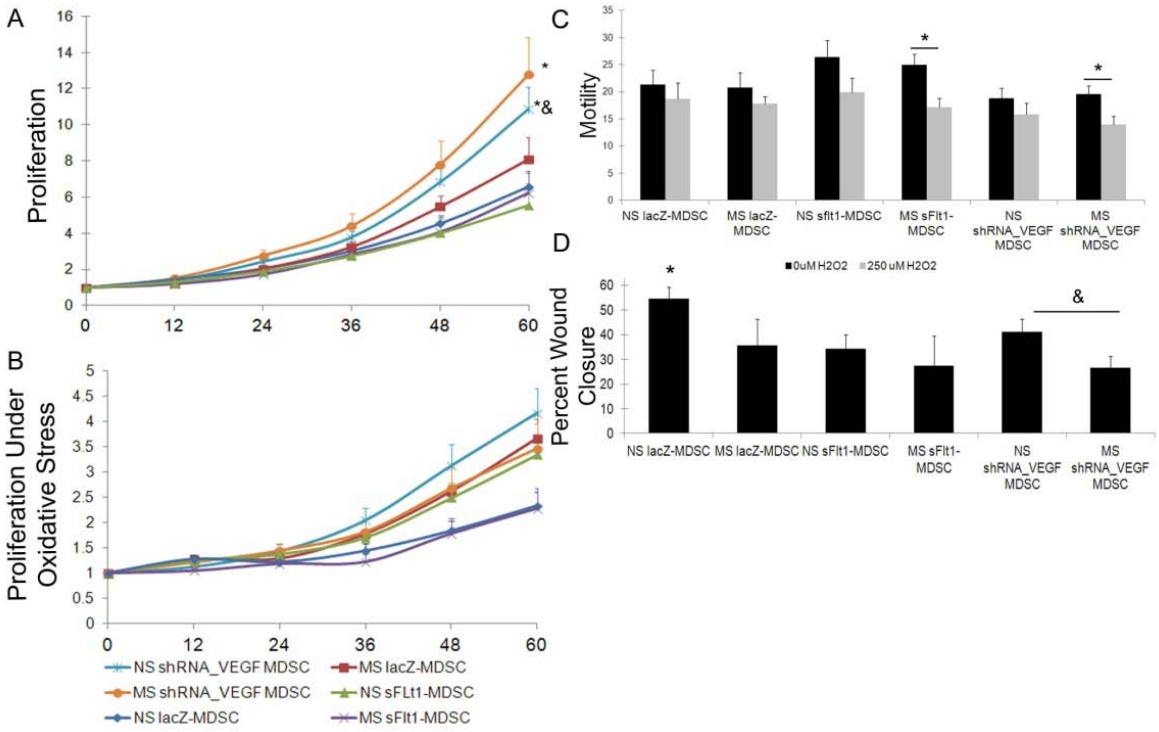

Figure 16: In vitro proliferation, motility and migration of MDSCs

MDSCs were seeded onto collagen-coated plates and put on a live cell imager. Bright field pictures were analyzed every 12 hours to determine cellular proliferation. (A) Proliferation under normal conditions in proliferation media (n=12, *P<0.05 to NS sFlt1-MDSC, $^{\&}$P<0.05 to MS sFlt1-MDSC). (B) Proliferation under oxidative stress conditions of 250 μM H_2O_2 in proliferation media. (C) Motility under normal and oxidative stress conditions. No significant differences between groups under normal conditions or under oxidative stress conditions (n=12 *P<0.05 between MS sFlt1-MDSCs grown under normal and oxidative stress conditions and between MS shRNA_VEGF MDSCs groups under normal and oxidative stress conditions). (D) NS lacZ-MDSCs closed a scratch wound more rapidly than all other groups (n=6, *P<0.05). There was a significant difference between shRNA_VEGF MS and NS wound closure ($^{\&}$P<0.05).

In vitro adhesion and myogenic differentiation of MDSCs is impaired by shRNA silencing VEGF, but not by sFlt1 expression

Adhesion was analyzed by plating MDSCs on collagen coated dishes and then rinsing away non-adherant cells after 30 minutes. The remaining attached cells were detected by crystal violet staining. There was significantly lower adhesion in the MS shRNA_VEGF MDSC groups compared to all other groups (Figure 17A, n=12 *P<0.05). The NS shRNA_VEGF group also had significantly lower adhesion than all groups except the MS lacZ-MDSC and MS

shRNA_VEGF MDSC groups (Figure 17A, n=12, $^{\&}P<0.05$). Blocking VEGF with shRNA decreased the adhesion of MDSCs and this was augmented after mechanical stretch. To determine the myogenic capacity of these cells, we compared the ability of genetically modified MDSCs mechanically stimulated or non-stimulated to form myotubes in low serum differentiation media for 5 days. Subsequently we fixed and stained cells for fast-skeletal myosin heavy chain (fsMHC) and quantified the myogenic index, the number of fsMHC-positive nuclei normalized to the total number of nuclei. We observed drastically fewer fsMHC-positive myotubes formed by shRNA_VEGF-MDSCs compared to the other groups (Figure 17B, n=15 *P<0.05). Altogether, these results suggest that the low dystrophin positive engraftment of shRNA_VEGF MDSCs may be due to a deficiency in myogenesis, possibly related to an autocrine reduction of VEGF, as there was no deficiency in the myogenesis of the sFlt1-MDSCs.

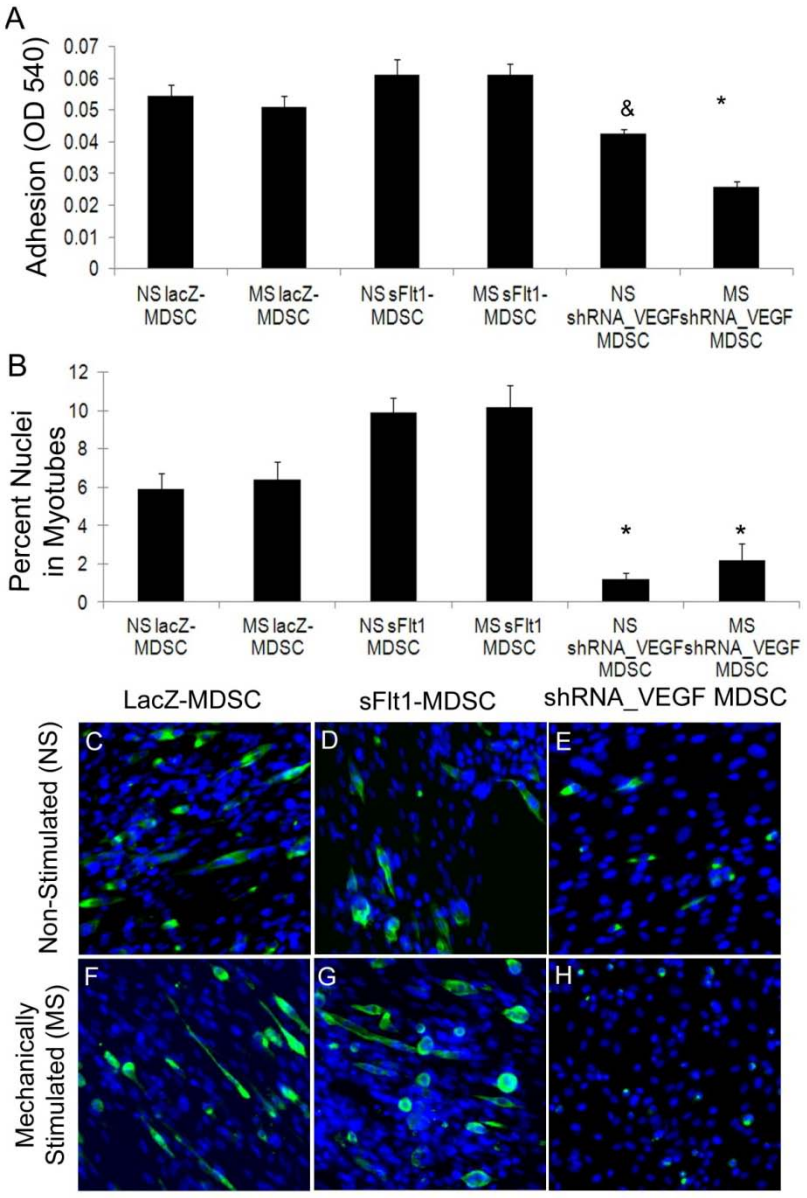

Figure 17: In vitro adhesion and myogenesis of MDSCs

(A) Adhesion was analyzed by quantifying how many cells remained attached to collagen coated flasks after 30 minutes of culture. The adhesion of MS shRNA_VEGF MDSCs was significantly lower than all other groups (n=12, *P<0.05). The adhesion of NS shRNA_VEGF MDSCs were significantly lower than all other groups except MS lacZ-MDSC ($^{\&}$P<0.05). (B) MDSCs were seeded onto collaged-coated plates and changed to differentiation medium after 24 hours in PM. After 5 days myotube formation was assessed by staining for fast skeletal myosin heavy chain (fsMHC). The percentage of fsMHC positive nuclei was determined, and there was decreased myotube formation in the shRNA_VEGF MDSC groups (n=13 *P<0.05 to NS/MS MDSC, NS/MS sFlt1-MDSC). (C-E) NS lacZ-MDSC, sFlt1-MDSC, shRNA_VEGF MDSC. (F-H) MS lacZ-MDSC, sFlt1-MDSC, shRNA_VEGF MDSC

4.4 DISCUSSION

Stem cell therapy has enormous potential for tissue regeneration (Cossu et al. 2007; Segers et al. 2008). However, to further improve this therapy, methods to harness stem cell potential and overcome obstacles such as limited differentiation and survival of transplanted cells are necessary. This study focused on understanding the underlying mechanism(s) of mechanical stimulation through VEGF blockage in order to further enhance the function of stem cells in tissue repair and regeneration.

Cells are often subjected to mechanical stimulation in their native environment: hearts beat, lungs inflate, muscles stretch, and cells react to the resultant mechanical load. Mechanical stretch is a powerful stimulus for a broad spectrum of cellular responses including growth, differentiation, motility, remodeling, and gene expression (Trepat et al. 2007; Shyu 2009; Li et al. 2011). Previous studies demonstrated that mechanically stimulated MDSCs expressed more VEGF and were able to repair the infarcted murine heart more effectively than non-mechanically stimulated MDSCs, which was attributed, at least in part, to an increase in angiogenesis in the peri-infarct area (Cassino et al. 2012).

Additionally, blocking VEGF with sFlt1-MDSCs abrogated the benefits of MDSCs in both skeletal muscle regeneration and cardiac repair (Payne et al. 2007; Deasy et al. 2009). Unlike gene therapy where excessive, continuous VEGF expression may cause disorganized vascular structures and is consequently detrimental to tissue repair, (Payne et al. 2007; Franco et al. 2011) mechanical loading physiologically pre-conditions MDSCs and therefore avoids unwanted side-effects of the genetically enhanced VEGF-expressing MDSCs. These studies indicate a critical magnitude of VEGF secretion by MDSCs that can be induced through appropriate mechanical stimulation during the tissue repair.

Due to the important role of VEGF in MDSC-mediated tissue regeneration, we examined the effect of this crucial growth factor on MDSC therapeutic function by inhibiting both host and donor-derived VEGF in sFlt1-MDSCs and specifically reducing the donor-derived VEGF in shRNA_VEGF-MDSCs. SFlt1 binds VEGF with the same affinity and specificity of the full length receptor but does not initiate signaling as it is not cell-associated due to the lack of a tyrosine domain. SFlt1 both sequesters VEGF and hetero-dimerizes with receptors, blocking VEGF signaling in two ways (Kendall et al. 1993; Kendall et al. 1996). VEGF is expressed uniformly in adult murine skeletal muscle, (Maharaj et al. 2006) therefore sFlt most likely reduces concentration of total VEGF in the extracellular space but does not completely abrogate it.

Previous studies have shown a decrease in fibrosis and muscle necrosis with sustained VEGF secretion from gene or cell therapy (Messina et al. 2007). However, when MS-MDSCs were transplanted into an infarcted heart they did not significantly affect fibrosis levels compared to NS-MDSC (Cassino et al. 2012). We examined collagen formation in muscles implanted with sFlt1-MDSCs, shRNA_VEGF-MDSCs, or lacZ-MDSCs, and found increased fibrosis in all groups where VEGF was blocked. This was independent of mechanical stimulation, and appears to be associated with the overall reduced levels of VEGF. Additionally, we did not observe a decrease in fibrosis in the mechanically stimulated groups, which is consistent with our previous results in the heart (Cassino et al. 2012) and may be attributed to the transient and reversible increase in VEGF secretion through mechanical pre-conditioning which may not sufficient to affect the long-term remodeling process.

Angiogenesis is a critical aspect of tissue repair, and VEGF is a potent growth factor involved in this process(Byrne et al. 2005). We observed when lacZ-MDSCs were mechanically

stimulated, their potential to promote angiogenesis increased compared to un-stimulated lacZ-MDSCs. However, when VEGF secretion was blocked with sFlt1 or shRNA, there was no difference in angiogenesis between the MS and NS groups. This suggests that the favorable response to mechanical stimulation is abrogated regardless of the method of VEGF blockage, and that VEGF secretion by donor cells is a significant factor affecting the increase in angiogenesis

Although VEGF is well-known as a mediator of angiogenesis, there is increasing evidence suggesting a role in other cellular functions, including skeletal muscle regeneration. For example, intramuscular administration of rAAV-VEGF into the *mdx* mouse promoted skeletal muscle regeneration and enhanced muscle function (Messina et al. 2007). Also, Deasy el al. demonstrated that MDSCs expressing varying levels of VEGF had greater numbers of centrally-nucleated fibers compared to control MDSCs (Deasy et al. 2009). In the current study, we demonstrated that blocking VEGF resulted in decreased numbers of centrally-nucleated fibers. VEGF can also prevent death of donor cells; pre-treating the hind limb muscles of mice with VEGF prior to myoblast transplantation resulted in reduced death of donor cells and improved their engraftment (Bouchentouf et al. 2008). Moreover, Arsic et al. observed that VEGF promoted the fusion of myogenic cells to form myotubes and protected them from apoptosis (Arsic et al. 2004).

In this study, we observed an effect on the differentiation of transplanted MDSCs when VEGF was decreased with shRNA. There were significantly fewer dystrophin-positive fibers in the shRNA_VEGF-MDSC transplantation groups compared to the lacZ-MDSC and sFlt1-MDSC groups, indicating that VEGF produced by the transplanted cells is important for their function and capacity to regenerate myofibers in dystrophic muscle. This result is in accord with previous

studies where VEGF had an effect on autocrine myogenic differentiation; VEGF-null embryonic stem cells had reduced capacity to differentiate into skeletal muscle (Bryan et al. 2008). Furthermore, C2C12 cells transduced with AAV-sFLt1 had reduced in vitro myotube formation compared to controls (Bryan et al. 2008). However, in another study, C2C12 cells treated with VEGF or a small molecule to block receptor tyrosine kinase activity showed no difference in myotube differentiation(Germani et al. 2003). After VEGF blockage, we examined myogenic differentiation capacity in vitro and found that shRNA_VEGF-MDSCs formed fewer myotubes than lacZ-MDSCs and sFlt1-MDSCs, which is–consistent with our in vivo findings. Taken together, these results indicate a significant role for VEGF signaling in myogenic differentiation and muscle regeneration.

VEGF has been shown to act in an autocrine manner and has a protective/survival effect on many cell types including endothelial cells, embryonic stem cells, hematopoietic stem cells (HSCs) and myoblasts (Gerber et al. 2002; Germani et al. 2003; Brusselmans et al. 2005; Byrne et al. 2005). When HSCs were treated with small molecule inhibitors that blocked internal VEGF receptor signaling, their colony formation was significantly decreased, but treatment with sFlt1 did not have an affect (Gerber et al. 2002). Autocrine VEGF signaling was also shown to be critical for vascular homeostasis. When VEGF was conditionally knocked out in endothelial cells, there was an increase in endothelial cell death with no reduction in serum VEGF, indicating that paracrine VEGF levels could not compensate for the lack of VEGF within the endothelial cells (Lee et al. 2007). The fact that shRNA_VEGF-MDSCs had a reduced capacity for myogenic differentiation but sFlt1-MDSCs had normal differentiation potential suggests the possibility of an internal autocrine mechanism of VEGF signaling in MDSC differentiation. Angiogenesis and tissue regeneration require cells to proliferate and migrate (Zachary 2003).

Therefore, to further investigate the effect of blocking VEGF and mechanical stimulation on fundamental cellular functions of MDSCs, we examined in vitro cell proliferation, migration, motility, and adhesion. Our results indicate there is no defect in the proliferation of shRNA_VEGF-MDSCs. Previous studies have shown increased VEGF levels increased cellular proliferation (Deasy et al. 2009). In our study, mechanical stimulation resulted in trends of increased proliferation for each group compared to it's non-stimulated counterpart. We observed no change in cellular motility after mechanical stimulation or blockage of VEGF. Interestingly, we recorded a decrease in cellular motility after both mechanical stimulation and exposure to oxidative stress when VEGF was blocked, indicating that a combination of stresses on the MDSCs resulted in impaired motility. In addition, it has been reported that blocking VEGF decreased migration of muscle cells (Bryan et al. 2008). We observed similar results with MDSCs as there was decreased cell migration in the groups in which VEGF was blocked. Cellular adhesion is necessary for many biological and therapeutic processes such as angiogenesis and cellular therapy (Lavdas et al. 2011). In previous studies, VEGF induced an increase in adhesion between cardiomyocytes and extracellular membrane (Takahashi et al. 1999) and promoted endothelial cell adhesion(Hutchings et al. 2003). In our study, we observed a decrease in adhesion of MS and NS shRNA_VEGF-MDSCs, which may have contributed to their decrease in differentiation into dystrophin-positive myofibers.

In conclusion, we demonstrated that inhibiting VEGF secretion from MDSCs blocked the beneficial effects imparted by mechanical pre-conditioning of these cells prior to cell transplantation including reduced differentiation capacity in vitro and in vivo as well as decreased angiogenesis in the transplantation area. This study highlights the importance of donor-derived VEGF in MDSC-mediated muscle regeneration as well as suggests a potential role

of internal autocrine VEGF signaling in MDSC myogenic differentiation. Future studies should involve exploring the mechanisms of these signaling pathways and dissecting out the differences between paracrine and autocrine VEGF signaling in MDSCs.

5.0 CHARACTERIZATION OF DYSTROPHIC MDSCS

5.1 INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited neuromuscular disorder characterized by progressive muscle weakness due to loss of the membrane stabilizing dystrophin protein. Dystrophin is part of a protein complex responsible for the maintenance of cellular integrity and muscle cell signaling (Hoffman et al. 1987). Muscle membrane instability leads to cycles of muscle fiber necrosis and subsequent regeneration. Eventually, healthy muscle is replaced with fibrotic and fatty tissue which accounts for much of the muscle weakness seen in these patients, who are wheelchair bound by age eleven and eventually die from cardiac or respiratory failure in their early 30's (Hoffman et al. 1987).

The extensive muscle damage characteristic of DMD initially activates muscle satellite cells, resident stem cells that reside beneath the basal lamina. These progenitors are initiated in response to injury, exercise, and disease. At the beginning of a DMD patient's life, damaged muscle fibers are regenerated by native stem cells. However, after some time (about 5 years) the stem cell store is exhausted and the quality of cells is compromised; at this point the patient will begin to show proximal muscle weakness (Hoffman et al. 1987).

Intriguingly, the extra-ocular muscles of aged and muscular dystrophy patients are spared from the pathological sarcopenia and degeneration seen in the rest of the muscles

(Kallestad et al. 2011). Extra-ocular muscles develop separately from somite-derived muscle and continuously remodel throughout life, maintaining a population of activated satellite cells, even later in life (Kallestad et al. 2011). One of the reasons that dystrophic skeletal and cardiac muscles degenerate may be that their satellite cell pool is not maintained in this same manner.

It was initially hypothesized that replacing dystrophin through gene or cell therapy would cure this debilitating disease. However, gene replacement strategies have largely been un-successful for many reasons, one being the size of dystrophin (427 kDa) and the difficulty of delivering such a large protein. A cell-based therapy is challenging, as skeletal muscle is the most abundant tissue in the body and is composed of hundreds of millions of post-mitotic nuclei (Cossu et al. 2007). It would be difficult to deliver the necessary cells to all of the affected muscles of the patient, both because of the large number of donor cells required and the systemic dissemination to all of the muscles and not just spotty clusters as is usually observed following local injection. Current management of DMD uses steroid therapy to reduce inflammation; however, obviously this treatment does not replace dystrophin. Replacing dystrophin would increase the integrity of the damaged muscle cell membrane, but it would not replenish the stem cell pool

This study used two mouse models of muscular dystrophy. The first is the *mdx* mouse which arose from a spontaneous nonsense mutation in the dystrophin gene. *Mdx* mice lack the dystrophin protein, just like their human counterparts; however, the severity of the disease is much less in this mouse model compared to the human phenotype; *mdx* mice have a normal lifespan and do not develop cardiac complications until very late in life, unlike DMD patients who die from respiratory or cardiac failures in their late 20s.

An intriguing difference between the *mdx* mouse and the DMD patient is that humans have much shorter telomeres than mice (Sacco et al. 2010). When *mdx* mice are crossed with mice lacking telomerase activity their pathology becomes much more severe and more similar to the human disease (Sacco et al. 2010). Muscle stem cells isolated from these mice display a proliferation deficit both in vitro and in vivo. These results suggest that stem cell exhaustion in an important part of the pathology of DMD.

Another difference between the *mdx* mouse model and human DMD patients is the up-regulation of utrophin in *mdx* mice. Utrophin is a structural protein very similar to dystrophin, which, under normal circumstances, is up regulated during development. However, during normal adult life utrophin is confined to the neuromuscular junction (Khurana et al. 1991), but is co-distributed in cardiac muscle (Grady et al. 1997). It is possible that in the *mdx* mouse utrophin compensates for dystrophin, thus resulting in the more modest pathology of the *mdx* mouse (Tinsley et al. 1998; Janssen et al. 2005). We have begun to study the *mdx*/utrophin-/- (DKO-/-) mouse in which both dystrophin and utrophin are knocked out (Grady et al. 1997). DKO mice have a much more severe pathology than their *mdx* counterparts. DKO mice have a shorter life span, kyphosis, severe muscle wasting and cardiomyopathy. Their symptoms more closely align with those of human DMD patients.

Table 2: dKO vs *mdx*

	DKO	*mdx*
Genotype	Dys-/-, Utr -/-	Dys -/-, Utr+/+
Phenotype	Diabling, lethal, closer to patients	Mild Utrophin compensation
Muscle Wasting	Severe	Mild, pseudo hypertrophy
Kyphosis	Severe	Mild
Heart Failure	Severe	Mild
Life-threatening	Severe	mild
Lifespan	3 months	normal

In this study we aimed to compare wt-MDSCs with MDSCs obtained from *mdx* and DKO mice. We found that wt and *mdx* MDSCs do not have differences in proliferation, differentiation, or VEGF secretion. We compared DKO-/- MDSCs and DKO heterozygous (+/-) MDSCs and found that DKO-/- MDSCs had decreased proliferation, differentiation, and cell survival capabilities compared to DKO+/- MDSCs. Finally, we pre-treated DKO MDSCs with mechanical stimulation and increased their proliferation capabilities.

5.2 METHODS

Animal studies: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

As such, all animal studies conform to the Guide for the Care and Use of Laboratory Animals as published by the National Institute of Health.

MDSC isolation: As previously described**,** the pre-plate technique was used to isolate MDSCs from the skeletal muscle of three week old normal C57BL6 mice, *mdx* mice (Jackson, Bar Harbor, ME) and dystrophin-utrophin knockout (DKO) mice (Gharaibeh et al. 2008). MDSCs were cultured in proliferation media (PM) containing Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 10% horse serum (HS), 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical, Westbury, NY).

Cell survival under oxidative stress: MDSCs were plated in PM at 10 cells/mm^2 in a 24-well collagen type-I coated plate. Twenty-four hours later, the media was switched to PM with propidium iodide (PI, 1:500, Sigma, St. Louis, MO), or PM containing 100 or 250 μmol/L hydrogen peroxide (H_2O_2) with PI. The plates were next placed onto a previously described live cell imaging system (LCI, Kairos Instruments, LLC, Pittsburgh, PA) (Drowley et al. 2010), and fluorescent and bright field images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageJ software (NIH, Bethesda, MD). Cell proliferation was determined by counting the number of cells present in the bright field images at 12 hour intervals. Cell survival was determined by counting the number of PI-positive cells (dead cells) in the fluorescent images at 12 hour intervals and subtracting the dead cells from total cell number.

Differentiation: MDSCs were plated at 50 cells/mm^2 on a 24-well collagen type-I coated plates. Twenty-four hours later, the media was changed to DMEM + 2% FBS. After 1,2,3 or 5 days the plates were stained with mouse anti-fast skeletal myosin heavy chain antibody

(fsMHC, 1:400; Sigma,) and 4',6-diamidino-2-phenylindole (DAPI, for nuclei). The percentage of nuclei in fsMHC+ myotubes compared to total DAPI positive nuclei was quantified to assess myotube formation.

VEGF secretion: MDSCs were plated in PM at 105 cells/mm^2 in a 6-well collagen type I coated plates. Prior to collection, the media was switched to DMEM with 1% penicillin/streptomyocin for 24 hours. Enzyme-linked immunosorbent assays (ELISAs) for mouse VEGF (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's instructions and as previously described (Payne et al. 2007). VEGF levels were normalized to cell number.

Mechanical Stimulation: MDSCs were cultured on bioflex plates-- flexible 6-well culture plates coated with collagen type I (105 cells/mm^2, Flexcell Intl. Corp.). After 12 hours of culture, an FX-4000T strain unit subjected the cells to 10% equibiaxial strain with a 0.5 Hz sine wave for 24 hours (Byrne et al. 2005; Payne et al. 2007). Control MDSCs were cultured on the same plates without strain. The mechanical stimulation parameters were chosen based on previous studies which indicated increased VEGF secretion under these conditions in vitro (Gruden et al. 1999; Seko et al. 1999; Payne et al. 2007).

5.3 RESULTS

There are no differences in proliferation or cell survival between wt and *mdx* MDSCs: Three week old *mdx* and wt MDSCs were tested for differences in proliferation and survival. MDSCs were plated on collagen coated flasks and placed on a live cell imaging

system. The cells were cultured either in proliferation media or proliferation media supplemented with hydrogen peroxide, to model oxidative stress. In order to analyze proliferation, cell numbers were counted every 12 hours with image J software. For survival, total cell number was counted as well as the number of PI positive dead cells. There was no difference in proliferation under normal or stress conditions between the two cell types (Figure 18A-B, $P > 0.05$). Also, there was no difference in cell survival when wt and *mdx* MDSCs were grown in PM supplemented with either 100 or 250 µM H_2O_2 (Figure 18C-D, $P > 0.05$). From these results we conclude that there are no differences in in vitro proliferation or survival between the two cell types under the conditions that were examined.

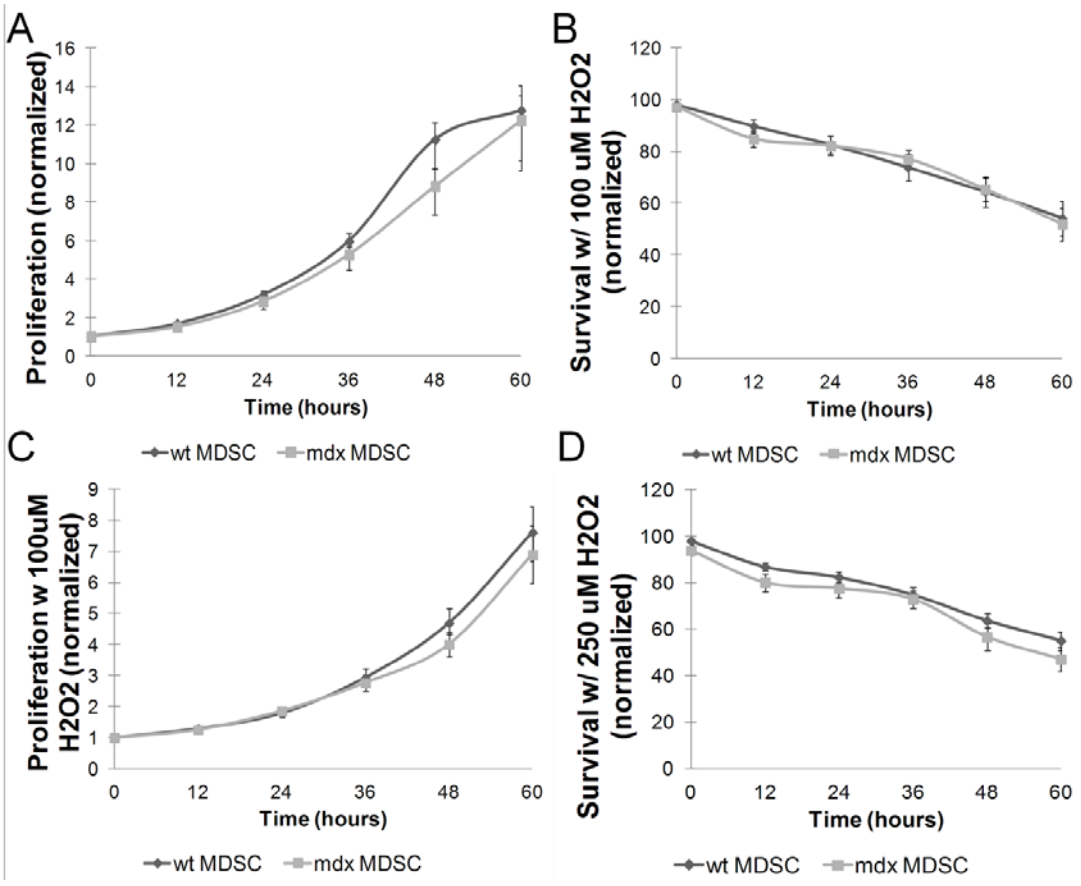

Figure 18: Proliferation and survival of wt and mdx MDSCs

Wt and *mdx* MDSDCs were grown in PM (A), PM supplemented with 100 uM H_2O_2 (B,C) or PM supplemented with 250 uM H2O2 (D). They were analyzed on a live cell imaging system for proliferation and survival. n=18 per group.

There are no differences in myotube formation between wt and *mdx* MDSCs: We also tested the differentiation capabilities of the two cell types. To differentiate the MDSCs they were grown in low-serum media for 1, 2, 3 or 5 days and myotube formation was quantified with fsMHC staining by counting the number of fsMHC positive nuclei compared to the total number of nuclei. Nuclei were stained with DAPI. We found no differences in myotube formation between *mdx* and wt MDSCs at any of the time points tested (Figure 19A, P>0.05). Figure 18B-C show representative images of fsMHC (green) stained myotubes from *mdx* and wt MDSCs after 5 days in 2% serum media (DAPI is in blue).

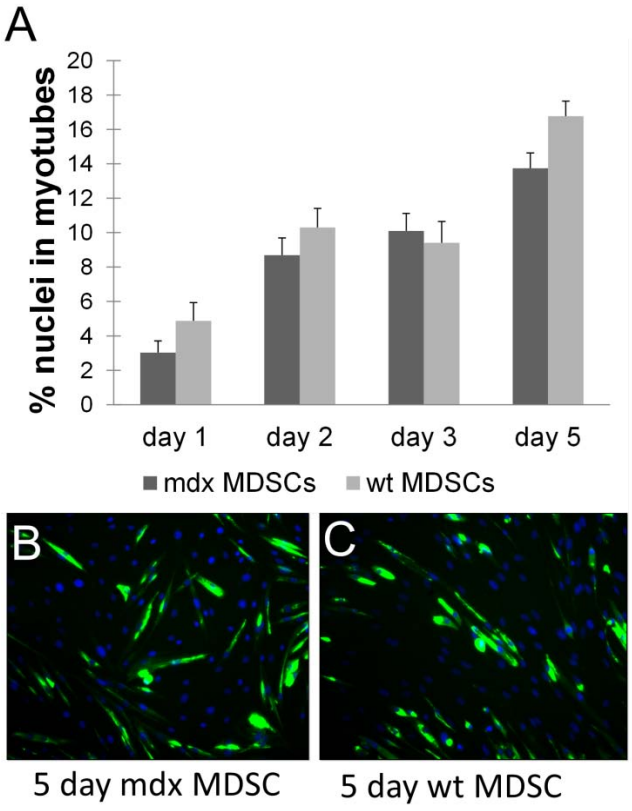

Figure 19: Myotube formation of mdx and wt MDSCs

Mdx and wt MDSCs were grown in low-serum media for 1,2,3 or 5 days and stained for fast skeletal myosin heavy chain (fsMHC) (to mark differentiated myotubes) and DAPI. The number of fsMHC positive nuclei in myotubes was quantified n=18. (A). Representative figures of fsMHC staining are shown at 5 days of *mdx* (B) and wt (C) MDSCs.

There is no significant difference in VEGF secretion between wt and *mdx* MDSCs: Many of the regenerative effects of cellular therapy are due to paracrine effects such as angiogenesis. We investigated growth factor secretion of vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis and found that although on average wt MDSCs secrete more VEGF than *mdx* MDSCs, there was a large range of VEGF secretion in wt MDSCs and no significant difference between their VEGF levels (Figure 20, P=0.09).

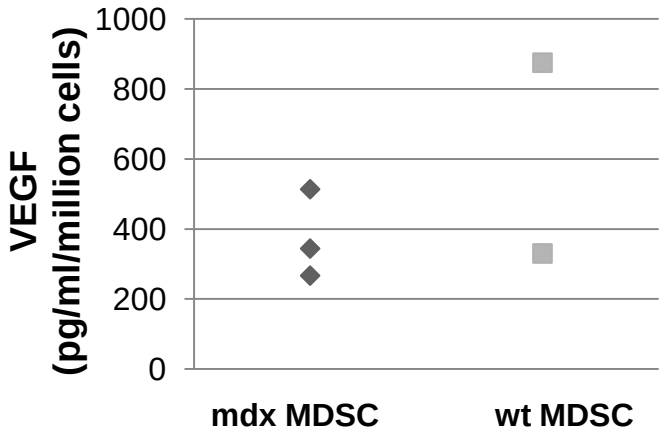

Figure 20: VEGF secretion of mdx and wt MDSCs

Mdx and wt MDSCs were compared for their VEGF secretion. Cells were grown in low serum media for 24 hours and media was analyzed for VEGF by ELISA n=6 wt MDSC, n=9 mdx MDSC.

DKO homozygous MDSCs show reduced capacity for differentiation, proliferation and survival compared to their heterozygous counterparts: Since *mdx* mice are known to have a less severe form of muscular dystrophy compared to their human counterparts, we shifted our focus to a more pronounced model of muscular dystrophy, DKO mice. We compared proliferation, survival, and differentiation of MDSCs from 6 week old dystrophin/utrophin double knockout mice (DKO-/-) and dystrophin/utrophin+/- mice (DKO+/-). DKO-/- MDSCs showed significantly impaired proliferation capabilities compared to DKO+/- MDSC (Figure 21A,

P<0.05). They also had decreased survival (Figure 21C, P<0.05). After 5 days, DKO+/- MDSCs formed significantly fewer myotubes compared to DKO-/- MDSCs (Figure 21B, P<0.05). This is represented in figure 21D which shows myotubes formation of DKO -/- and +/ MDSCs as represented by fsMHC staining (red) normalized to dapi (blue).

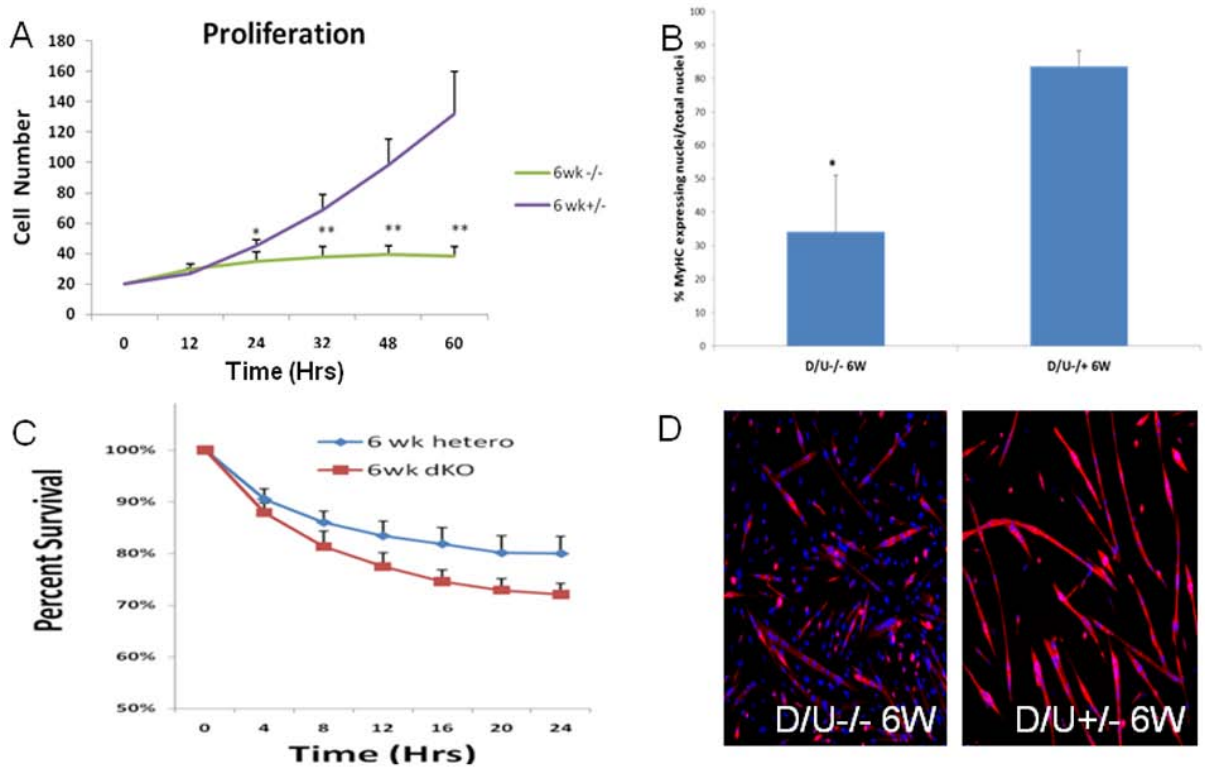

Figure 21: Proliferation and differentiation of DKO MDSCs

MDSCs from 6 week old heterozygous and homozygous DKO MDSCs were compared for proliferation n=6 (A), cell survival n=12 (C) and differentiation n=6 into myotubes as shown by fast skeletal myosin heavy chain staining (red) and DAPI for nuclei (blue) (B and D).

Mechanical stimulation does not affect differentiation of DKO MDSCs: It has previously been shown that pre-conditioning muscle cells can positively affect their capacity for cardiac repair (Byrne et al. 2005). If there is a defect in DMD muscle progenitor cells it would be beneficial for autologous therapies to improve stem cell performance if cells are not able to be

isolated from patients at an early time point before stem cell exhaustion sets in. Therefore, to examine the effects of mechanical stimulation, we mechanically stimulated the DKO-/- and DKO+/- MDSCs for 24 hours and evaluated their differentiation and proliferation capabilities. We found no difference in differentiation after 2 or 4 days in differentiation media (Figure 22).

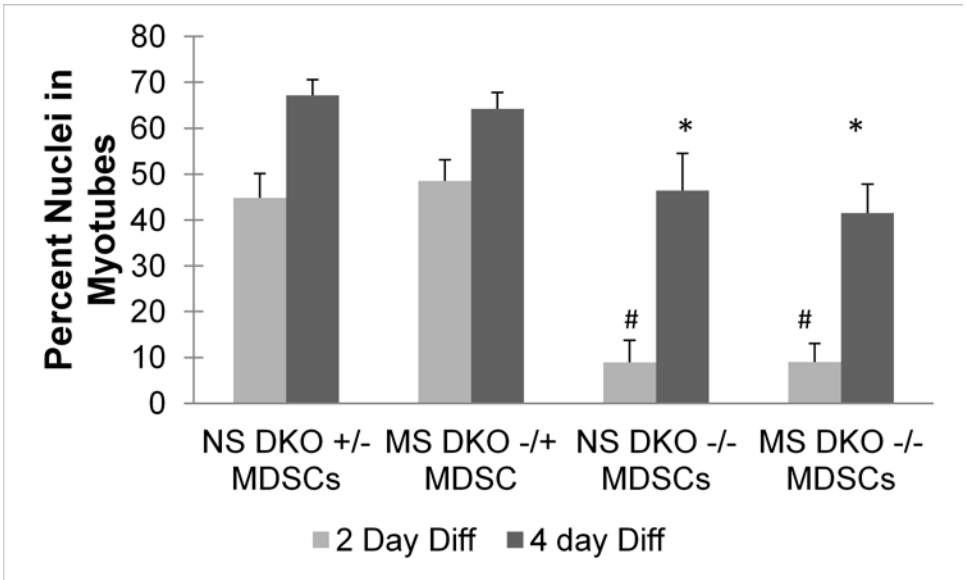

Figure 22: Myotube formation after Mechanical Stimulation of dys/utr MDSCs

DKO-/- and DKO+/- MDSCs were mechanically stimulated for 24 hours at 10% equibiaxial strain with a 0.5 Hz sine and then seeded onto 24 well plates. After 24 hours the media was changed to low serum differentiation media and the cells were grown in these conditions for 2 or 4 days. Myotubes were stained with fast skeletal myosin heavy chain (fsMHC) and nuclei with DAPI. The percentage of fsMHC positive nuclei over total nuclei was quantified and the results are shown n=12. The DKO-/- MDSCs had decreased myogenic differentiation compared to their DKO+/- MDSC counterparts at each time point (n=12, $*,^{\#}P<0.05$)

Mechanical stimulation increases proliferation of DKO MDSCs: When we tested proliferation in growth media and proliferation under oxidative stress with and without mechanical stimulation there was an increase in survival and proliferation of both DKO-/- and DKO+/- MDSCs after mechanical stimulation. This increase in proliferation was significant in the stretch DKO+/- group under normal proliferation conditions (Figure 23A) and proliferation under oxidative stress (Figure 23B). This shows that it is possible to improve the proliferation of murine dystrophic muscle stem cells.

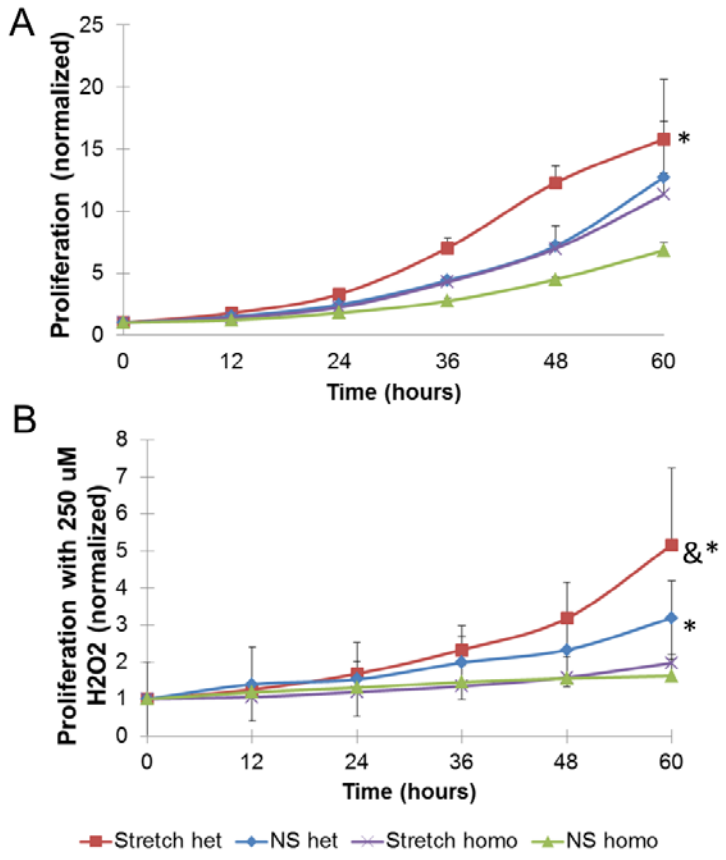

Figure 23: Proliferation and survival of DKO MDSCs

MDSCs were grown in (A) PM and (B) PM supplemented with 250 uM H2O2 to normal oxidative stress. When grown in PM MS DKO+/- cells had significantly greater proliferation compared to NS DKO-/- MDSCs (n=12-18*p<0.05). When grown under oxidative stress MS and NS DKO het MDSCs had significantly greater proliferation than NS DKO-/- MDSCs (*P < 0.05) and MS DKO+/- MDSCs had significantly greater proliferation compared to MS DKO-/- MDSCs (n=12-18 &P < 0.05).

DKO mice have greater cardiac fibrosis compared to *mdx* mice: A challenge of using the *mdx* mouse as a model for DMD is their lack of cardiomyopathy until a very late stage (about 2 years)(Quinlan et al. 2004). Therefore, we tested DKO+/- mice for fibrosis, a pathological hallmark of cardiomyopathy. At 10 weeks and 14 weeks, respectively, the *mdx* and DKO+/- mice had similar level of fibrosis around their left ventricle as shown by Masson trichrome staining (Figure 24 A,B). However, the fibrosis levels in a 17 month *mdx* heart were similar to the levels in a DKO+/- heart of only 8 months (Figure 24 C,D). This suggests that DKO+/- mice

develop cardiomyopathy at a more rapid rate than *mdx* mice, and are a better model to study dystrophic-mediated cardiomyopathy.

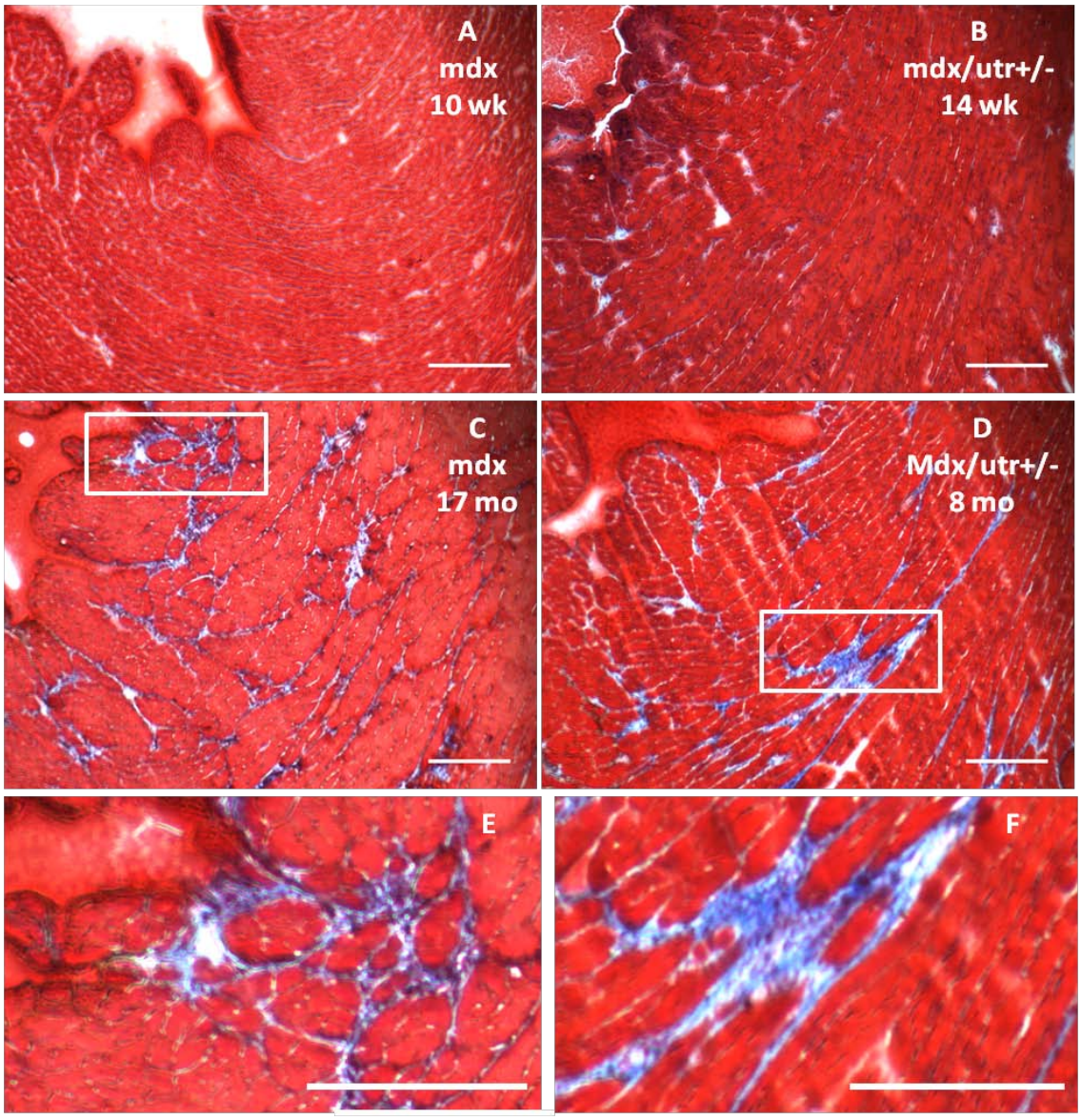

Figure 24: Fibrosis in mdx and DKO+/- hearts

Mdx and DKO+/- hearts were stained with Masson Trichrome which marks muscle (red) and collagen deposition (blue). (A) mdx hearts at 10 weeks of age. (B) mdx/utr+/- hearts at 14 weeks of age. (C) mdx heart at 17 months of age. (D) DKO+/- hearts at 8 month of age. (E) The boxed area in C at higher magnification. (F) The boxed area in D at higher magnification. Scale bar = 0.1 mm

5.4 DISCUSSION

Despite recent advances, there is still no cure for DMD. Over the past 20 years research has focused on replacing dystrophin using gene therapy in the *mdx* mouse model. In this study, we suggest that the DKO-/- mouse model more closely follows the human disease due to defects in muscle cell function and greater cardiac fibrosis. There is strong evidence that stem cell dysfunction plays a role in the pathology of dystrophic muscle and we have found that there is a difference in MDSC proliferation and myogenic differentiation between *mdx* MDSCs and DKO MDSCs.

We have shown that there are no differences in proliferation, survival, differentiation and VEGF secretion of *mdx* MDSCs compared to wt MDSCs. This concurs with recent data indicating that although there may be a stem cell deficiency in DMD patients, the *mdx* mouse has fully functional satellite cells (Sacco et al. 2010). A superior regenerative response due to longer telomeres may compensate for the lack of dystrophin, presenting a mild phenotype compared to their human counterparts (Sacco et al. 2010). This indicates that dystrophin deficiency is not the only pathological mechanism of DMD, and that under the right circumstances pathology may be improved without dystrophin replacement. Satellite cell renewal is a critical part of disease progression in muscular dystrophy. Therefore, if satellite cell function can be improved, or the satellite cell pool can be renewed, then dystrophic muscle function may be enhanced even without restoring dystrophin.

Although our results demonstrate that *mdx* MDSCs function normally, this is not the case for DKO MDSCs. DKO-/- MDSCs have decreased cell survival, proliferation and myotube formation compared to DKO+/- MDSCs. This is interesting as muscle stem cell deficiency correlates with a decreased lifespan and the severe pathology of the DKO mouse. The DKO-/-

mouse has a life span of 4-14 weeks, and the DKO+/- mouse lives for 12-14 months. Utrophin deficiency in these mice may result in increased cycles of degeneration and regeneration, thus aging the muscle stem cells in a similar manner to DMD patients (Grady et al. 1997).

Cardiomyopathy and respiratory failure are the main causes of death in patients with DMD. Defects include decreased systolic and diastolic function, impaired perfusion and conduction, degeneration of the atria, ventricle and conduction system exhibit degeneration, dilation of the saroplasmic reticulum, disordered myo-filaments, and abnormal mitochondrial structure (Grady et al. 1997). As such, it is critical to have a model in which to study dystrophic cardiomyopathy. To this aim, we have preliminary data to support increased fibrosis in the DKO+/- mouse at 8 months of age that is of a similar level in an *mdx* mouse at 17 months. All together, these findings suggest that the *mdx* mouse is not a good model for stem cell exhaustion in muscular dystrophy and that the pathology of the *mdx* mouse does not mimic the human disease. The DKO mouse may be a better model as it presents with symptoms similar to human DMD patients, such as stem cell exhaustion and cardiomyopathy. The lack of the membrane stabilizing utrophin protein probably leads to more mechanical damage, thus more death and fibrosis and subsequently more regeneration, thus exhausting stem cells at an earlier time point.

Ultimately, the purpose of this line of investigation is to study the possibility of autologous stem cell transplantation for muscular dystrophy patients; it is crucial to understand the stem cells that come from these patients. If autologous transplantation is truly going to be possible we will likely have to enhance the cells before transplantation. Webster and Blau showed that even at 2 years of age myoblasts from a DMD patient had significantly fewer doubling times compared to a healthy control patient with no known muscle degenerative diseases (Webster et al. 1990). Our results suggest that mechanical stimulation may be a

possible option to enhance proliferation of dystrophic MDSCs, which is particularly important in light of the decreased proliferation of human DMD patient muscle cells.

Future work could involve studying human stem cells from DMD patients. We have shown that dystrophic MDSC proliferation can be increased by mechanical stimulation, therefore, a critical next step would be to repeat these experiments with human DMD cells. Future work would also involve studying DKO stem cells in greater detail. They appear to suffer from exhaustion similar to human DMD cells, even though it is known that mice have longer telomeres than humans (Sacco et al. 2010). Is there a difference in telomere length between *mdx* and DKO MDSCs or does the lack of utrophin and increased muscle degeneration cause the decrease in function of DKO MDSCs?

6.0 CONCLUSIONS

"Stem cells would be well suited for regeneration if they clung to the exuberance of youth while also maintaining the self-control that comes with maturity."

Mihsin, Siddiqu, Collins and Sussman Circulation Research 2011

Cell therapy has enormous potential to treat disease and repair damaged tissue. However, it has not yet met the overwhelming clinical need. Stem cells from a variety of tissues and sources produce modest benefits for muscle regeneration and cardiac repair; however, optimization is necessary to improve efficacy (Mohsin et al. 2011). The aim of this thesis was to explore ways to improve stem cells for muscle and cardiac repair, specifically looking at the role of improving cell survival with antioxidant pretreatment and the role of vascular endothelial growth factor (VEGF) on mechanical stimulation pre-treatment. For model systems, we concentrated on myocardial infarction and muscular dystrophy. The three aims of this thesis were each focused on ways to improve cell therapy, which will be expounded upon in the following chapter, as well as exploring what is the "next generation" of cellular therapy and the hurdles that must be overcome for safe, efficient and effective muscle repair and regeneration.

After delivery, the ideal stem cell would 1) survive and adhere to the tissue, 2) migrate to the site of injury, and 3) recruit endogenous stem/progenitor cells or directly contribute to host repair and rejuvenation. There are multiple ways a stem cell can potentially modulate damaged

tissue, such as inducing angiogenesis, decreasing fibrosis, modulating the immune response such that there is a repair phenotype, finding a niche and establishing itself as a "resident" stem cell or differentiating into functional resident cell types (i.e. electrically integrated cardiomyocytes in the realm of cardiac repair). A large limitation of the study of cell therapy is that it is difficult to address more than one of these issues at a time, when in reality to be successful most of these conditions should be met. The field still has a long way to go, but progress is being made.

The first aim of this thesis addressed the primary barrier a donor cell faces when implanted into host tissue: it must survive. Globally, about 1-5% of cells survive in the heart after transplantation, and although there is variation, this is largely independent of cell type (Laflamme et al. 2005). When cells are injected into an ischemic environment most of them do not survive very long. Therefore, the first hurdle that must be overcome for effective cell therapy is that cells should be more resistant to the stresses that they are presented with when implanted into such an environment. Improvements on cell survival should prove beneficial because the longer cells persist in the tissue, the greater chance they have to contribute to repair through paracrine or other mechanisms.

In the first aim, we hypothesized that the reason MDSCs repaired the heart and skeletal muscle in a more effective manner than myoblasts was due to their superior cell survival and higher levels of antioxidants. Therefore, we increased the levels of antioxidants in myoblasts with N-acetylcysteine (NAC) and decreased them with diethyl maleate (DEM) in order to attempt to reach MDSC levels of survival. We found that even though early proliferation and survival was increased in the myo+NAC group, at 6 weeks post injection there was a trend toward greater cardiac function in the myo+NAC group but it was not significantly greater than the myo group, and there was no difference between the two groups as far as fibrosis and

angiogenesis. However, in the myo+DEM group the cardiac function, fibrosis, and angiogenesis levels were not significantly different from PBS, indicating that depleting the cells of antioxidants took away the functional benefits of decreased fibrosis, increased angiogenesis, and greater cardiac function imparted by myoblasts onto the cardiac tissue. All together these results indicate that antioxidant levels effect the survival of not only stem cells but more committed cell populations as well. However, more work needs to be done to reach the proper level of antioxidants to significantly improve myoblast transplantation therapy Another possible conclusion to draw from the study is that cell source is as important as cell survival. When MDSCs are treated with the same levels of antioxidants their repair capacity is increased. It is possible that since myoblasts are a more committed and differentiated cell type their capacity for repair is limited. Therefore, in the remainder of our studies we used MDSCs.

A characteristic related to the survival of transplanted cells is adherence. If donor cells do not have the proper machinery to adhere to the tissue, or if there is no place in the tissue for them to adhere (due to large amounts of inflammatory or fibrotic tissue), then they will simply die. A large contributor to cell death in cell based cardiac repair is anoikis, which is programmed cell death induced by the loss of matrix attachments (Song et al. 2010). Increased levels of ROS in damaged or diseased tissue hinder cell adhesion and thus the effectiveness of cellular therapy (Taddei et al. 2012). In vitro, MSCs subjected to H_2O_2 induced stress had reduced mRNA levels of the β-2 and α-V integrins. Treatment with NAC partially rescued integrin mRNA levels, indicating that antioxidant treatment of stem cells may affect their integrin levels and ability to resist anoikis following transplantation (Song et al. 2010). Also, plasminogen activator inhibitor 1 (PAI-1) has been shown to have a negative effect on MSC attachment to matrices. PAI-1 is primarily a regulator of plasminogen activators (uPA and tPA),

which modulate the conversion of plasminogen to plasmin (Cesarman-Maus et al. 2005). Yet, there was no difference in the ability of wt and PAI-null MSCs to produce plasmin. However, when PAI-1 was blocked in MSCs, cell survival was improved and when PAI-1 was added to MSC media, the cells detached from the matrix (Copland et al. 2009). This was most likely due to fact that PAI-1 competes with the urokinase receptor (uPAR) for binding to vitronectin (Deng et al. 2001) and seems to act as an anti-adhesive molecule for MSCs. PAI-1 also affects integrin mediated cell adhesion and migration but the molecular basis of this phenomena is unkown (Kjoller et al. 1997). Thus, modulating integrin levels may decrease anoikis and improve cellular retention and survival.

The second challenge to successful cell therapy is proper dissemination of transplanted cells and homing to areas of injury. Often, cellular migration (either into or out of the engraftment area) is hindered by a layer of fibrotic tissue surrounding the engrafted cells. In cardiac therapy this will make it more difficult for donor cells to electrically connect with the host in order to produce a steady pulse and avoid arrhythmias. Preliminary clinical trials for cardiac repair using myoblasts were terminated early due to concerns about life-threatening arrhythmias (Menasche et al. 2001). Until this concern is satisfactorily met it may be necessary to implant defibrillators along with cell therapy. In skeletal muscle therapy the fibrotic barrier tends to keep injected cells all in one spot, when wide spread migration and dissemination through the tissue would be beneficial for global tissue regeneration. Therefore, in order to achieve optimal cell therapy it will be necessary to overcome the limitations of fibrotic tissue barriers and cell dissemination.

Finally, once transplanted cells have survived and arrived at a location of tissue damage, they can begin to modulate the microenvironment. Examples of beneficial effects from

transplanted cells include inducing angiogenesis, affecting the survival of surrounding host tissue, or recruiting endogenous progenitor cells (Mummery et al. 2010). One process necessary to rejuvenate damaged tissue is angiogenesis; damaged tissue is often lacking in blood and nutrients and the more rapidly a blood supply is brought to the tissue, the sooner regeneration can begin. One way to encourage angiogenesis is to pre-treat donor cells with mechanical stimulation, which we have shown increases VEGF secretion (Payne et al. 2007). In aim number 2 we explored the role that mechanical stimulation has on VEGF expression from MDSCs and the resultant angiogenesis and fibrosis after injection with treated MDSCs into dystrophic muscle. To accomplish this we blocked VEGF in two different ways, by transducing with sFlt1, which blocked any VEGF in the tissue, both from host and donor, as well as transducing MDSCs with shRNA_VEGF, which decreased VEGF secretion from the transplanted MDSCs only. We found that mechanically stimulating wt MDSCs increased VEGF expression but stimulation of shRNA_VEGF MDSCs or sFlt1-MDSCs did not affect VEGF secretion. Angiogenesis was increased in the transplantation area of MS-MDSCs by quantification of CD31 per dystrophin positive fiber, but this increase in angiogenesis was not seen in the MS-sFlt1 group, indicating that VEGF is necessary for the increase in angiogenesis seen with mechanical stimulation. Also of note was that transplantation with shRNA_VEGF MDSCs resulted in significantly fewer dystrophin positive myofibers, indicating that VEGF, possibly in an internal autocrine manner, is also affecting transplantation and differentiation of MDSCs into dystrophic tissue. This was further confirmed by significantly decreased in vitro differentiation capacities of shRNA_VEGF MDSCs compared to wt and sFlt1-MDSCs. This indicates that VEGF is critical not only for MDSC mediated angiogenesis but also MDSC donor cell differentiation.

Finally, an important consideration for cell therapy is the progenitor cells that are already in the tissue, the "niche" or resident progenitor cells. Both cardiac and skeletal muscles have native populations of cells able to self-renew and differentiate into resident tissue type cells for routine maintenance or in response to injury. Skeletal muscle progenitor cells are satellite cells which reside underneath the basal lamina of the muscle and are activated in response to injury and disease (Wallace et al. 2009). Cardiac progenitor cells are more controversial but it has been established that they do exist, although there is not universal agreement as to how to identify them or whether they reside in the heart or are a form of circulating progenitor cells possibly from the bone marrow (Beltrami et al. 2001; Hsieh et al. 2007; Bergmann et al. 2009).

Recent publications have shown that resident stem cells are depleted in number or function with the occurrence of disease or aging (Webster et al. 1990; Bockhold et al. 1998). Once such disease is Duchenne muscular dystrophy, which we have established is caused by lack of dystrophin but is propagated by resident stem cell dysfunction. In aim 3 we demonstrate that the progenitor cells of wt mice and the *mdx* mouse had the same proliferation, differentiation, and survival. However, the stem cells of the more severe mouse model, the DKO mice, had impaired MDSC survival and differentiation. We also demonstrated that this correlates with a more severe cardiac pathology, with increased fibrosis in DKO hearts compared to similar aged *mdx* mouse hearts. This is very relevant for the human disease, as DMD patients most often succumb to cardiac complications as early as their 20's (Deconinck et al. 2007).

Resident stem cells may be a powerful force for tissue regeneration. If resident stem cells can be replaced or rejuvenated through cell or small-molecule therapy this would be a major coup for diseases such as DMD or heart failure, both of whose resident stem cell populations are

in-sufficient to overcome the disease. Efforts to optimize cell therapy are necessary to improve transplantation outcomes for both ischemic cardiac cell therapy and muscular diseases.

7.0 FUTURE WORK

There is ample room for improvements in cell therapy research. In the first aim we concluded that a more sustained release of antioxidants such as could be achieved by genetic modulation of myoblasts or MDSCs may be beneficial to increase cell survival after transplantation. This theory has been well documented in MSCs (Gnecchi et al. 2005; Tang et al. 2005). It would also be reasonable to predict that an antioxidant other than NAC may be beneficial for use with muscle cells. Some examples would include XJB (Fink et al. 2007; Vella et al. 2011), vitamin K (Li et al. 2003), or taurine (Aruoma et al. 1988). It would also be interesting to combine the VEGF producing angiogenic potential of mechanical stimulation with the survival advantage imparted by antioxidant stimulation.

To follow up aim 2, it would be interesting to explore the effect of other growth factors besides VEGF on mechanical stimulation. We demonstrated that VEGF had an effect on angiogenesis in the donor engraftment area as well as differentiation of donor cells, both important aspects of cellular therapy. However other aspects of tissue regeneration are equally important, such as survival of donor cells, recruitment of native stem/progenitor cells, and factors affecting remodeling and fibrosis. In our study, we did not see an affect of transplantation of MS-MDSCs on fibrosis; it would be interesting to determine if the protocol could be modified in order to increase both VEGF and factors that affect remodeling. For example, in human patellar tendon fibroblasts, matrix metalloproteinase 1 (MMP-1) expression was positively affected by

the level of mechanical stimulation (Yang et al. 2005), indicating the different protocols can affect MMPs, which degrade extracellular matrix proteins. Modulating local inflammation can also affect cell transplantation outcomes. Limiting monocyte chemo-attractant protein 1 (MCP-1) was shown to improve cardiac function (Ohnishi et al. 2007). This could be another factor to examine in order to improve therapy.

Aside from limiting inflammatory cells, recruiting stem cells and progenitor cells could be beneficial to therapy (Chavakis et al. 2008). Stromal-cell derived factor-1 (SDF-1) recruits hematopoietic stem cells (HSCS), endothelial progenitor cells (EPCs), cardiac stem cells (CSCs) and mesenchymal stem cells (MSCs) while hepatocyte growth factor (HGF) and fibroblast growth factor (FGF-2) recruit cardiac stem cells (CSCs) (Penn et al. 2008). In fact, HGF was released from muscle satellite cells upon mechanical stimulation (Tatsumi et al. 2002). Both of these factors would be interesting to assay following mechanical stimulation of MDSCs.

Another strategy would be to examine the effect of mechanical stimulation on other cell types, such as cardiomyocytes or endothelial cells. For example, VEGF is also up-regulated in mechanically stimulated cardiomyocytes (Schmelter et al. 2006). Further exploration of the role of VEGF on MDSC myogenic differentiation and engraftment would also provide further insight into the effects seen after transplantation of those cells. Research into the autocrine signaling ability of MDSCs could be started by blocking internal VEGF signaling with small molecules (Gerber et al. 2002) and then assaying myogenic differentiation, as well as assaying phosphorylation of VEGF receptors on MDSCs. It would also be relevant to study the role of VEGF on survival and apoptosis of MDSCs as both of these cellular functions are affected by VEGF (Arsic et al. 2004; Bouchentouf et al. 2008). Finally, examining the effect of VEGF

blockage on differentiation into other cell lineages, such as adipogenic, chondrogenic and osteogenic would be interesting.

Intriguingly, myogenic transcription factor MyoD mRNA is increased after mechanical stimulation (Kendall et al. 1994), and MyoD has been shown to have the ability to regulate VEGF expression.(Bryan et al. 2008) This suggests a possible role for MyoD in regulating VEGF after mechanical stimulation and would be a fascinating future line of investigation.

Another interesting link is between VEGF and adhesion, which is integral for cells to persist and migrate after injection (Zachary 2003). VEGF induced activation of focal adhesion kinase (FAK) in rat cardiomycytes (Takahashi et al. 1999). Also, up-regulation of FAK was shown to be protective of cardiomyoctes after ischemia-reperfusion injury in-vivo (Cheng et al. 2012).

As for the study of resident stem cells in DMD, and the use of these cells to improve cardiac repair, there is still much that we do now know. To further improve our knowledge, dystrophic cells could be injected into the circulatory system of sick DKO mice and histology and functional analysis performed on the heart. It may be necessary to pre-treat these cells before injections with regimens of mechanical stimulation or antioxidant pre-treatment, or potentially small molecules to make homing to the heart more efficient. It would probably do more harm than good to inject cells directly into the diseased heart as an injection itself will cause damage. Another option would be to inject small molecules that activate resident stem cells.

A logical progression of this work is to study human DMD MDSCs. If their survival and proliferation could be improved upon with mechanical stimulation or antioxidant treatment this would be of clinical benefit for autologous cell therapy.

Finally, a general future direction would be to combine therapies to improve stem cells for transplantation, ie. to improve both their potential for angiogenesis and cardiac differentiation before implantation into the infarcted heart. This may be achieved with a combination of growth factor, small molecules, and conditioning pre-treatments.

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