CELLULAR CARDIOMYOPLASTY BASED ON MUSCLE STEM CELLS: IMPLICATIONS FOR THERAPY

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Heart disease is the leading cause of death in the world and cellular cardiomyoplasty is an emerging therapeutic option to repair damaged myocardium. Muscle-derived stem cells (MDSCs) have been shown to have an improved regenerative capacity in bone, cartilage, and skeletal muscle when compared to myoblasts. After implantation into ischemic hearts, MDSCs display high levels of engraftment, induce neoangiogenesis, prevent cardiac remodeling, and elicit significant improvements in cardiac function. Notably, diversity in MDSC behavior has been associated with innate sex-related differences in skeletal muscle, bone, and cartilage. These results suggest that differences in inherent stem cell characteristics, including sex and age, could account for some of the outcome variability noted in clinical trials. Therefore we examined the effect of sex-related differences of MDSCs in cardiac repair. Transplantation of both cell sexes significantly improved cardiac function in comparison to saline. We also found that with increasing age, the proliferation and differentiation abilities of MDSCs decreased while their survival under stress conditions and vascular endothelial growth factor (VEGF) secretion remained unchanged. Our *in vivo* study demonstrates that the age of MDSCs does not impact their regenerative capacity but increasing the age of the host leads to decreased repair, which

implies that the majority of age-related decreases in repair are due to changes in the microenvironment. We then examined methods to improve the efficacy of cell transplantation via preconditioning strategies. We first investigated the effects of mechanical stimulation on increasing the secretion of VEGF, which plays a major role in effecting cardiac repair after cell transplantation. Mechanical preconditioning significantly increased VEGF secretion, angiogenesis, and cardiac function after myocardial infarction, suggesting that this method of cell preconditioning could increase therapeutic efficacy. Another major issue with cell therapy is low cell survival after implantation. We hypothesized that increasing the level of antioxidants in MDSCs prior to transplantation could increase survival and therefore improve functional cardiac repair. We found that antioxidant pretreatment increased cell survival, cardiac function, and angiogenesis, and decreased scar formation. These pretreatment strategies have the potential to significantly improve the efficacy of cell transplantation and enhance the outcomes of heart disease patients.

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DESCRIPTORS

Cell Transplantation

Cardiac Repair

Muscle Derived Stem Cells

Mechanical Stimulation

Antioxidants

Sex

Age

NOMENCLATURE

ANOVA, analysis of variance

BMP-4, bone morphogenetic protein 4

CD34, cluster of differentiation 34

c-kit, cluster of differentiation 117

cTnI, cardiac troponin I

DAPI, 4, 6'-diamidino-2-phenylindole

DEM, diethyl maleate

DMEM, Dulbecco's modified Eagle medium

EDA, end diastolic area

ELISA, enzyme-linked immunosorbant assay

ESA, end systolic area

FAC, fractional area change

FS, fractional shortening

fsMHC, fast skeletal myosin heavy chain

GSH, reduced glutathione

H&E, hematoxylin and eosin

LV, left ventricle

MDSC, muscle derived stem cells

MI, myocardial infarction

NAC, N-acetylcysteine

Pax-7, paired box gene 7

PBS, phosphate buffered saline

PM, proliferation medium

Sca-1, stem cell antigen 1

SCID, severe combined immunodeficiency

sFLT-1, soluble fms-like tyrosine kinase 1

TGFβ3, transforming growth factor beta 3

TNF-α, tumor necrosis factor alpha

VEGF, vascular endothelial growth factor

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1.0 INTRODUCTION

1.1 CARDIAC REPAIR

Heart disease is the number one cause of death in the world, and the cost associated with heart disease and loss of productivity in the United States in 2007 was over $400 billion [1]. Myocardial infarctions (MI) are one of the most common forms of heart disease and occur when an artery supplying blood to the heart becomes occluded [2]. The cells in the affected area no longer receive oxygen and nutrients, which leads to cell death. Dead cells are replaced by non-contractile scar tissue, consisting primarily of collagen type I, which does not have the same mechanical properties as native tissue [3]. The buildup of scar tissue results in a reduction of cardiac output and over time can lead to end-stage heart failure. The adult vertebrate heart has a limited capacity to regenerate following injury and therapies to address this problem are lacking [4]. Currently, the standard therapy for progressive heart failure is heart transplantation, which is less than ideal due to low numbers of available organs, risk of complications, and the need for chronic immunosuppression [5, 6]. Other treatment options include surgery, drug treatments, and implantation of mechanical devices, but all of these therapies have significant drawbacks and the development of new treatments to address the growing problem of heart disease is therefore essential.

Cellular cardiomyoplasty, the transplantation of exogenous cells, is gaining recognition as an alternative method for cardiac repair [7]. The repair seen with cellular transplantation into the myocardium does not appear to be due to differentiation of the implanted cells but rather a paracrine effect that reduces the spread of scar tissue and allows the host cells to repair [8]. The ideal cell type for cellular cardiomyoplasty are stem cells, which are readily available from various tissues, can easily be expanded for implantation, and allow for the possibility of autologous transplantation. Many cell types have been examined in cardiac transplantation with some degree of efficacy, but these therapies simply limit the decline of heart function rather than restoring cardiac output to undamaged levels [9-14].

1.2 STEM CELLS

Stem cells are characterized by their ability to self-renew, differentiate down multiple lineages, and potentially by their ability to survive stress [15, 16]. Stem cells are used by the body to replace lost or damaged tissue, and have been recently used as a therapeutic treatment after injury. There are a wide variety of stem cell sources, including hematopoietic stem cells [17], cardiac progenitor cells [18, 19], bone marrow derived cells, mesenchymal cells [9, 20], embryonic stem cells [12, 21], side population cells [22], induced pluripotent stem cells [23], and skeletal muscle cells [24]. Skeletal muscle derived stem cells (MDSCs) are one of these populations, and are distinct from skeletal myoblasts and satellite cells [25, 26].

Skeletal muscle contains a cellular hierarchy [26, 27]. The mature muscle cell is the multi-nucleated muscle fiber, which is surrounded by satellite cells that are within the basal lamina but outside the sarcolemma [28]. Satellite cells remain quiescent in the muscle until an

external stimuli such as injury occurs, at which point they re-enter the cell cycle and proliferate [29]. Following this increase in numbers, the satellite cells then differentiate into myoblasts, which subsequently fuse to form new myofibers [29, 30].

Distinct from both the satellite cell and myoblast populations are the MDSCs [26]. MDSCs are isolated based on their adhesion characteristics to collagen coated flasks [31]. A skeletal muscle biopsy is digested using dispase, collagenase, and trypsin, and the resulting single cell suspension is plated onto a series of collagen-coated flasks [31]. The cells that are slowest to adhere contain the MDSC population, and after passaging to remove other cells, the long term progenitor cells of the muscle, MDSCs, remain (Figure 1.1) [26, 31]. These cells can be passaged to greater than 250 population doublings without any significant changes to their cell characteristics [32].

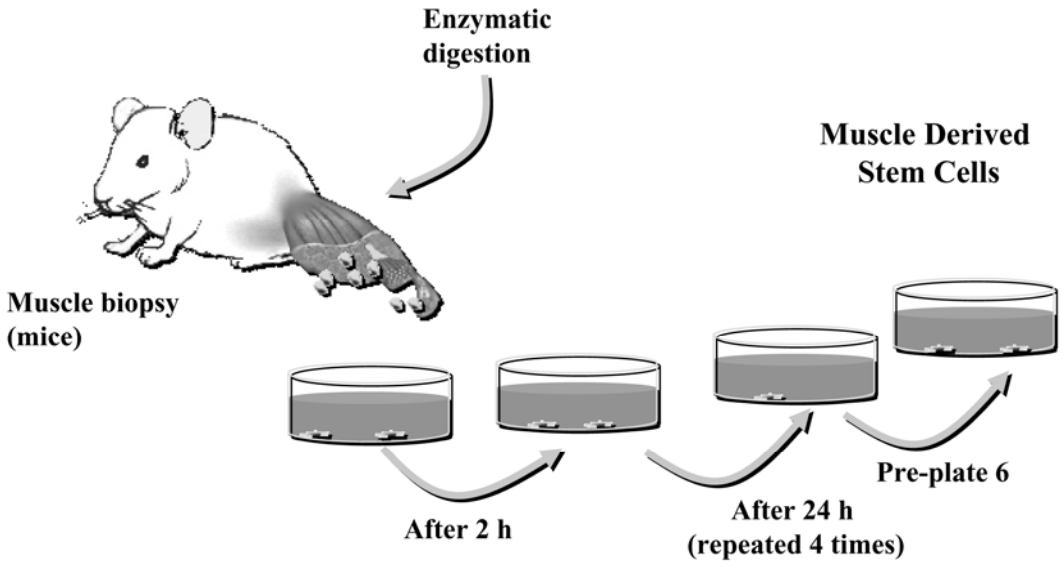

Figure 1.1 The preplate technique for isolating muscle-derived stem cells

In vivo studies provide additional evidence for the stem cell nature of MDSCs. MDSCs differentiate into multiple lineages, self-renew, and regenerate bone, cartilage, muscle, nerve, and cardiac tissue [33-36]. In a lethally irradiated mouse model, implantation of a subpopulation of MDSCs expressing cluster of differentiation 117 (c-kit) and Sca-1 is able to rescue the animal [37]. MDSCs also are at least partially immune-privileged, as transplantation with MDSCs results in dystrophin expression in *mdx* mice (dystrophin-deficient) over 3 months after injection [26]. Potential human counterparts to MDSCs have been isolated based on cell-surface markers. These cells, myogenic endothelial cells (myoendo), co-express myogenic (CD56) and endothelial (CD34 and CD144) markers [24]. When compared to myogenic cells (CD56 + only), myoendo cells are superior for repair in both skeletal and cardiac muscle, similar to MDSCs [14, 24].

Although MDSCs are similar to satellite cells in their regeneration abilities in skeletal muscle, MDSCs are a separate population of cells that expresses distinct markers and phenotypes. Satellite cells, whether active or quiescent, typically express paired box gene 7 (Pax-7) [38] whereas MDSCs are more heterogenous but express stem cell antigen 1 (Sca-1) consistently, and often express cluster of differentiation 34 (CD34). Pax-7 and Sca-1 positive cells have not been found to colocalize in skeletal muscle, providing further evidence that satellite cells and MDSCs are distinct subpopulations [39]. MDSCs also appear to be more primitive than satellite cells, as they have the ability to differentiate into a broader number of cell types, thus displaying greater plasticity [40, 41]. Based on these results, the hierarchy of cells in skeletal muscle is, from most primitive to most differentiated: MDSCs, satellite cells, myoblasts, and multi-nucleated myofibers.

1.3 APPLICATIONS OF MUSCLE DERIVED STEM CELLS

MDSCs are used in a variety of applications for cellular therapy. MDSCs are particularly attractive for cell therapy purposes because they are easy to isolate, are adult-derived, are able to self-renew, can differentiate down multiple lineages, express greater levels of important paracrine factors (i.e. VEGF) than more differentiated counterparts such as myoblasts, and are, at least to some degree, protected from the typical immune response after implantation [16, 32, 33]. There is also the potential for autologous transplantation with MDSCs, which would bypass the need for immunosuppression. There are many potential uses for MDSC therapy, and promising results have been obtained in bone, cartilage, skeletal and cardiac muscle, and nerve repair [34, 36, 42]. Currently, autologous human MDSCs are in clinical trials for urinary incontinence [43] and phase I trials for cardiac repair after a myocardial infarction will begin in the near future.

1.3.1 Bone/Cartilage

There are a variety of conditions that would be treatable with cellular therapy using MDSCs, including non-union fractures or critical sized defects (unable to repair without therapeutic intervention). Since formation of new bone with the appropriate mechanical properties is critical for bone repair, research has focused on scaffolds and cells that release paracrine factors to induce osteogenesis. Previous work has shown that MDSCs transduced with bone morphogenetic protein 2 (BMP-2) and placed into a fibrin gel prior to implantation into a critical sized skull defect improves repair above BMP-2 in a fibrin gel [44]. MDSCs are also able to repair chondrogenic defects when transduced with bone morphogenetic protein 4 (BMP-4) and transforming growth factor beta 3 (TGFβ3), with an increase in repair seen when vascular

endothelial growth factor (VEGF) secretion is blocked by cell pretreatment with soluble fms-like tyrosine kinase 1 (sFLT-1) to prevent bone growth into the new articular cartilage [45].

1.3.2 Nerve

MDSCs have been shown to repair critical-sized nerve defects. This regeneration appears to occur mainly through paracrine mechanisms, as MDSCs are found around the repaired nerve rather than participating directly in repair through differentiation into nerve cells. The repair is functional, as after treatment rats treated with MDSCs as opposed to saline are able to regain motor control to a significantly greater extent [46].

1.3.3 Skeletal Muscle

After injury, regeneration of the muscle and scar tissue formation both occur [47]. However, the collagen is disorganized and is not always replaced by functional skeletal muscle tissue over time, resulting in weakness and a tendency for reinjury in that location [47, 48]. MDSCs are significantly better at reducing scar tissue formation and increasing angiogenesis than myoblasts or saline injection [42]. In addition, MDSCs can differentiate into mature myofibers and thereby participate directly in the regeneration process. Moreover, treatment of MDSCs with suramin or decorin, both antifibrotic agents, further increases functional healing by increasing differentiation and downregulating myostatin expression [49-51].

1.3.4 Cardiac

Cellular therapy for cardiac repair after a myocardial infarction is beneficial with cells derived from a variety of cell sources, including cardiomyocytes, smooth muscle cells, hematopoietic stem cells, and skeletal muscle derived cells such as myoblasts and MDSCs [6, 9, 52-54]. All of these cell types have demonstrated some efficacy, but the extent is limited and is far from complete healing [4]. Some issues that need to be addressed in order to improve cell therapy include cell survival post-transplantation, neoangiogenesis, electromechanical integration, and differentiation into cardiomyocytes [55].

MDSCs have been shown to be superior to myoblasts for repair after a myocardial infarction in preclinical models, with better maintenance of cardiac function, increased angiogenesis, and decreased formation of scar tissue [34]. The importance of paracrine factors, particularly VEGF, has also been explored [56]. Increased VEGF secretion improves cardiac function, and blocking VEGF decreases cardiac repair, but the dose is important, as too much VEGF causes hemangiomas [57]. In myoendo cells, the potential human counterparts to MDSCs, similar results have been obtained, with increased cardiac function and angiogenesis and decreased scar tissue formation when compared to myogenic cells or saline [14, 24].

1.4 PROJECT OBJECTIVES:

1.4.1 Basic Science

1.4.1.1 Objective 1: Evaluate MDSCs for sex differences in cardiac repair

The sex of cells has been shown in some cases to affect cellular therapy, and previous results from our laboratory indicate that cell sex influences repair in skeletal muscle, cartilage, and bone regeneration. These experiments demonstrate that female MDSCs are better at engrafting into skeletal muscle, while male cells are more osteogenic and chondrogenic [58, 59]. Other groups have shown that cell sex is also a factor with mesenchymal stem cell activation, myocyte death, and cardiac repair, with female cells improving heart function to a greater extent [60-62]. These results suggest that sex differences are dependent on the organ system. Knowing the effects of cell sex could influence the development of cellular therapies and answer an important basic science question.

1.4.1.2 Objective 2: Evaluate MDSCs for age differences in skeletal and cardiac muscle repair

Stem cells have the ability to proliferate and self-renew far beyond the Hayflick limit, which constrains more differentiated cells to a limited number of divisions before entering quiescence [63]. There are subpopulations of cells within the skeletal muscle that have been shown to resist aging, and MDSCs may also have this ability [64]. However, this age resistance is not

permanent, and extensive aging can affect even stem cells. Previous work has demonstrated that with increasing age, a reduction in potency and differentiation capacity is seen in a variety of stem cell types [65, 66]. The level of cellular senescence also increases in stem cells with age and this is likely correlated to increasing amounts of DNA damage which forces the cells out of the cell cycle [67]. There is some debate as to the cause of these changes including if they are directly related to the cell or to the microenvironment [63, 68, 69], although it is most likely a combination of both factors. Earlier work with MDSCs has shown that with up to 200 population doublings there is no decrease in the regenerative capacity of the cells [32]. The marker phenotype was also preserved over long term expansion, and no difference was seen in the population doubling time [32]. However, expansion beyond 200 population doublings begins to result in altered cell behavior, including lower regeneration capacity and myogenic differentiation [32]. As many of the potential cellular therapy patients would be aged, understanding the effects of aging on the environment and stem cells could improve therapy.

1.4.2 Improvement of Cell Therapy

Though there are significant benefits to cellular therapies using MDSCs, including the possibility of autologous transplantation, the lack of ethical issues, and the ease of use, cell transplantation therapies can be further improved. Functional repair is not complete in many organ systems following cell transplantation and this could be due to low cell survival after transplantation. Methods to increase cell survival and paracrine factor expression could significantly improve repair by increasing the involvement of host cells in the repair process. Potential methods

include preconditioning the cells prior to transplantation to increase survival or paracrine factor expression.

1.4.2.1 Objective 3: Determine effect of mechanically preconditioning MDSCs prior to transplantation

Mechanical stimulation has been shown to have a wide variety of effects on cells, from gene expression to morphology [70, 71]. Paracrine factor expression, and in particular VEGF secretion, has been shown to be increased after mechanical stretch [72]. VEGF secretion is critical for cardiac repair, with increased levels resulting in improved functional recovery [57] [73]. If VEGF secretion is blocked with sFLT-1, cardiac repair is decreased to the level of saline controls [57]. This method of pretreatment to increase angiogenesis and cardiac repair has the potential for translation to clinical therapies, as mechanical stimulation of cells has the additional benefit of causing no long-term or permanent effects on the treated cells.

1.4.2.2 Objective 4: Determine effect of treatment of MDSCs with antioxidants prior to transplantation

The local environment after injury is less than optimal due to the presence of oxidative, inflammatory, and ischemic stresses [2]. This harsh environment contributes to the low cell survival following transplantation and the limited functional recovery observed clinically [11]. MDSCs have been shown to survive stress and transplantation to a greater degree than myoblasts, and MDSCs express higher levels of antioxidants, in particular glutathione, a major

cellular antioxidant [16]. When antioxidant levels in MDSCs are decreased, a reduction in regeneration is observed [16]. We hypothesized that treatment with antioxidants to increase cellular levels prior to transplantation could have a significant impact on cell survival, thereby increasing functional repair. Similar to mechanical preconditioning, the effects of antioxidant pretreatment are limited in cells and the therapy has significant potential to increase the efficacy of cell transplantation in clinic.

1.4.3 Clinical Relevancy

The gold standard of cell transplantation is autologous cell injection to bypass the immune response seen with allograft or xenograft transplantations and thereby eliminate the need for immunosuppressive therapy. Answering basic science questions about the properties of stem cells, such as the effect of sex and age of the cells, is critical for understanding the field before the therapy can become widely accepted. In addition, methods to improve transplantation that are safe, effective, and relatively easy to implement would improve functional recovery and potentially bypass the need for whole heart transplantation.

2.0 SEX OF MUSCLE STEM CELLS DOES NOT INFLUENCE POTENCY FOR CARDIAC CELL THERAPY

2.1 INTRODUCTION

Heart disease is the leading cause of death in the world, and a recent estimate from the American Heart Association stated that 1 in 3 American adults have a form of heart disease [74]. Heart disease includes both congenital and acquired cardiomyopathies as well as other types of damage, including ischemic injury from myocardial infarction. For most patients, the final therapeutic option for end-stage heart failure is heart transplantation, though this is not always a viable option due to low organ availability and the co-morbidities associated with long-term immunosuppression [4]. Limited donor organ availability and the low regenerative capacity of the adult heart following injury [75] has stimulated both basic science research and clinical trials to validate tissue engineering approaches to repair the injured and failing human heart.

Cellular cardiomyoplasty is an emerging therapeutic option that has gained recognition as a strategy to repair damaged myocardium and potentially reverse many debilitating heart diseases [4]. To date, a wide variety of cell types have been examined for suitability for cardiac repair, including skeletal myoblasts [52, 76], smooth muscle cells [7], fibroblasts [77], resident myocardial progenitors [10, 18, 19], mesenchymal stem cells [9, 20], hematopoietic stem cells [13, 17], and embryonic stem cells [12]. There have also been numerous clinical trials using cell

transplantation to repair the ischemic heart, but there were low efficiencies of transplanted cell survival, and concerns regarding increased risk of clinical arrhythmias continue to be under investigation.

Recently a population of murine muscle-derived stem cells (MDSCs) has been shown to have an improved regenerative capacity in bone and skeletal/cardiac muscles when compared to satellite cells or myoblasts [26, 34]. MDSCs are a unique cell population whose characteristics, including marker profile, proliferation and differentiation kinetics, and regenerative capacity, are distinct from myoblasts [25, 32]. After implantation into ischemic hearts, MDSCs display high levels of engraftment which persist over time, induce neoangiogenesis, prevent cardiac remodeling, and elicit significant improvements in cardiac function [34]. A human counterpart to MDSCs has been isolated based on cell surface markers with the human muscle-derived cells co-expressing myogenic and endothelial markers. These cells have been shown to be better than purely myogenic or endothelial cells for both skeletal muscle repair [24] and cardiac repair following myocardial infarction [14].

The variable results noted following cellular transplantation in most organ systems could be due to a variety of factors, including differences in cell populations and treatment regimens, as well as other intrinsic characteristics, including the sex of the cells and recipient. The association between sex and cardiac morbidity and mortality is well-established. The prognosis for males with heart disease is worse than for premenopause females [78, 79], and cardiovascular disease in women is less common and has a later average age of onset [80, 81]. Sex-related differences in stem cell behavior have been noted for the efficiency of endothelial progenitor cells colony formation, mesenchymal stem cell activation and function, MDSC-triggered skeletal muscle repair, and stress-triggered MDSC gene expression [58, 61, 62, 82]. These results

suggest that while the microenvironment plays a critical role in stem cell repair, differences in inherent stem cell characteristics, including sex, could account for some of the outcome variability noted in clinical trials. Characterizing subtle but clinically relevant factors that influence the biology of cells used for cardiac therapy is especially important to optimize clinical translation.

Our laboratory has recently shown that female MDSCs are superior to male cells for repairing skeletal muscle [58]. Transplantation of wild-type female cells resulted in greater number of dystrophin(+) myocytes in the gastrocnemius muscle of *mdx* mice (model for Duchenne muscular dystrophy lacking expression of dystrophin), regardless of the sex of the recipient [58]. Female recipients were also shown to have more dystrophin(+) myocytes after transplantation than male recipients with either cell sex. When exploring MDSC-induced osteogenesis, male MDSCs displayed more robust osteogenic and chondrogenic differentiation *in vitro* and *in vivo* versus female cells, regardless of the sex of the recipient [59, 83]. Based on these results, we hypothesized that there would also be a sex difference in MDSC-induced cardiac regeneration, which was supported by a recent study demonstrating sex differences in mesenchymal stem cells for cardiac repair [61]. In the current study we investigated transplantation efficiency of MDSCs in *mdx* mice (mouse model of Duchenne muscular dystrophy) and in a SCID mouse model of myocardial infarction to determine if donor cell sex or recipient sex would influence cardiac engraftment and regeneration/repair. Our results indicate that cell and host sex is not a critical factor for cardiac engraftment or repair using MDSCs.

2.2 MATERIALS AND METHODS

MDSC isolation: Muscle-derived stem cells were isolated from the skeletal muscle of three week old normal C57BL mice (Jackson) using the modified preplate technique as previously described [26, 31, 32]. Mice were anatomically sexed prior to isolation. Both male and female MDSCs (5 populations of each) were cultured in proliferation media (PM) containing DMEM (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 10% HS, 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical).

Intramyocardial cell transplantation to *mdx* mice: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Pittsburgh (protocol 14-06). *Mdx* mice (C57BL/10ScSn-Dmd^{mdx}) were bred in our institution's animal facility. Cell transplantation into *mdx* hearts was performed as previously described with less than 1% mortality[57, 84]. Briefly, each heart was exposed via a left thoracotomy, and $1x10^5$ MDSCs in 10µl phosphate buffered saline (five separately isolated populations of male and female cells) were injected into the left ventricular free wall of male or female *mdx* mice (12-16 weeks of age, N=6-12 animals per MDSC population). Two weeks after injection, the mice were sacrificed, the hearts harvested and flash-frozen in liquid nitrogen-cooled 2-methylbutane, and then cryosectioned to 10µm thickness.

Cell transplantation to infarcted myocardium: 50 male immunodeficient C57BL/6J-Prkdc^{scid} mice were bred for the current study. Infarcted mice were randomly allocated by a blinded investigator between the treatment groups (saline, female MDSCs, or male MDSCs), and $3x10^5$ cells total in 30µl saline was injected into the left ventricular free wall of male SCID mice (13-16 weeks of age) five minutes after the permanent ligation of the left coronary artery [34,

57]. Six weeks after cell injection, echocardiography was performed by a blinded investigator on the left ventricular short axis view at the mid-papillary muscle level as described previously [34, 57] to assess cardiac function, and the mice were euthanized and the hearts harvested, flash-frozen in 2-methylbutane, and cryosectioned [34, 57].

Engraftment: Cryosections were fixed in 4% paraformaldehyde and then stained to determine donor cell engraftment. For non-injury model in *mdx* mice, the number of dystrophin(+) myocytes was determined as described previously [25, 32]. Briefly, sections were stained for dystrophin with a rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 488 donkey anti-rabbit secondary antibody (1:200; Molecular Probes). Nuclei were revealed with 4'6-diamidino-2-phenylindol (DAPI) stain (100 ng/ml; Sigma) and sections were mounted with Gel Mount Aqueous Mounting Medium (Sigma). The engraftment capacity was determined by counting the number of dystrophin(+) fibers. In the infarction model, heart sections were stained with mouse anti-fast skeletal myosin heavy chain (fsMHC) antibody (1:400; Sigma) and Alexafluor 555 donkey anti-mouse secondary antibody (1:300; Molecular Probes) to examine engraftment of the different cell populations. Engraftment was determined by assessing the area of fsMHC(+) cells normalized to the area of the entire muscle section [32].

Identification of Endothelial Cells within the Engraftment of Transplanted Cells: The number of CD31+ cells in the engraftment area was determined by double-staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma) and rabbit anti-dystrophin primary antibody (1:400; Abcam), and Alexafluor 488 donkey anti-rabbit secondary antibody (1:200; Molecular Probes) and Alexafluor 555 donkey anti-rat secondary antibody (1:300; Molecular Probes) for the *mdx* mouse model. The rat anti-CD31 primary antibody (1:300; Sigma) and mouse anti-fsMHC antibody (1:400; Sigma) was used for the infarcted samples [34, 57, 84].

Blood vessel formation within the cell-injected areas was determined by the number of CD31(+) cells associated with dystrophin(+) cells for the *mdx* model or fsMHC(+) cells for the infarction model.

Collagen Staining: Infarcted heart sections were fixed with 1% gluteraldehyde for 2 minutes and stained with the Masson Modified IMEB Trichrome Stain Kit (IMEB, CA, USA). Trichrome staining was performed according to the manufacturer's guidelines and as previously described [34, 57]. The sections were then assessed for the percentage area of collagen in 5 sections per heart normalized to total muscle area within the section using ImageJ software (NIH).

Staining for Cardiac-Specific Markers: Sections from *mdx* samples were stained with rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 488 donkey anti-rabbit secondary antibody (1:300; Molecular Probes) and goat anti-cardiac troponin I (1:25,000; Scripps) with rabbit anti-goat Alexafluor 555 (1:200; Molecular Probes). Sections from the infarcted samples were stained with mouse anti-fsMHC (1:400; Sigma) and Alexafluor 488 donkey anti-mouse secondary antibody (1:300; Molecular Probes) and goat anti-cardiac troponin I (1:25,000; Scripps) with rabbit anti-goat Alexafluor 555 (1:200; Molecular Probes) and the number of double-positive cells were counted in 3 high powered fields per heart.

Analysis of Angiogenic Factor Secretion: Five male and female MDSC populations were plated in PM at 50,000 cells/well in 6-well collagen type 1 coated plates. Twenty-four hours later, the media was switched to DMEM with 1% penicillin/streptomyocin. Cells were then cultured for an additional 24 hours when the media was collected and flash-frozen until analysis. ELISA for mouse VEGF (R&D Systems) was performed according to manufacturer's

instructions and as previously described [57]. The VEGF levels were normalized to cell number as measured by hemacytometer.

Cell Survival following Oxidative Stress: Cells were plated in PM at 1000 cells/well in a 24-well collagen type 1 coated plate. Twenty-four hours later, the media was switched to PM with propidium iodide (PI, 1:500, Sigma) or PM containing 350 μM hydrogen peroxide with PI. The plates were then placed onto a previously described live cell imaging system (Automated Cell, Inc), and fluorescent and brightfield images were taken every 10 minutes in 3 fixed locations per well. These images were analyzed using ImageViewer software (ACI). Cell proliferation was determined by counting the number of cells present in the brightfield images at 12 hour time points for 60 hours. Cell survival was determined by counting the number of PI-positive cells in the fluorescent images at each 12 hr time point.

Microscopy: Florescence and brightfield microscopy were performed using either a Nikon Eclipse E800 microscope or Leica DMIRB inverted microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix). Image analysis was performed using Northern Eclipse software or Image J software (available from NIH).

Statistical analysis: Data are summarized as mean and standard deviation. Statistically significant differences between groups were determined using a t-test for *mdx* studies (Microsoft Excel) or 1-way or 2-way ANOVA (SigmaStat) as appropriate for the infarction model. In the event significant differences were detected using ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (Tukey test).

2.3 RESULTS

2.3.1 Sex-Related MDSC Fate Following Implantation into the Non-Ischemic *MDX* Heart

Survival and Engraftment of MDSCs in the *mdx* heart: To determine whether cell and recipient sex were critical determining factors in engraftment efficiency of dystrophin(+) cells in the myocardium, 5 male and 5 female MDSC populations were injected into the hearts of both male and female *mdx* mice. Two weeks after cell transplantation, dystrophin(+) myocytes were observed within the hearts of all animals injected MDSCs (Figure 2.1 A&B). The regeneration index (RI) revealed variability in MDSC engraftment in cardiac muscle, with cell populations varying between approximately 100 to over 2000 dystrophin(+) cells. Sex-matched experiments showed no sex-based difference in RI (P=0.68). Male cells/recipients had 1309 ± 295 dystrophin(+) cells and female cells/recipients had 1010 ± 181 dystrophin(+) cells. Sex of the recipient also showed no preferential effect on MDSC engraftment within the heart: 1086 ± 172 dystrophin(+) fibers in male recipients versus 1069 ± 146 dystrophin(+) fibers in female recipients (P=0.42, Figure 2.1C). When the sex of the cell was matched with that of the host, the number of dystrophin(+) myocytes was slightly increased (sex-matched: 1167 ± 178, sex-mismatched: 838 ± 107, *P*=0.12). Very low levels of differentiation of implanted MDSCs toward a cardiac phenotype, averaging less than 1% of injected cells, were seen with all MDSC populations, regardless of sex or number of dystrophin(+) myocytes, which is consistent with previously published results [13, 34]. Overall, there was no difference in the number of engrafted dystrophin(+) myocytes based either on the sex of the MDSCs or the sex of the recipient.

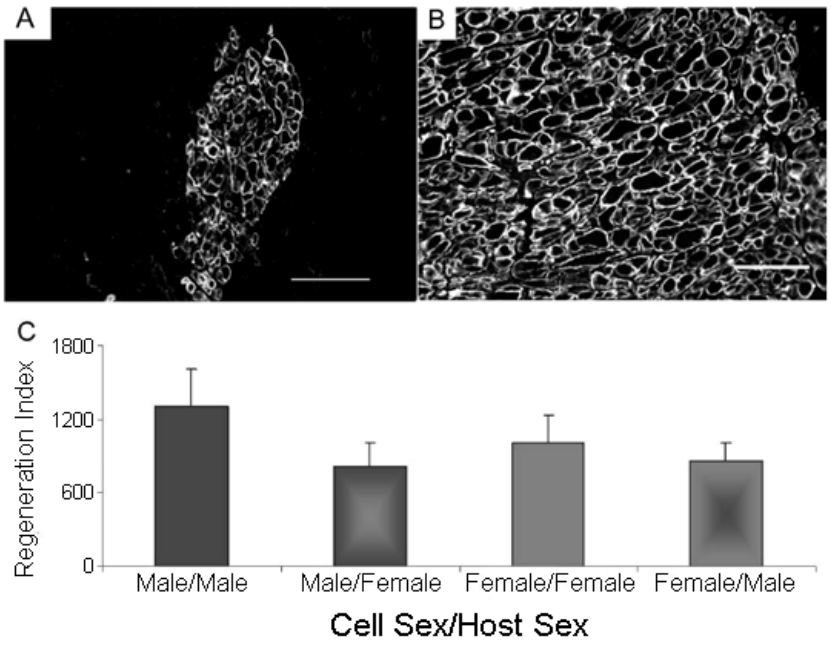

Figure 2.1 Engraftment of MDSCs is not dependent on sex of cells or host. (A) Poor engraftment (B) Good engraftment (C) Engraftment based on cell and host sex

Sex does not influence vascularization of MDSCs in the *mdx* heart: MDSC implantation triggers angiogenesis during cardiac remodeling following injury [57, 85]. At two weeks post-cell transplantation, we measured the number of endothelial cells within the dystrophin (+) cell engraftment zones for all animals injected with MDSCs (Figure 2.2 A,B). There was no difference between male and female cells (male cells: 17 ± 3 CD31(+) structures, female cells: 14 ± 2, Figure 2.2A). No differences were noted between the sex of the MDSCs or recipient with respect to the number of CD31(+) structures in the dystrophin(+) areas (male cells: 17 ± 3, female cells: 13 ± 2, P=0.26, male recipient: 15 ± 3, female recipient: 14 ± 3, P= 0.725, Figure 2B). We also noted no correlation between the density of dystrophin(+) myocytes and the number of endothelial cells (endothelial cells in high dystrophin(+) regions: 17 ± 3, endothelial cells in low dystrophin(+) regions: 12 ± 2, P = 0.23, Figure 2.2B). Thus, angiogenesis within

the implanted region did not appear to be affected by the sex of MDSCs and recipient mice or the efficiency of engraftment of MDSCs.

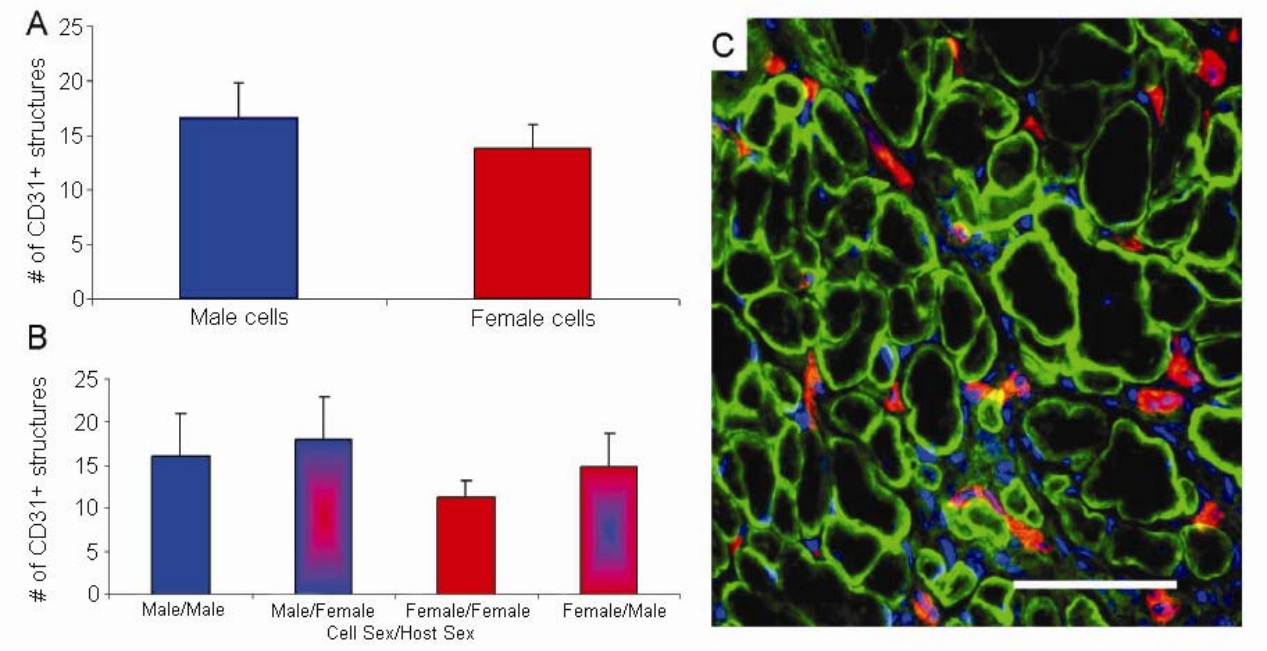

Figure 2.2 Vascularization is not dependent on cell or host sex. (A) Blood vessels based on cell sex (B) Blood vessels based on cell and host sex (C) Image of blood vessel formation in engraftment

2.3.2 Sex-Related MDSC Fate in a Myocardial Infarct Model

Cardiac Function: We measured cardiac function using transthoracic echocardiography in *SCID* mice 6 weeks following myocardial infarction and MDSC implantation. Animal groups were divided into recipients treated with male MDSCs, female MDSCs, or saline alone. We noted no difference in left ventricular fractional area change (FAC) between cell-injected hearts based on MDSC sex: however, male and female cells had increased FAC versus saline controls (23.2 ± 1.3% for male cells, 21.5 ± 1.1% for female cells, and 13.1 ± 0.8% for saline, $p<0.05$, Figure 2.3A). Post-infarction fractional shortening (male cells: 20.6 ± 1.2%, female cells: 19.1 ± 0.5%, and saline: 14.4 ± 0.8), another measure of LV contractility, and end diastolic area, which

measures dilatation of the heart (male cells: 15.0 ± 0.7%, female cells: 14.6 ± 0.6%, and saline: 17.8 ± 1.3%, $p<0.05$, Figure 2.3B) displayed similar therapeutic effects.

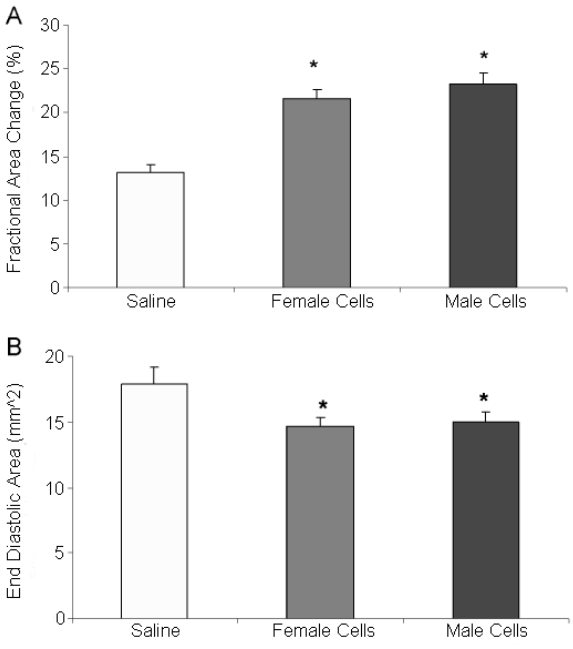

Figure 2.3 Cardiac function after injury is not influenced by cell sex. (A) Fractional area change (B) End diastolic area

Cardiac Scar Tissue Remodeling: Collagen deposition and scar formation following myocardial injury impairs systolic and diastolic function as well as functional recovery [86]. Using standard measures of cardiac scar formation (collagen staining by Masson's trichrome and calculation of the ratio of healthy LV to LV scar), we noted that saline-injected hearts had very large scar tissue area ratios 6 weeks following myocardial infarction (65.0 ± 5.1%, Figure 2.4A). MDSCs were found to have superior engrafting abilities versus saline injection, however, cell sex did not influence post-infarction scar inhibition, as all cell-injected groups had significantly smaller scar tissue area ratios (male cells: 35.2 ± 5.4%, Figure 2.4B, female cells: 35.7 ± 6.9%, Figure 2.4C, $p<0.05$ compared to saline, Figure 2.4D).

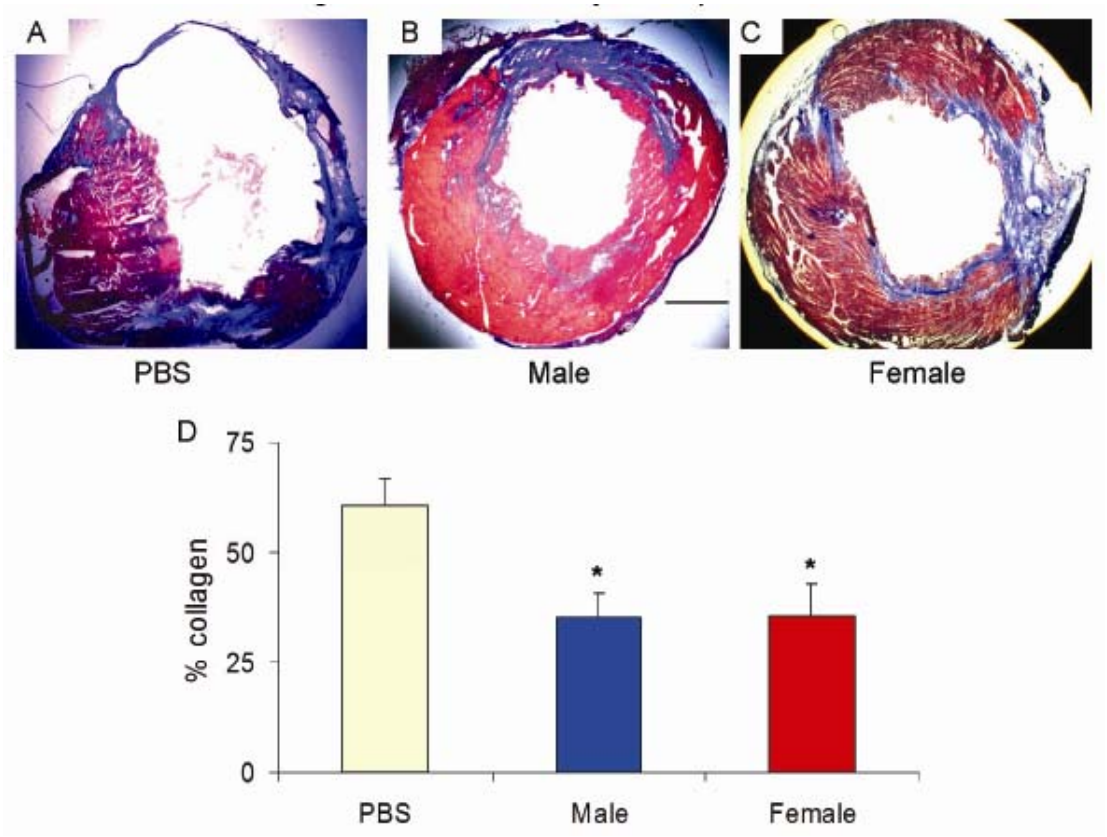

Figure 2.4 Scar tissue formation is not dependent on cell sex. (A) PBS (B) Male MDSCs (C) Female MDSCs (D) Scar tissue fraction

Sex-Related MDSC VEGF Secretion: We determined VEGF secretion into cell culture supernatant for 24 hours of serum starvation due to the critical role of VEGF in post-infarction angiogenesis. No difference in VEGF secretion between male and female MDSCs was noted (male: 623 ± 200 pg/ml/10^6 cells, female: 752 ± 143, P=0.58, Figure 2.5A). When subjected to oxidative stress via hydrogen peroxide treatment, all cells increased their VEGF production regardless of cell sex (male cells: 1387 ± 389 pg/ml/10^6 cells, female cells: 1240 ± 233).

Angiogenesis: No difference in blood vessel formation was noted in the engraftment areas based on MDSC sex (male cells: 10 ± 1 CD31(+) structures, female cells: 11 ± 1, P=0.37,

Figure 2.5B). However, MDSC injection induced greater levels of neovascularization than saline injection outside of the engraftment area at the 6-week time point regardless of cell sex (PBS: 34 ± 4 CD31(+) structures, male cells: 43 ± 7, female cells: 54 ± 9, Figure 2.5C). These results support the role for MDSCs in inducing neovascularization versus a recipient response.

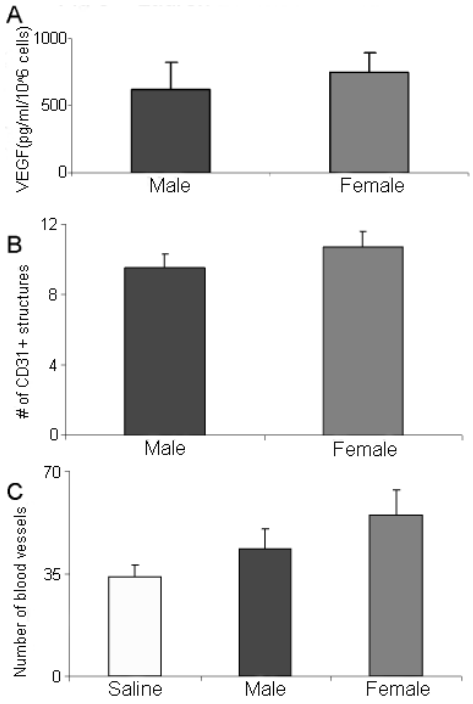

Figure 2.5 Angiogenesis after injury is not cell sex-dependent. (A) VEGF secretion (B) Angiogenesis in engraftment (C) Angiogenesis outside engraftment

Cell Survival after Oxidative Stress: MDSCs have a superior ability to survive oxidative stress compared to myoblasts [34], which may correlate to a higher regeneration capacity in skeletal and cardiac muscle. Male and female MDSCs display similar survival characteristics in response to oxidative stress (Figure 2.6A), which correlates to the similar levels of survival and cardiac engraftment for male and female cells seen *in vivo*.

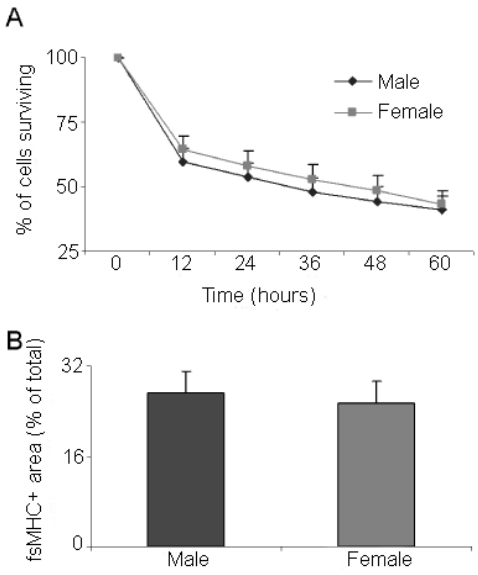

Figure 2.6 Cell sex does not alter survival *in vitro* or *in vivo*. (A) Cell survival under oxidative stress *in vitro* (B) Engraftment area

Donor Cell Engraftment and Differentiation: We noted a lack of difference in the degree of MDSC engraftment in post-infarction *SCID* LV myocardium based on the sex of implanted MDSCs at 6 weeks (male cells: 27.2 ± 3.8%, female cells: 25.2 ± 3.9 %, Figure 2.6B). As noted in previous studies, we found less than 1% of implanted MDSCs expressed cardiac markers (male cells: 0.2 ± 0.2%, female cells: 0.3 ± 0.1%), with the vast majority maintaining expression of skeletal muscle markers [34, 57]. The engraftment and regeneration noted in the infarction model differed from the non-infarcted *mdx* model, and the results may have been influenced by the time points examined, the different number of injected cells, and presence of injury in the infarction model.

2.4 DISCUSSION

Cell transplantation is considered to be a promising therapy for cardiac repair after injury, however, there is considerable debate regarding the mechanism and critical factors that impact outcome. Some groups have demonstrated that stem cells can differentiate into cardiomyocytes *in situ* [12], thereby enacting repair, whereas others have shown that paracrine mechanisms provide cardioprotection [8, 85]. Up until recently, cell sex was not considered to be a factor in determining outcome after cell transplantation, and results seem to be primarily dependent on injury model, cell type, and time points examined [61, 81]. Previous results from our laboratory suggested that cell sex could influence muscle, cartilage, and bone regeneration. These experiments demonstrated that female MDSCs are better at engrafting into skeletal muscle, while male cells are more osteogenic and chondrogenic [58, 59]. Other groups have shown that cell sex is also a factor in mesenchymal stem cell activation and cardiac repair, with female cell injection producing improved cardiac recovery [61, 62]. Based on these results, we hypothesized that female muscle-derived cells would be superior for cardiac repair.

We observed no difference in the engraftment capacity of MDSCs in the *mdx* heart based on sex. This result is in contrast to previously reported results on MDSC transplantation into skeletal muscle, and we hypothesize that this difference may be due to the different microenvironment found in the heart, including cyclic mechanical strain, paracrine factors, and the biochemical make-up of infarcted myocardium. We have demonstrated that transplantation of both male and female murine MDSCs attenuated functional deterioration after myocardial

infarction, and we observed that cardiac repair using MDSCs does not appear to be dependent on cell sex. There was no difference when examining cardiac function after cell transplantation, engraftment capacity, scar tissue formation, or co-expression of cardiac phenotype and angiogenesis between the male and female cells. These results suggest that the recovery of post-infarction LV function following MDSC implantation is not due to MDSC sex.

The original paradigm for the mechanism of cardiac cell-mediated therapy is that delivery of the appropriate stem cells would repair the damaged heart via active myocardial regeneration from transdifferentiation of the administered stem cells to the cardiac lineage [87]. However, increasing evidence has led to the recognition that paracrine effects are another key mechanism of cardiac repair. Consistent with this paradigm, MDSCs seem to enact repair through paracrine mechanisms and increasing survival and repair of the recipient cardiac tissue rather than by differentiating into cardiomyocytes. Recovery of cardiac tissue without cardiac differentiation has also been seen with bone marrow and hematopoietic stem cells used to treat ischemia reperfusion injuries and myocardial infarctions [8, 13]. Previous results have highlighted the importance of VEGF secretion in cardiac repair [57], and since both male and female MDSCs secrete similar levels of VEGF, this could help reconcile, at least in part, the lack of a sex difference seen in terms of capillary density in this study. When treating ischemic heart disease, reperfusion of the affected tissue is an essential goal of any therapy. The improved regenerative capabilities of MDSCs might be attributed to higher expression levels of antioxidants and/or increased VEGF production [57]. In the heart, cells do not need to differentiate into cardiomyocytes to improve heart function after injury, and this suggests that sex-based differences in MDSCs may only be seen when differentiation toward a specific lineage is required. This idea is supported by sex differences observed with MDSC transplantation for

skeletal muscle, where the cells had to differentiate toward skeletal muscle lineages to promote the healing process.

There are many issues to consider when investigating the influence of sex on stem cell regenerative potential, particularly for cardiac repair. Intrinsic and extrinsic cellular mechanisms regulate the balance of self-renewal and differentiation in stem cells [88]. Though we did not find sex differences with MDSCs for cardiac repair, other groups have shown that source sex is relevant for mesenchymal stem cells [61]. Though it was not examined in the mesenchymal cell study, we cannot exclude the possibility that a sex difference was seen with these cells due to differential differentiation toward a cardiac phenotype. Of note, we implanted MDSCs isolated from 3 week old mice, which could have influenced the results, and our results do not rule out a sex-related effect on regenerative efficiency for aged MDSCs in the heart.

In summary, there appears to be no sex-based difference for cardiac repair using young MDSCs. Further work needs to be done to determine if this lack of effect is related to age of cells or recipients, as well as to clarify the role differentiation plays in sex-related differences in repair. The lack of a sex difference in MDSCs for cardiac repair is promising for clinical translation. For autologous use, these results suggest that the potency of cells for clinical translation will theoretically be equivalent for both male and female patients. However, autologous transplantation it is not always feasible, and either sex of MDSCs can be used with beneficial effect.

2.5 ACKNOWLEDGEMENTS

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3.0 AGING HAS LITTLE EFFECT ON BEHAVIOR OF MUSCLE DERIVED STEM CELLS

3.1 INTRODUCTION

The ability of the body to heal after injury decreases significantly with age, and this leads to increasing health problems that need to be addressed by the medical community. There are two explanations for this age-related decline: either the cell populations within the organs that are responsible for repair after injury are decreasing and thus not as well able to enact repair or the environment is no longer optimal and causes a decrease in cell-mediated repair. Based on current research, it appears that both explanations play some role in aging and decreased stem cell function [32, 63]. Stem cells not only repair after injury, they are also responsible for replacing cells that are lost during normal activity. The population of stem cells decreases as some of the cells become quiescent, and DNA damage and apoptosis further reduces the available population of cells for repair [67, 69]. The environment also undergoes age-related changes, including reduced notch signaling and the presence of byproducts such as cleaved fibronectin, which can lead to cell death [65].

Skeletal muscle contains several cell types that have been used for repair, including myoblasts, satellite cells, and MDSCs. As skeletal muscles age, there is a reduction in the regenerative response, and after injury there is a slower and less effective repair response, with a

corresponding increase in fibrosis. Satellite cells have been extensively studied, and though there is an age-related decrease in cell number, there is also a decrease in functionality that impairs healing and regeneration. Further research has found that with the right environmental cues, aged satellite cells are able to regain a "younger" phenotype, which implies that it is the environment that has undergone the majority of age-related decline rather than the cells themselves. To further reinforce that hypothesis, a subpopulation of aged satellite cells has been shown to survive skeletal muscle aging, and these cells are able to repopulate damaged muscle in a manner similar to young satellite cells.

Here we show that MDSCs have the same ability to maintain a stem cell phenotype throughout the aging process. We isolated MDSCs from three different ages of mice: 3 weeks (young), 9 months (adult), and 18 months (aged). Cell survival under stress and vascular endothelial growth factor (VEGF) secretion are both unchanged, though proliferation and differentiation toward a skeletal muscle phenotype *in vitro* decreased with age. In comparison, *in vivo* MDSCs have the same ability to regenerate skeletal muscle regardless of their age, and cells isolated from aged animals have the same capacity for neoangiogenesis as cells isolated from young mice. There is an age-related decrease in regeneration in the host, and this further confirms that the environment of transplantation plays more of a role in the age-related decline in regeneration than the stem cells themselves.

3.2 MATERIALS AND METHODS

MDSCs were obtained from young (3 week old), adult (9 month old), and aged (18 month old) male C57BL/10J mice using the modified preplate technique described previously[31].

Proliferation: To assess cell proliferation, cells were plated at 1000 cells/well in proliferation medium and placed onto a previously described time-lapsed microscopic imaging system (Automated Cell Inc (ACI)) where brightfield pictures were taken every 10 minutes. Images were taken at 10X, and three pictures were taken per well with 3 wells for each cell type (3 young, 3 old). Cells were observed for 48 hours, and data was analyzed using ImageViewer software (ACI) and the number of cells in the brightfield image was counted in 12 hour increments.

Differentiation: To assess differentiation toward the myogenic lineage, cells were plated at 1000 cells/well in a 24-well plate in proliferation medium for 24 hours, and were then switched to fusion medium (DMEM, 2% FBS, 1% P/S). At 3 and 5 days post change to fusion media, cells were fixed in ice-cold methanol, washed, stained for fast skeletal myosin heavy chain and conterstained with DAPI. Microscopy was performed with a Leica DM IRB inverted microscope equipped with a Spot digital camera and Northern Eclipse software. Three images per well were taken for three wells of each cell type. After imaging, the amount of differentiation was determined by dividing the number of nuclei in myotubes by the total number of nuclei.

Intracardiac cell transplantation to *mdx* mice: The use of animals and the surgical procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Pittsburgh. *Mdx* mice (C57BL/10ScSn-Dmd^{mdx}) were bred at the institution's animal facility. Cell transplantation into *mdx* hearts was performed as previously described with less than 1% mortality. Briefly, each heart was exposed via a left thoracotomy, and $1x10^5$ MDSCs in 10µl PBS (three young male populations, 3 adult male populations, and 2 aged male cell populations) were injected into the left ventricular free wall (3

and 15 months of age, N=3-4 animals of each age per MDSC population). At 2 weeks post-injection the mice were sacrificed, the hearts harvested and flash-frozen in liquid nitrogen-cooled 2-methylbutane, and then cryosectioned at 10μm.

Intramuscular cell transplantation to *mdx* mice: Cell transplantation into *mdx* skeletal muscle was performed as previously described[58]. Briefly, each gastrocnemius muscle was injected with $1x10^5$ MDSCs in 10μl PBS (three young male populations, 3 adult male populations, and 2 aged male cell populations) into differentially aged mice (3 and 12 months of age, N=3-4 animals of each age per MDSC population). At 2 weeks post-injection the mice were sacrificed, the skeletal muscles harvested and flash-frozen in liquid nitrogen-cooled 2-methylbutane, and then cryosectioned at 10μm.

Number of dystrophin(+) myocytes: Cryosections were fixed in 4% paraformaldehyde and then stained to determine donor cell engraftment. For non-injury model in *mdx* mice, the number of dystrophin(+) myocytes was determined as described previously. Briefly, sections were stained for dystrophin with a rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 488 donkey anti-rabbit secondary antibody (1:200; Molecular Probes). Nuclei were revealed with 4'6-diamidino-2-phenylindol (DAPI) stain (100 ng/ml; Sigma) and sections were mounted with Gel Mount Aqueous Mounting Medium (Sigma). The engraftment capacity was determined by counting the number of dystrophin (+) fibers.

Identification of blood vessels within the engraftment of transplanted cells: The number of blood vessels in the engraftment area was determined by double-staining with rat anti-CD31 primary antibody (1:300; Sigma) and Alexafluor 488 donkey anti-rabbit secondary antibody (1:200; Molecular Probes), as well as rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 555 donkey anti-mouse secondary antibody (1:300; Molecular Probes)

for the *mdx* mouse model. Blood vessel formation within the cell-injected areas was determined by the ratio of CD31(+) cells associated with dystrophin(+) cells.

Analysis of angiogenic factor secretion: Two or three populations of each age group of MDSC populations were plated in PM at 50,000 cells/well in 6-well collagen type 1 coated plates. Twenty-four hours later, the media was switched to DMEM with 1% penicillin/streptomyocin. Cells were then cultured for an additional 24 hours when the media was collected and flash-frozen until analysis. ELISA for mouse VEGF (R&D Systems) was performed according to manufacturer's instructions and as previously described[57]. The VEGF levels were normalized to cell number as assessed by hemocytometer.

Microscopy: Florescence and brightfield microscopy were performed using either a Nikon Eclipse E800 microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix) or a Leica DMIRB inverted microscope with a Retiga digital camera and Northern Eclipse software. Image analysis was performed using Northern Eclipse software or Image J (available from NIH).

Statistical analysis: The mean and standard deviation will be obtained for all measured values, and statistical significance between the groups will be determined by a t-test (Microsoft Excel) or 1-way or 2-way ANOVA (SigmaStat) as appropriate. In the event of a significant ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (Tukey test).

3.3 RESULTS

Increased MDSC donor age leads to decrease in *in vitro* skeletal muscle differentiation and proliferation: The proliferation rate of young and adult MDSCs are similar, but cells isolated

from aged animals (18 months) have a reduced rate at later time points (Figure 3.1A, * is $p<0.05$ to young and adult cells). With increasing age, MDSCs differentiate toward skeletal muscle *in vitro* to a reduced extent as assessed by the number of nuclei in myotubes (Figure 3.1D, * is $p<0.05$ to young cells, ** is $p<0.05$ to adult cells).

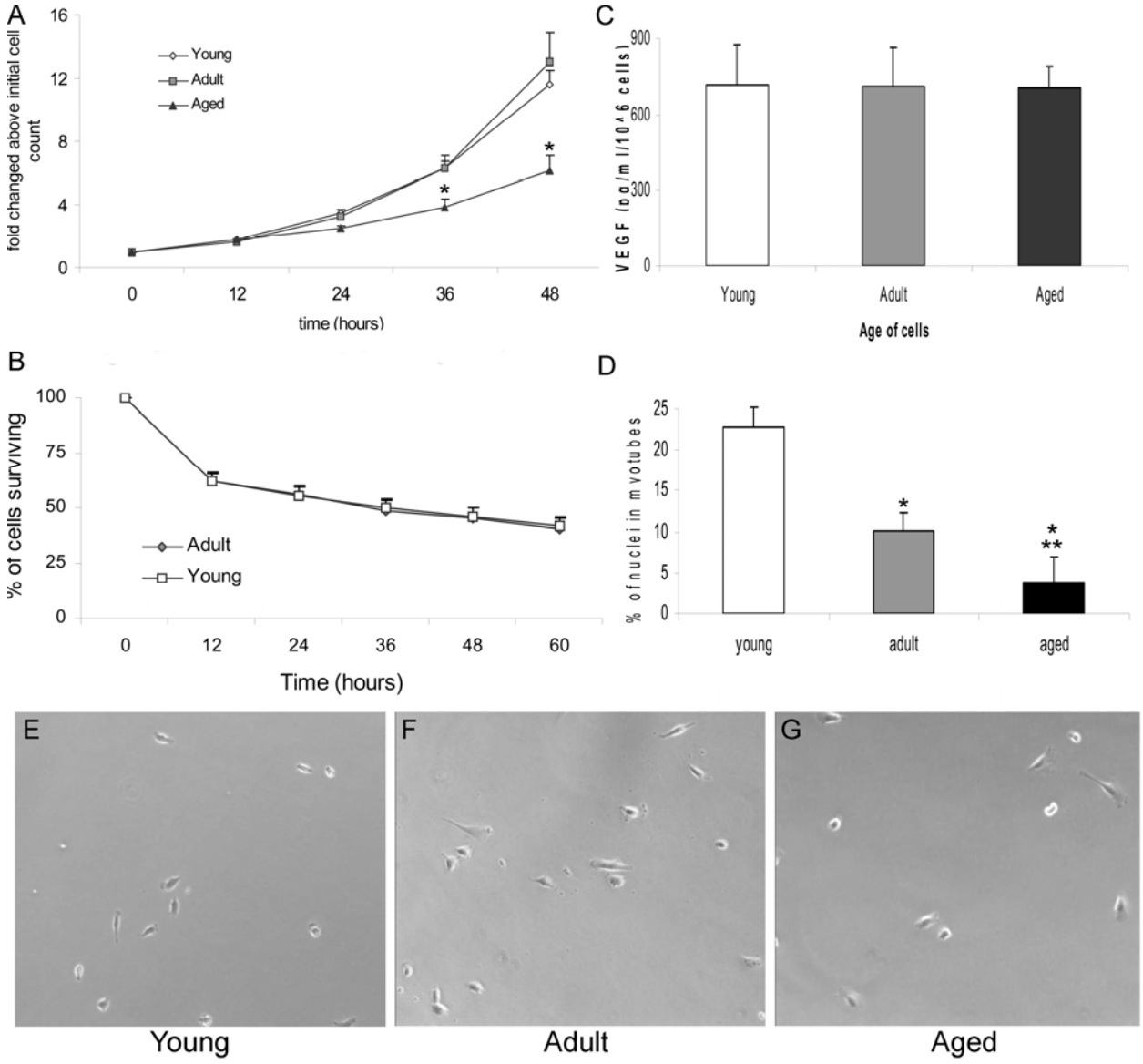

Figure 3.1 *In vitro* characteristics change with MDSC age. (A) Proliferation (B) Cell survival under oxidative stress (C) VEGF secretion (D) Differentiation (E, F, G) Young, adult, and aged MDSC morphology

***In vitro* cell survival, VEGF secretion, and morphology are unaffected by age of donor cells:** Cell survival under oxidative stress was similar in the young and adult populations, but was decreased in the aged population, implying that there is a lowered survival capacity in older cell populations (Figure 3.1B). VEGF secretion between the different age groups was also similar (Figure 3.1C). No difference in the morphology of the cells (Figure 3.1E,F,G) was visible.

Engraftment and host regeneration in *mdx* skeletal muscle is unaffected by cell age, but host age plays a role: At two weeks post cell implantation, dystrophin positive cells were counted for the largest engraftment area in skeletal muscle. No difference was seen in the regeneration index based on cell age, but the age of the host may play a role, with older hosts having a trend towards lower regenerative capacity (Figure 3.2A, P=.08 between young cells in young and aged hosts, P=0.17 between adult cells in young and aged hosts). When regeneration of host cells was examined in skeletal muscle using centrally-located nuclei in myofibers, there was no difference based on cell age, but increasing the age of the host significantly decreased host regeneration (Figure 3.2C, * is $p<0.05$ to aged host).

Angiogenesis is unaffected by cell or host age: To observe the role angiogenesis plays in this response, the number of CD31 positive microvessels within the engraftment areas of the skeletal muscles of animals injected with MDSCs of different ages was quantified. Angiogenesis was similar between all age groups examined, and no difference was seen based on host age (Figure 3.2B).

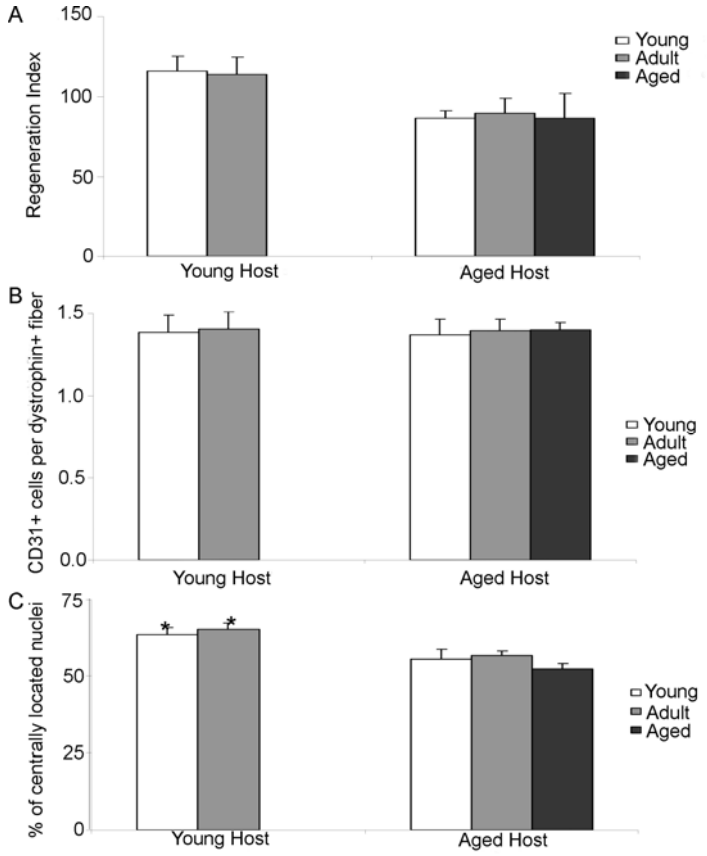

Figure 3.2 *In vivo* regeneration and angiogenesis with MDSCs of varying ages. (A) Regeneration (B) Angiogenesis (C) Host regeneration

Engraftment and angiogenesis in *mdx* cardiac muscle is unaffected by cell age: At two weeks post cell implantation, dystrophin positive cells were counted for the largest engraftment area in cardiac muscle. No difference was seen in the regeneration index based on cell age, though only the young and adult populations were examined (Figure 3.3A). The number of CD31 positive microvessels within the engraftment areas of the heart of animals injected with MDSCs of different ages was quantified. Angiogenesis was similar between the two age groups (Figure 3.3B).

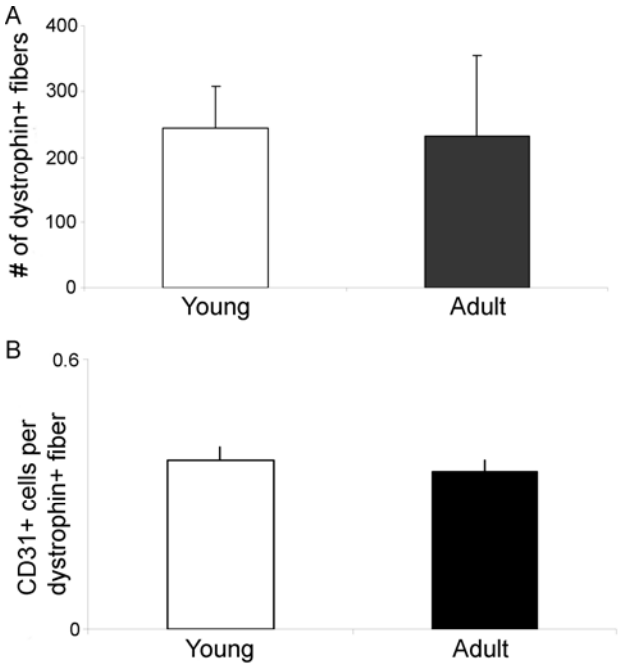

Figure 3.3 Cardiac regeneration unaffected by cell age. (A) Number of dystrophin+ fibers (B) Angiogenesis

3.4 DISCUSSION

It has previously been shown that stem cell function decreases with age, but whether intrinsic or extrinsic factors play the determining role has yet to be elucidated. However, there has been increasing evidence that it is the stem cell niche in skeletal muscle which suffers the majority of deleterious effects of aging rather than the stem cells themselves. There remains some disagreement as to the effects of increasing age on the cells, with some observations reporting that differentiation is reduced, while others noting that proliferation or regeneration is affected more severely. Our current results aid in clarifying this issue for muscle-derived stem cells. Specifically, we investigated the performance of the cells *in vitro* and their regeneration capacity *in vivo*. These cells appear to resist, but not completely escape, the effects of aging. Differentiation is significantly decreased with increasing age, and proliferation is also decreased. On the other hand, VEGF secretion and the regeneration capacity of the cells *in vivo* appear unchanged. To provide more evidence that it is indeed the milieu that is affected by aging, the age of the host had more of an effect on host regeneration and the number of dystrophin positive fibers than the age of the cells.

Aging is a relentless process, though the effects are not typically seen until the accumulation of changes is great enough to impair function. We have confirmed here that the MDSC population of skeletal muscle does exist at all ages and can be isolated through the preplate technique. We have documented the phenotype of different ages of MDSCs, including morphology, proliferation, differentiation, and VEGF secretion. Using *in vivo* experiments, we have examined the regeneration capacity of these cells in skeletal and cardiac muscle. Despite the phenotypic changes seen *in vitro*, *in vivo* MDSCs do not appear to be deleteriously affected by age, with the regeneration capacity remaining similar between the age groups examined.

4.0 MECHANICAL LOADING OF STEM CELLS IMPROVES TRANSPLANTATION OUTCOME: UNIQUE ROLE OF LOADING HISTORY

4.1 INTRODUCTION

Stem cell transplantation is a prospective therapy for the regeneration of numerous tissues and medical conditions, including heart disease, muscular dystrophy, diabetes, hemophilia, multiple sclerosis, Parkinson's and others [5, 89, 90]. Cardiac disease, particularly myocardial infarction, is a serious health issue worldwide [1]. Cell therapy for cardiac repair is a growing area of research, with a wide variety of cell types under investigation [4-6]. Progenitor and stem cells can be derived from nearly every tissue in the body, but skeletal muscle derived cells have shown particular promise due to the potential for autologous transplantation, ease of isolation, and high resistance to ischemia. We have previously isolated a population of early myogenic progenitors called muscle-derived stem cells (MDSCs) based on selective adherence to collagen coated flasks [26, 31] that have been effective in transplantation approaches. MDSCs are multipotent and have been shown to differentiate along skeletal, cardiac and smooth muscle, bone, cartilage, tendon, nerve, endothelial and hematopoietic lineages [26, 33, 34, 91, 92]. MDSCs regenerate cardiac and skeletal muscle in a model of Duchenne muscular dystrophy, and significantly improve cardiac function above myoblasts in a myocardial infarction model [34, 84].

While stem cell transplantation has produced promising results, additional work is needed to improve the repair process. One main hurdle that needs to be overcome in order to improve cell transplantation is low cell survival post-transplantation, which is the likely cause for limited functional repair. This may due to initial loss during transplantation and subsequent biological cell death [93], including immunological issues and poor survival of the injected cells [94, 95]. The response of cells *in vivo* is influenced by factors in the cellular environment such as the extracellular matrix, growth factors, cytokines, and mechanical loading. For example, when vascular endothelial growth factor (VEGF) secretion is increased, improved cardiac repair is seen after a myocardial infarction [57]. The activation of signaling pathways by mechanical preconditioning of skeletal myoblasts promoted their survival by release of paracrine factors that increase angiomyogenesis in the infarcted heart [96].

Most cells and tissues experience forces within the body [97], and mechanical and structural cues have been shown to affect cell behavior and play a critical role in tissue physiology, including cell growth, migration, gene expression, cell signaling, and survival [97, 98]. However, no consensus has been reached as to the role mechanical stimulation plays in the regeneration process. One area which has shown recent promise is the concept of examining the effects of mechanically preconditioning cells on biological responses [70, 99-101]. Skeletal muscle is known to respond to mechanical stimulation through its functional demands and can regulate the production of structural proteins including actin and myosin [102]. Mechanical stretch plays an important role in the proliferation of mesenchymal stem cells [103], and a recent report demonstrates that the application of cyclic stretch induced the proliferation of satellite cells and inhibited their differentiation into myotubes [104]. The effects of mechanical preconditioning on biochemical and structural responses suggests the mechanical loading history

experienced by cells *in vitro* or *in vivo* may affect cellular behavior to a greater extent than previously thought.

In these experiments, we show that the loading history of MDSCs plays a role on *in vitro* characteristics as well as *in vivo*, including transplantation into undamaged cardiac and skeletal muscle in *mdx* mice (dystrophin-deficient) and into infarcted myocardium. We investigated the effects of mechanically preconditioning cells *in vitro* in order to identify important molecular pathways that regulate this biologic effect. We analyzed paracrine factor secretion, resistance to oxidative stress, proliferation, and differentiation toward skeletal muscle. We then examined whether mechanically stimulating (MS) MDSCs *in vitro* prior to implantation could improve regeneration *in vivo*, and found that compared to non-stretched (NS) MDSCs, mechanical loading increases angiogenesis, donor cell engraftment as assessed by the number of dystrophin positive cells, and host regeneration in uninjured *mdx* mice. These results are evident from the cellular to tissue levels, indicating that it is not just a local cell mechanotransduction event. In a mouse model of myocardial infarction, MS MDSCs decreased the negative cardiac remodeling to a greater extent than NS MDSCs or saline alone. Through these studies, we found that mechanical preconditioning has a significant effect on angiogenic factor secretion and improves stem cell transplantation primarily through paracrine mechanisms.

4.2 MATERIALS AND 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 Children's Hospital of Pittsburgh of UPMC (University of Pittsburgh Medical Center, protocol 14/06).

MDSC isolation and cell culture under mechanical stimulation: Muscle-derived stem cells were isolated from the skeletal muscle of three week old normal C57BL/10J mice (Jackson Laboratories) using the modified preplate technique as previously described [31]. MDSCs were cultured in proliferation media (PM) containing DMEM (Invitrogen), 10% FBS (Invitrogen), 10% HS (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.5% chick embryo extract (Accurate Chemical). MDSCs were cultured on flexible collagen-coated culture plates (100,000 cells/well, Flexcell Intl. Corp.). After 12 hours of culture, an FX-4000T strain unit was used to subject the cells to 10% equibiaxial strain with a 0.5 Hz sine wave for the designated time period as previously described [57]. Control MDSCs were cultured as described without strain. These mechanical stimulation conditions were chosen based on our previous study which indicated increased vascular endothelial growth factor secretion under these conditions *in vitro* [57, 72, 105].

Cell transplantation to *mdx* mice: *Mdx* mice (C57BL/10ScSn-Dmd^{mdx}) were obtained from Jackson Laboratories (Bar Harbor, ME) and bred at the institution's animal facility (Protocol 33/00). Cell transplantation into *mdx* hearts was performed as previously described [84]. Briefly, each heart was exposed via a left thoracotomy, and $1x10^5$ MDSCs in 10µl phosphate buffered saline (PBS) were injected into the left ventricular free wall of male and female *mdx* mice (12-15 weeks of age). In the same animal $1x10^5$ MDSCs in 10 µl PBS were injected into the left GN muscle. At 2 weeks post-injection the mice were sacrificed, the hearts and GN muscles were harvested and flash-frozen in liquid nitrogen-cooled 2-methylbutane, and then cryosectioned into 10 µm sections.

Engraftment in *mdx* mice: Cryosections were fixed in 10% formalin and then stained to determine donor cell engraftment. Engraftment was determined as described previously [34, 84].

Briefly, sections were stained for dystrophin with a rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 594 donkey anti-rabbit secondary antibody (1:200; Molecular Probes). Nuclei were revealed with 4',6-diamidino-2-phenylindol (DAPI) stain (100 ng/ml; Sigma) and sections were mounted with Gel Mount Aqueous Mounting Medium (Sigma). The regeneration index was determined by counting the number of dystrophin-positive-fibers per $1x10^5$ cells in the section for the largest engraftment in each sample.

Cell transplantation to infarcted myocardium: 50 male immunodeficient C57BL/6J-Prkdc^{scid} mice were bred for the current study. Infarcted mice were randomly allocated between the treatment groups (saline, NS MDSCs, or MS MDSCs), and $3x10^5$ cells total in 30µl saline was injected into the left ventricular free wall of male SCID mice (13-16 weeks of age) five minutes after the permanent ligation of the left coronary artery [34, 57]. Two, six, and twelve weeks after cell injection, echocardiography was performed on the left ventricular short axis view at the mid-papillary muscle level as described previously [34, 57] to assess cardiac function. The mice were then euthanized and the hearts harvested after being flash-frozen in 2-methylbutane, and cryosectioned [34, 57].

Host Regeneration in skeletal muscle: Muscle sections were stained with hematoxylin and eosin. Muscle regeneration was assessed by counting the number of centronucleated myofibers in 3 randomly chosen high-powered fields for each muscle. A blinded observer performed the analyses.

Staining for blood vessels within the engraftment in *mdx* mice: The number of blood vessel in the engraftment area was determined by double-staining with CD31 (an endothelial cell marker) and dystrophin as described previously [57, 84]. Briefly, sections were stained for CD31 with a rat anti-CD31 primary antibody (1:300, BD Pharmingen) and Alexafluor 555 goat

anti-rat secondary antibody (1:300, Molecular Probes). The ratio between CD31 positive structures and dystrophin fibers was used to assess blood vessel formation within the cell injected area.

Cell survival under oxidative stress: Preconditioned cells were plated in PM at 1000 cells/well in a 24-well collagen coated plate then assessed for cell survival as previously described [24]. Briefly, twenty-four hours after plating the media was switched to PM or PM containing 400 μM hydrogen peroxide with propidium iodide (PI, 1 μl/500 μl media, Sigma). The plates were then placed onto a live cell imaging system (Automated Cell, Inc). Fluorescent and brightfield images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageViewer software (ACI). Cell survival was determined by counting the number of PI-positive cells in the fluorescent images and the number of cells present in the brightfield images at 12 hour time points.

***In vitro* VEGF expression:** Cells were mechanically stimulated as described above in DMEM with 1% penicillin/streptomyocin for 24 hours. Conditioned media was collected for each sample and flash frozen. VEGF secretion was quantified by an ELISA for mouse VEGF according to the manufacturer's instructions (R&D Systems) and normalized to cell number.

***In vitro* proliferation:** After exposure to mechanical strain, cells were replated on 24-well collagen coated plates, then placed onto a previously described [24] live cell imaging system (ACI), and fluorescent and brightfield images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageViewer software (ACI) and the number of cells was counted at 12 hour intervals to track proliferation rates.

***In vitro* differentiation toward skeletal muscle**: Differentiation toward skeletal muscle was assessed as previously described [106]. Cells were plated on 24-well plates after mechanical

stimulation, and 24 hours later the media was changed to DMEM + 2% serum. At ten days, the plates were stained with a mouse primary antibody for fast skeletal myosin heavy chain (1:500, Sigma), Alexafluor 488 donkey anti-mouse secondary antibody (1:400; Molecular Probes), and DAPI (for nuclei). The percentage of nuclei in myotubes was quantified for three images per well of three wells per treatment. This indicates the extent of fusion of MDSCs into multinuclear myotubes, an indication of differentiation toward skeletal muscle [32].

Identification and quantification of transplanted MDSCs: Cryosections were fixed in 4% paraformaldehyde and then stained to determine donor cell engraftment. Heart sections were stained with mouse anti-fast skeletal myosin heavy chain (fsMHC) antibody (1:400; Sigma) and Alexafluor 555 donkey anti-mouse secondary antibody (1:300; Molecular Probes) to examine engraftment of the different cell populations. The number of engrafted cells was determined by assessing the area of fsMHC(+) cells normalized to the area of the entire muscle section.

Identification of endothelial cells within the engraftment of transplanted cells: The number of CD31+ cells in the engraftment area was determined by double-staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma) and Alexafluor 555 donkey anti-rat secondary antibody (1:300; Molecular Probes) and mouse anti-fsMHC antibody (1:400; Sigma) and Alexafluor 488 goat anti-mouse (1:200; Sigma). Blood vessel formation within the cell-injected areas was determined by the number of CD31(+) cells associated with fsMHC(+) cells for the infarction model.

Scar area fraction: Infarcted heart sections were fixed with 1% gluteraldehyde for 2 minutes and stained with the Masson Modified IMEB Trichrome Stain Kit (IMEB). Trichrome staining was performed according to the manufacturer's guidelines and as previously described

[34, 57]. The sections were then assessed for the percentage area of collagen in 5 sections per heart normalized to total muscle area within the section using ImageJ software (NIH).

Staining for cardiac-specific markers *in vivo*: Sections from the infarcted samples were stained with mouse anti-fsMHC (1:400; Sigma) and Alexafluor 488 donkey anti-mouse secondary antibody (1:300; Molecular Probes), and goat anti-cardiac troponin I (1:25,000; Scripps) with rabbit anti-goat Alexafluor 555 (1:200; Molecular Probes). The numbers of double-positive cells were counted in 3 high powered fields per heart.

Microscopy: Fluorescence and brightfield microscopy were performed using either a Nikon Eclipse E800 microscope (Nikon Corp.) or a Leica DMIRB inverted microscope (Leica Microsystems) with a Retiga digital camera (Q Imaging) and Northern Eclipse software (version 6.0, Empix Imaging, Inc.). Image analysis was performed using Northern Eclipse software.

Statistical analysis: The mean and standard deviation were obtained for all measured values, and statistical significance between the groups was determined by a t-test in the case of comparison between 2 groups or 1-way ANOVA when comparisons were done between 3 groups. In all cases, $\alpha=0.05$ and data was normally distributed. In the event of a significant ANOVA, the Tukey test was used for post-hoc analysis. Data shown is mean ± standard error of the mean. (SPSS v. 16.0, SPSS Inc.)

4.3 RESULTS

***In vitro* proliferation and resistance to oxidative stress is increased but differentiation of MDSCs is not affected by mechanical stretch:** The proliferation rate of MS MDSCs after

mechanical stimulation (white squares) was similar to the proliferation rate of NS cells (black diamonds) at early time points, but at 48 hours MS cells proliferated at a significantly higher level (* is $p<0.05$, Figure 4.1A). The *in vivo* milieu after transplantation is harsh, with oxidative stress playing a significant role, and cells that are better able to survive this stress could have a preferential and beneficial effect on repair. MS cells (black diamonds) showed similar levels of survival under oxidative stress to NS controls (white squares, Figure 4.1B). In addition, we explored differentiation toward skeletal muscle *in vitro*. Differentiation toward skeletal muscle was unaffected by mechanical stimulation (Figure 4.1C).

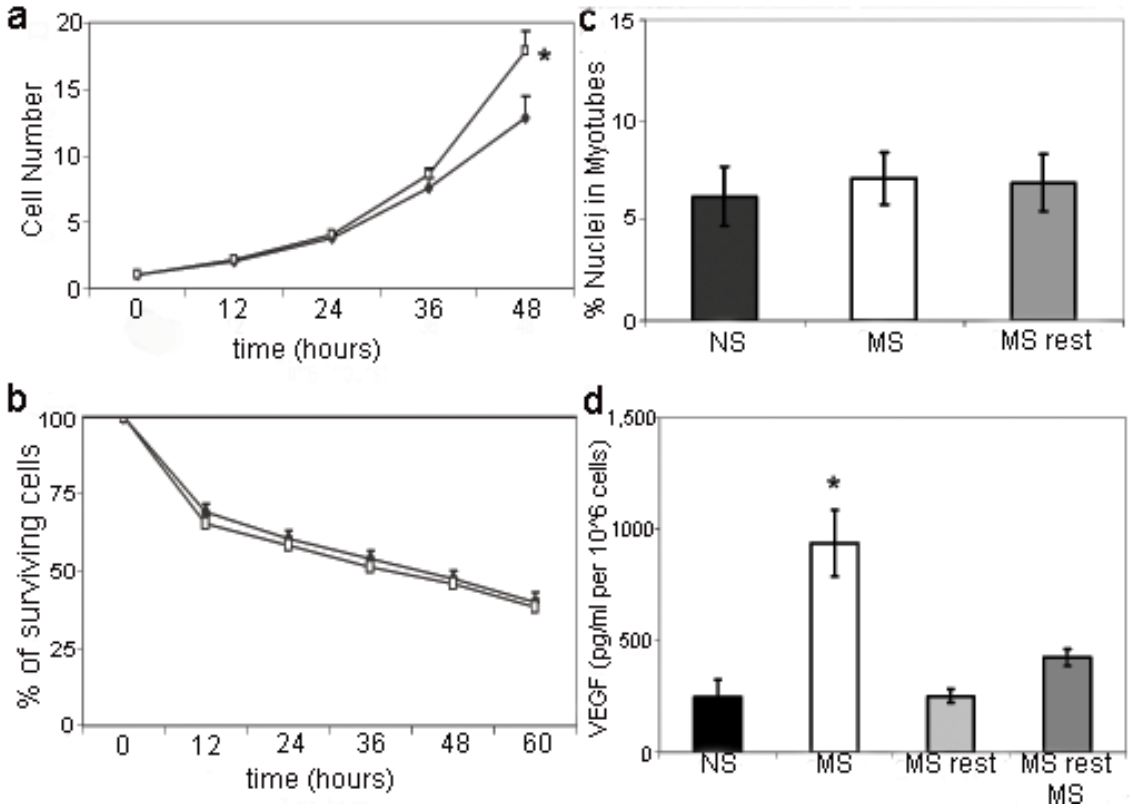

Figure 4.1 *In vitro* MDSC characteristics with mechanical stimulation. (A) Proliferation (B) Cell survival (C) Differentiation (D) VEGF secretion

Increased expression of vascular endothelial growth factor (VEGF) after mechanical stretch: Previous results have shown that mechanically stimulating MDSCs increases VEGF secretion [57]. We extended these experiments to examine the temporal effect of mechanical stimulation on VEGF secretion. Our results confirm that *in vitro* mechanical stimulation of MDSCs increases VEGF expression (937 pg/ml ± 148, Figure 4.1D) but demonstrates that this increase is transient, as VEGF levels return to the level of NS cells (248 ± 80 pg/ml) after 24 hours of static culture following mechanical stimulation (254 ± 30, MS rest). We then restimulated the cells for 24 hrs with the same mode of stimulation, and cells re-exposed to mechanical stimulation (MS rest MS) show a slight increase in VEGF (426 ± 36), but this increase was much lower than the initial activation levels. These results provide insight into the time-dependent effects of mechanical stimulation as VEGF secretion is related to mechanical loading history.

Mechanical stimulation significantly improves MDSC engraftment in the *mdx* heart: We explored the effects of the loading history through mechanical preconditioning on stem cell response. At two weeks post cell implantation in the heart, dystrophin positive cells were counted for the largest engraftment area. Compared to non-stimulated cells (0hr, Fig 4.2A), increased numbers of dystrophin positive cells were observed after mechanical stimulation for 24 hours (Fig 4.2B). The number of dystrophin positive cells, indicating the regeneration index, was significantly higher in the hearts injected with cells stretched for 24 hours (1120 ± 230 cells) when compared to 0hr controls (380 ± 150 cells, Fig 2C, n=9 per treatment, $p<0.05$). Cells mechanically preconditioned for only 4 hours also showed increased dystrophin expression upon transplantation into the heart (620 ± 200 cells) but this was not statistically significant (Fig 4.2C). To observe the role angiogenesis plays in this response, the number of CD31 positive

microvessels within the engraftment areas was quantified. No difference in the number of microvessels was observed for cells stimulated for 24 hours(0.30 ± 0.02 microvessels/dystrophin + cell, Fig 4.2D) when compared to 0hr cells (0.45 ± 0.13 microvessels/dystrophin + cell). Though angiogenesis alone does not explain this differential engraftment, other factors such as increased proliferation or paracrine factor secretion may explain the increased regeneration capacity of preconditioned MDSCs.

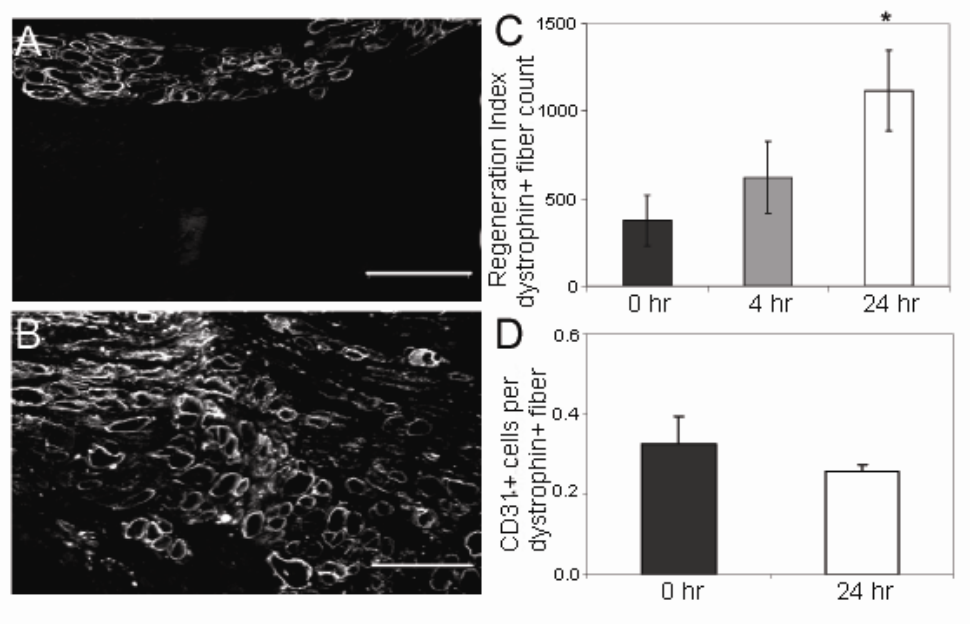

Figure 4.2 Mechanical preconditioning increases engraftment but does not affect angiogenesis. (A) Unstimulated MDSC engraftment (B) 24hr mechanically stimulated MDSC engraftment (C) Regeneration Index (D) Angiogenesis

Mechanical stimulation significantly improves MDSC engraftment in *mdx* skeletal muscle: At two weeks post cell implantation, dystrophin positive cells were counted for the largest engraftment area in skeletal muscle. An increase in the regeneration index was observed in the skeletal muscles transplanted with MS cells (30 ± 20 dystrophin + cells) compared to NS cells (14 ± 7 dystrophin + cells) but this was not statistically significant (Fig 4.3A, n=9 per

treatment). When regeneration of host cells was examined in skeletal muscle using centrally-located nuclei in myofibers, muscles that had been implanted with MS cells had significantly higher levels of regenerating host myofibers (76 ± 1%) than NS controls (68 ± 1%, Figure 4.3B, n=6 per treatment, $p<0.05$). To observe the role angiogenesis plays in this response, the number of CD31 positive microvessels within the engraftment areas of the skeletal muscles of animals injected with MS MDSCs was quantified. Angiogenesis was significantly increased with mechanical stimulation of cells prior to injection (1.8 ± 0.1 CD31+ microvessels/dystrophin + cell, Fig. 4.3C) when compared to NS cells (1.3 ± 0.1 microvessels/dystrophin + cell).

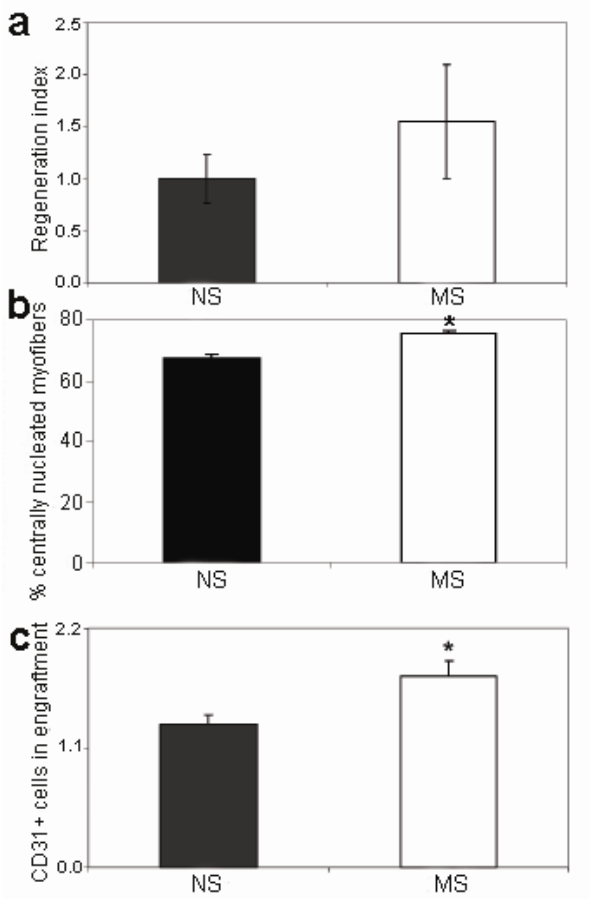

Figure 4.3 Mechanical preconditioning of MDSCs improves cell transplantation to skeletal muscle. (A) Regeneration Index (D) Host regeneration (C) Angiogenesis

Mechanical preconditioning maintains cardiac function after myocardial infarction: The most critical endpoint in cell transplantation for cardiac repair is heart function. Fractional shortening is a measure of heart contractility and hearts treated with MS cells had significantly higher levels of fractional shortening (2wk: 28 ± 1%, 6wk: 24 ± 1%, 12wk: 24 ± 1%) than NS cells (2wk: 19 ± 1%, 6wk: 19 ± 1%, 12wk: 18 ± 1%) or saline alone (2 wk: 16 ± 2%, 6wk: 15 ± 1%, 12wk: 15 ± 1%, Figure 4.4A). Fractional area change is another measure of systolic cardiac function and cells that had been preconditioned with mechanical stimulation (2wk: 27 ± 1%, 6wk: 28 ± 1%, 12wk: 27 ± 1%) had higher fractional area change than NS cells (2wk: 21 ± 1%, 6wk: 24 ± 1%, 12wk: 24 ± 1%), which were in turn higher than saline (2wk: 15 ± 1%, 6wk: 15 ± 2%, 12wk: 18 ± 1%, Figure 4.4B).

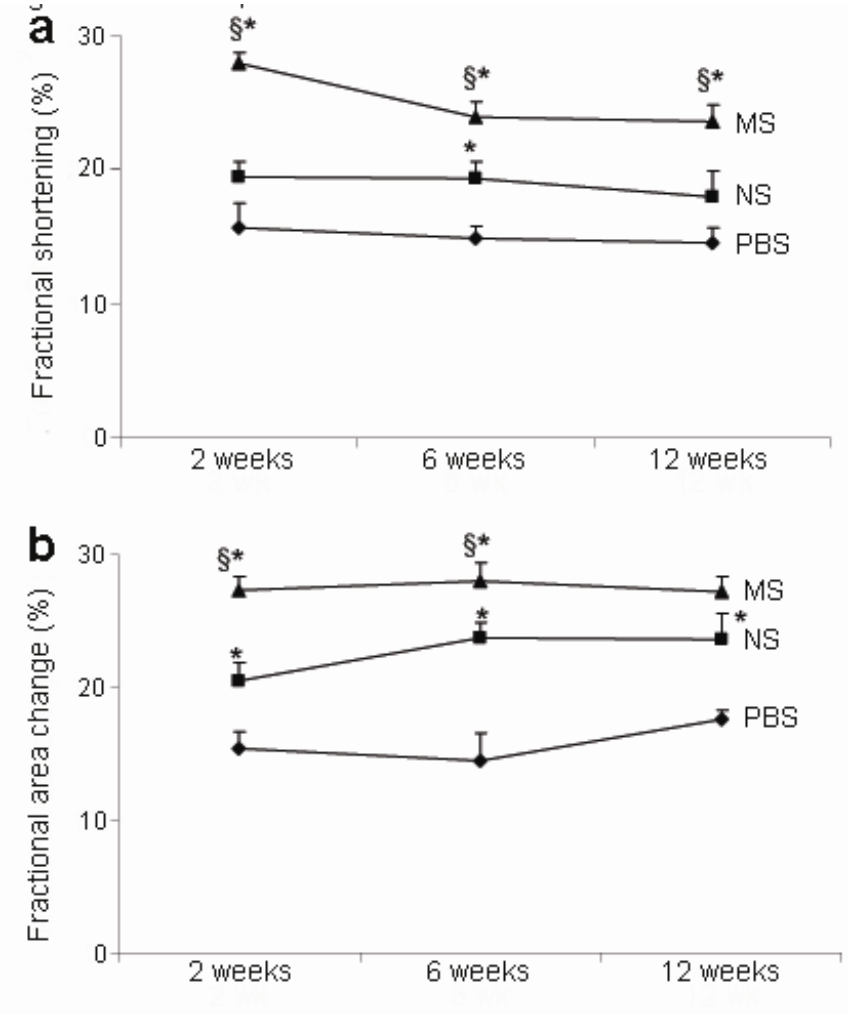

Figure 4.4 Cardiac function is improved after mechanical preconditioning of MDSCs. (A) Fractional shortening (B) Fractional area change

		2 weeks	6 weeks	12 weeks
MS	FS	28 ± 1%, n=13, P=0.01 to NS, P=0.01 to saline	24 ± 1%, n=13, P=0.01 to NS, P=0.01 to saline	24 ± 1%, n=8, P=0.049 to NS, P=0.01 to saline
	FAC	27 ± 1%, n=13, P=0.01 to NS, P=0.00 to saline	28 ± 1%, n=13, P=0.049 to NS, P=0.01 to saline	27 ± 1%, n=13, P=0.16 to NS, P=0.01 to saline
NS	FS	19 ± 1%, n=13, P=0.16 to saline	19 ± 1%, n=13, P=0.02 to saline	18 ± 1%, n=11, P=0.38 to saline
	FAC	21 ± 1%, n=13, P=0.03 to saline	24 ± 1%, n=13, P=0.01 to saline	24 ± 1%, n=11, P=0.11 to saline
Saline	FS	16 ± 2%, n=8	15 ± 1%, n=14	15 ± 1%, n=5
	FAC	15 ± 1% n=8	15 ± 2%, n=14	18 ± 1%, n=5

Table 4.1: Fractional shortening and fractional area change data for the three treatment groups

Mechanical stimulation significantly increases neoangiogenesis after myocardial infarction: Similar to the *in vitro* results showing increased VEGF secretion after mechanical stretch, a significant difference was observed between NS MDSCs (14 ± 1 blood vessels/mm^2), MS MDSCs (21 ± 1 blood vessels/mm^2), and saline (9 ± 1 blood vessels/mm^2) neoangiogenesis in the peri-infarct region at 12 weeks (Figure 4.5A). A similar trend was found when examining angiogenesis in the engraftment region, however this difference was not statistically significant (P=0.11, Figure 4.5B).

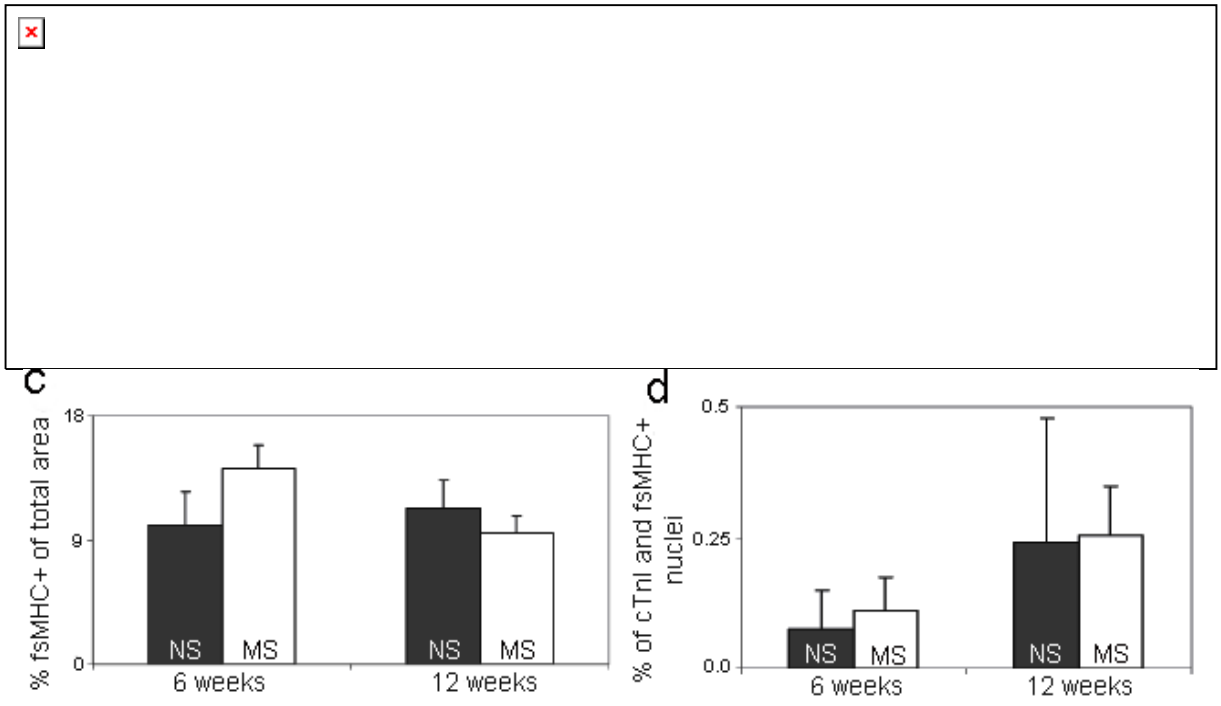

Figure 4.5 *In vivo* effects of mechanically preconditioning MDSCs prior to transplantation. (A) Angiogenesis in peri-infarct region (B) Angiogenesis in engraftment (C) Engraftment area (D) Cardiac differentiation

Mechanical stimulation does not affect cell survival and differentiation *in vivo*: When examining cell survival *in vitro* under oxidative stress, no difference was found between MS and NS MDSCs. Similar results were observed with cell survival and engraftment after myocardial infarction, with no difference between the two cell treatments (Figure 4.5C).

Previously we have found only low levels of differentiation toward a cardiac phenotype *in vivo*, and that was confirmed here, with less than 1 out of 100 injected cells co-expressing cTnI and fsMHC at both 6 and 12 weeks after injection, with no significant difference between MS and NS cells (Figure 4.5D). These results imply that the increase in cardiac function seen with mechanically stimulated cells is not related to a direct contribution via differentiation toward a cardiac phenotype.

Mechanical stimulation has effect on scar area: To detect the outcome of transplantation for mechanical stretched cells, scar area fraction was analyzed by looking at the amount of collagen compared to cardiac muscle, which affects contractility. Compared to NS cells (6 week: 66 ± 2%, 12 weeks: 57 ± 7%), a decrease in scar tissue formation was observed in the MS MDSC group at both 6 and 12 weeks (6 weeks: 58 ± 6%, 12 weeks: 45 ± 5%), though the difference was not statistically significant (P=0.28 at 12 weeks, Figure 4.6A). Both MDSC-injected groups had significantly lower levels of scar tissue than saline controls (6 weeks: 80 ± 5%, 12 weeks: 76 ± 2%).

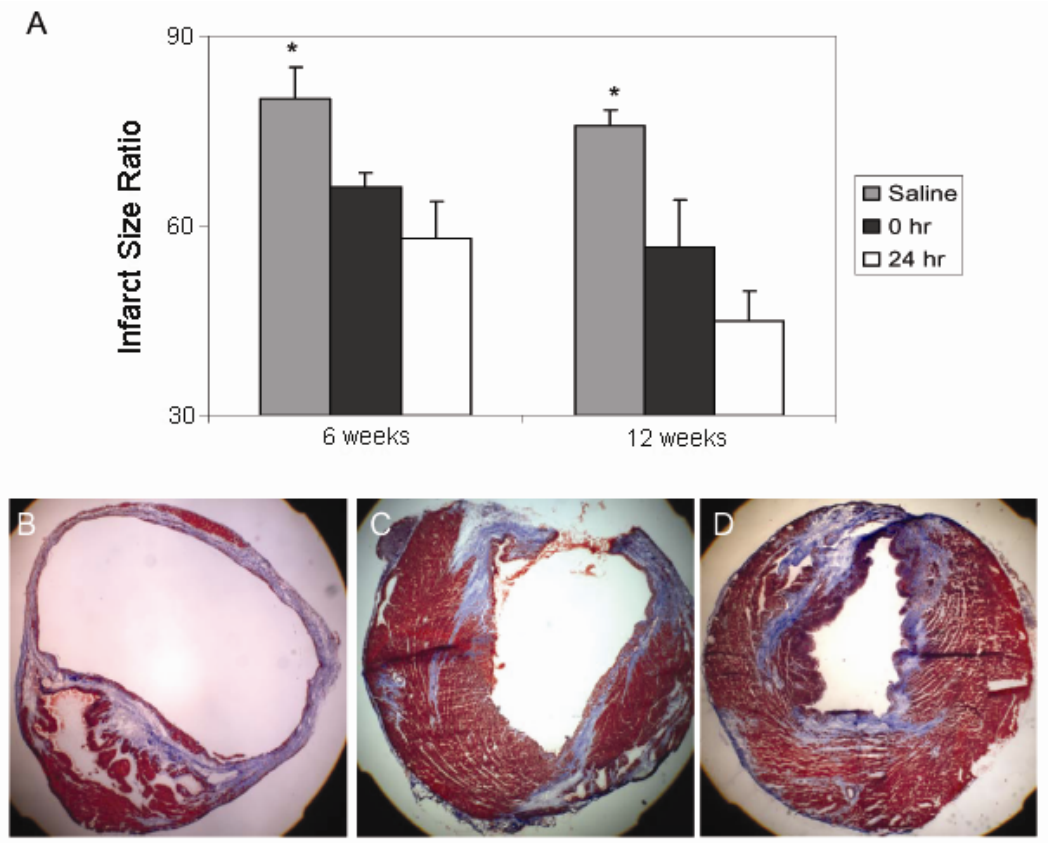

Figure 4.6 Scar tissue formation is decreased with mechanical preconditioning of MDSCs. (A) Scar tissue fraction (B) PBS (C) Non-stimulated MDSCs (D) Mechanically stimulated MDSCs

4.4 DISCUSSION

The overall goal of this study was to investigate the effects of preconditioning MDSCs with mechanical stimulation prior to stem cell transplantation. We demonstrate that mechanical preconditioning causes behavioral changes in MDSCs both *in vivo* and *in vitro*, and can result in significantly improved cardiac function after myocardial infarction. We hypothesized that

transplanting MDSCs that were exposed to mechanical loading prior to injection would result in higher numbers of dystrophin positive cells in *mdx* skeletal and cardiac muscle as well as increased repair of a myocardial infarction. Studying the effects of mechanical stimulation on skeletal and heart muscle was based on a number of previous studies showing mechanically governed responses. Application of mechanical stretch to cultured adult rat muscle satellite cells resulted in release of hepatocyte growth factor (HGF) and accelerated entry into the cell cycle [71]. In addition, in a co-culture system with rat neonatal cardiomyocytes, skeletal muscle stem cells can transdifferentiate into cardiomyocytes where mechanical loading was necessary for this differentiation [107].

We explored cell behavior *in vitro*, and we observed an increased secretion of VEGF after mechanical stimulation, which may partially explain the improvement in MS MDSC engraftment and repair and fits with previous results [57]. Kasper et al [73] showed that conditioned medium from mesenchymal stem cells subjected to mechanical loading increased sprouting of endothelial cells by stimulating an angiogenic promoting environment. Results from our VEGF secretion study also demonstrate that the effect of mechanical stimulation is time-dependent, indicating that loading history control may be important to achieve ideal regeneration. Interestingly, the increased secretion of VEGF, while being significant at 24 hours, was reversible as the growth factor levels returned to close to baseline levels within 24 hours after cessation of mechanical stimulation. Just as with spatiotemporal control of chemical factors being critical in regeneration, this indicates that the timing of the mechanical stimulation could be just as important. As cells and the body are controlled by tightly regulated spatiotemporal processes, it has been shown by numerous groups that it is not just the addition or removal of stimulation [108, 109], but rather the controlled timing of the stimuli which produces distinct

results [110]. The tight regulation and timing demonstrated by these results suggest that the mechanical loading history can predispose the MSDCs toward specific responses that are advantageous for cell transplantation. This is an essential point in this study since it shows that adding mechanical stimulation prior to injection into a mechanical environment is both a valid means of increasing VEGF secretion and improves transplantation results. The last part of the study examined implanting MS cells into skeletal muscle. An increase in regeneration and angiogenesis was seen, similar to the results in the infarcted heart, showing that mechanical stimulation prior to implantation is useful for cellular therapy for more than one organ system.

We examined the biologic effect of MS MDSCs on improving regeneration *in vivo*, and found that compared to NS MDSCs, mechanical loading increases angiogenesis, donor cell engraftment (as assessed by the number of dystrophin positive cells), and host regeneration in uninjured *mdx* mice. We then explored the effect of MS cells after transplantation into a model of myocardial infarction and noted that the benefits of mechanical preconditioning became more pronounced. After injection with MS MDSCs, cardiac function was improved above NS cells, and significantly above saline alone. The improvement above NS cells is likely due to the significant increase in angiogenesis, previously shown to be critical in cardiac repair after injury and disease [57, 111]. Previous reports suggest that VEGF influences myoblast migration and survival and increases skeletal muscle repair during ischemia [112, 113]. These mechanically governing loading history results are critical as it is not just the addition of mechanical stimulation, but fine tuning the spatiotemporal stimulation that results in an advantageous response. This control over the mechanical environment can lead to radically different responses in a diversity of areas, including growing functional arteries [110].

The most noteworthy finding of this study is that controlling the loading history through prestimulation with mechanical stretch ameliorates the negative remodeling and subsequent decrease in cardiac function normally seen after myocardial infarction to a greater degree than NS MDSC treatment. In summary, examining the loading history through mechanical preconditioning of muscle derived stem cells increases the understanding of stem cell biology along with providing a potential method to improve cell transplantation for treatment of disease and injury. The experiments reported here show that the release of VEGF is the critical factor in mechanical activation and that MS cell-induced angiogenesis plays a role in improved maintenance of cardiac function after injury. Further study is necessary to fully optimize the mechanical preconditioning regimen.

4.5 ACKNOWLEDGEMENTS

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5.0 CELL PRETREATMENT WITH N-ACETYLCYSTEINE: IMPLICATIONS FOR CELL SURVIVAL AND THERAPY

5.1 INTRODUCTION

Stem cells are a promising cell type for tissue repair after injury because they can differentiate into multiple lineages, proliferate long-term, and allow for the potential of autologous transplantation. Another emerging stem cell characteristic is ability to survive stress, which is of critical importance for cell transplantation because the natural *in vivo* environment is imbalanced due to injury, and both inflammatory and oxidative stresses are present [15, 95]. Current research has shown that differentiated cells are susceptible to damage from environmental stresses that stem cells can avoid [114]. This increased resistance to stress seen in stem cells is likely involved with the levels of antioxidants found in the cells [16].

Increased resistance to stress has been seen with a variety of stem cell types, including endothelial progenitor cells (EPCs) and cardiac side population cells [22], and this has been linked directly to antioxidants levels [114, 115]. EPCs were shown to be less sensitive to oxidative-stress induced apoptosis than more differentiated endothelial cells, express significantly higher levels of antioxidant enzymes, and have upregulated levels of DNA repair [114, 116]. Muscle derived stem cells (MDSCs) have lower levels of stress-induced death when compared to myoblasts [16, 34]. Oxidative stress is also involved in the regulation of telomere-

shortening, and so it is possible that additional protection against this type of stress could prevent dysfunction and senescence [115]. Other research has shown that antioxidant treatment can reduce apoptosis in a variety of cell types, including neural and cardiac cells [117-119]. Interventions that increase longevity are often associated with increased antioxidant activity and improved resistance to oxidative stress, which provides further evidence that stress resistance is a characteristic that is inherent to stem cells [120].

The level of antioxidants becomes critical only under stress or injury conditions. The levels of antioxidants are tightly regulated within the cell, and a small change in redox state can have a significant effect on cell behavior, stem cell characteristics, and survival [117]. Oxidative stress is common in the local tissue area after injury, and can cause apoptosis and senescence of cells, and tissue ischemia causes a depletion of antioxidants. Cells with higher antioxidant levels can survive stress to a greater extent [16], and treatment with antioxidants could protect against these deleterious effects of stress and ischemia. An inverse relationship has been found in the heart between glutathione content, the predominant cellular antioxidant, and the extent of injury after ischemia/reperfusion [15]. N-acetylcysteine (NAC) is a direct antioxidant as well as a cysteine precursor for glutathione synthesis [121]. Previous research has shown that NAC increases proliferation [122], decreases cell death from extrinsic stresses as well as a loss of trophic factors [118], protects against oxidative stress both *in vitro* and *in vivo* [123, 124], has antifibrotic effects [125], and can increase heart function in a mouse model of Duchenne muscular dystrophy [126]. NAC is currently used clinically to treat hepatic acetaminophen toxicity and as a treatment for diseases characterized by low glutathione levels, including COPD [123, 127-130].

Results of cell transplantation with muscle-derived cells, as with all other cell types, has been plagued with low survival efficiencies and limited functional improvement [6]. We have previously identified a population of muscle cells, termed muscle-derived stem cells (MDSCs), which are easily accessible, multipotent, and can proliferate long-term and survive stress at significantly higher rates than myoblasts [26, 33, 92]. We have previously shown that muscle-derived stem cells (MDSCs) significantly reduce post-infarction ventricular remodeling compared to myoblasts after myocardial infarction [34]. Since the environment following transplantation is less than optimal with oxidative and inflammatory stresses, increasing antioxidant levels in the cells prior to implantation could increase survival. Increased cell survival could increase functional repair, which would make cell transplantation an increasingly viable alternative to current therapies. In order to confirm that antioxidants were the deciding factor in the repair process, we used diethyl maleate (DEM) to reduce levels of intracellular glutathione, thereby decreasing antioxidant levels [16, 34], and NAC treatment to increase antioxidant levels. Here we investigated a potential combination therapy using both stem cells and NAC in order to have a synergistic effect on cardiac repair. We demonstrate that cells treated with antioxidants such as NAC have a therapeutic advantage over untreated cells. We have also shown that the levels of antioxidants in the cells impacts cell behavior and regeneration after transplantation.

5.2 MATERIALS AND 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 14/06).

MDSC isolation: Muscle-derived stem cells were isolated from the skeletal muscle of three week old normal C57BL mice (Jackson) using the modified preplate technique as previously described [26, 31, 32, 42]. Mice were anatomically sexed prior to isolation. MDSCs were cultured in proliferation media (PM) containing DMEM (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 10% HS, 1% penicillin/streptomycin, and 0.5% chick embryo extract (Accurate Chemical).

Cell treatments: Diethyl maleate (DEM, Sigma) was used to deplete intracellular levels of reduced glutathione (GSH), and NAC (Sigma) was used to increase GSH levels. Cells were treated with 50μM DEM or 10mM NAC in PM for 24 hours, and then the cells were used for VEGF ELISA, live cell imaging, or cell injection.

Differentiation: MDSCs were plated at 5000 cells per well on a 24-well collagen coated plate. 24 hours later, the media was changed to DMEM + 2% serum + 100μM hydrogen peroxide + 50μM DEM or 10mM NAC. At five days, the plates were stained with mouse anti-fast skeletal myosin heavy chain antibody (fsMHC, 1:400; Sigma) and DAPI (for nuclei), and the percentage of nuclei in myotubes was quantified.

Analysis of Angiogenic Factor Secretion: MDSCs were plated in PM at 50,000 cells/well in 6-well collagen type 1 coated plates and treated with PM, 50μM DEM, or 10mM NAC. Twenty-four hours later, the media was switched to DMEM with 1% penicillin/streptomyocin. Cells were then cultured for an additional 0, 48, 96, or 144 hours, at

which time the media was collected and flash-frozen until analysis. ELISA for mouse VEGF (R&D Systems) was performed according to manufacturer's instructions and as previously described [57]. The VEGF levels were normalized to cell number as assessed by hemocytometer.

Cell Survival under oxidative/inflammatory stress: Cells were plated in PM at 1000 cells/well in a 24-well collagen coated plate. Twenty-four hours later, the media was switched to PM, PM containing 500 μM hydrogen peroxide with propidium iodide (PI, 1:500, Sigma), or PM containing 10ng/ml TNF-α. Each well was left untreated as a control or treated with 50μM DEM or 10mM NAC. The plates were then placed onto a previously described live cell imaging system (Automated Cell, Inc), and fluorescent and brightfield images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageViewer software (ACI). Cell proliferation was determined by counting the number of cells present in the brightfield images at 12 hour time points. Cell survival was determined by counting the number of PI-positive cells in the fluorescent images at each time point.

Cell transplantation to infarcted myocardium: 40 male immunodeficient C57BL/6J-Prkdc^{scid} mice were used in the current study (Jackson). Infarcted mice were randomly allocated between the treatment groups (saline, untreated MDSCs, NAC-treated MDSCs, or DEM-treated MDSCs), and $3x10^5$ cells total in 30μl saline was injected into the left ventricular free wall of male SCID mice (14-18 weeks of age) five minutes after the permanent ligation of the left coronary artery. Prior to injection and two and six weeks after cell implantation, echocardiography was performed on the left ventricular short axis view at the mid-papillary muscle level as described previously [34, 57] to assess cardiac function, and the mice were euthanized and the hearts harvested, flash-frozen in 2-methylbutane, and cryosectioned.

Identification of Endothelial Cells within the Infarct Area: The number of CD31+ cells in the infarct area was determined by staining tissue sections with rat anti-CD31 primary antibody (1:300; Sigma) and donkey anti-rat Alexafluor 594 secondary antibody (1:300, Sigma). Blood vessel formation within the cell-injected areas was determined by counting the number of CD31(+) cells per high powered field in 3 fields per heart.

Collagen Staining: Infarcted heart sections were fixed with 1% gluteraldehyde for 2 minutes and stained with the Masson Modified IMEB Trichrome Stain Kit (IMEB, CA, USA), which stains both collagen (blue) and muscle (red). Trichrome staining was performed according to the manufacturer's guidelines. The sections were assessed for the percentage area of collagen in 5 sections per heart normalized to total muscle area within the section using ImageJ (NIH).

Staining for Cardiac-Specific Markers: Sections were stained with mouse anti-fsMHC (1:400; Sigma) and Alexafluor 488 donkey anti-mouse secondary antibody (1:300; Molecular Probes) and goat anti-cardiac troponin I (1:25,000; Scripps) with rabbit anti-goat Alexafluor 555 (1:200; Molecular Probes) and the number of double-positive cells were counted in 3 high powered fields per heart.

Staining for Proliferation: Sections were stained with mouse anti-fsMHC (1:400; Sigma) and Alexafluor 488 donkey anti-mouse secondary antibody (1:300; Molecular Probes) and rabbit anti-Ki-67 (1:250; Abcam) and Alexafluor 555 goat anti-rabbit (1:200; Molecular Probes). The number of ki-67 positive nuclei was quantified in 3 high powered fields per heart.

Cell transplantation to injured skeletal muscle: 20 male *mdx* mice were used in the current study. All mice were injected with cardiotoxin (1μg in 20μl PBS) in both left and right gastrocnemius muscles. Two weeks after injury, mice were randomly allocated between the

treatment groups (saline, untreated MDSCs, NAC-treated MDSCs, or DEM-treated MDSCs), and $1x10^5$ cells total in 20µl saline was injected into the left and right gastrocnemius muscle. Two weeks after cell injection, the mice were euthanized and the muscles harvested, flash-frozen in 2-methylbutane, and cryosectioned.

Engraftment and host regeneration in skeletal muscle: Cryosections were fixed in 10% formalin and then stained to determine donor cell engraftment. Engraftment was determined as described previously[34, 84]. Briefly, sections were stained for dystrophin with a rabbit anti-dystrophin primary antibody (1:400; Abcam) and Alexafluor 594 donkey anti-rabbit secondary antibody (1:200; Molecular Probes). Nuclei were revealed with 4',6-diamidino-2-phenylindol (DAPI) stain (100 ng/ml; Sigma) and sections were mounted with Gel Mount Aqueous Mounting Medium (Sigma). The regeneration index was determined by counting the number of dystrophin-positive-fibers per $1x10^5$ cells in the section for the largest engraftment in each sample. For muscle regeneration, muscle sections were stained with hematoxylin and eosin. Muscle regeneration was assessed by counting the number of centronucleated myofibers in 3 randomly chosen high-powered fields for each muscle. A blinded observer performed the analyses.

Staining for Blood Vessels within the Engraftment in skeletal muscle: The number of blood vessel in the engraftment area was determined by double-staining with CD31 (an endothelial cell marker) and dystrophin as described previously [57, 84]. Briefly, sections were stained for CD31 with a rat anti-CD31 primary antibody (1:300, BD Pharmingen) and Alexafluor 555 goat anti-rat secondary antibody (1:300, Molecular Probes). The ratio between CD31 positive structures and dystrophin fibers was used to assess blood vessel formation within the cell injected area.

Inhibiting MEK pathway: Cells were plated in PM at 1000 cells/well in a 24-well collagen coated plate. Twenty-four hours later, the media was switched to PM containing 500 μM hydrogen peroxide with propidium iodide (PI, 1:500, Sigma) and PD98059 (MEK inhibitor, Cayman, 25 μM). Each well was left untreated or treated with 50μM DEM or 10mM NAC. The plates were then placed onto a previously described live cell imaging system (Automated Cell, Inc), and fluorescent and brightfield images were taken every 10 minutes in 3 locations per well. These images were analyzed using ImageViewer software (ACI). Cell survival was determined by counting the number of PI-positive cells in the fluorescent images at 12 hour time points.

Western blots: Cells were treated for 15 minutes with 25 μM PD, 10 mM NAC, 10 mM NAC + μM PD, or left untreated. Nuclear and cytoplasmic extracts according to kit instructions (Pierce) and protein content was quantitated using a spectrophotometer. 20 μg of protein was added to each lane. Blots were blocked with 5% non-fat milk. Antibodies to phospho p44/42 and p44/42 (1:500, Cell Signaling) were incubated for two hours at room temperature. Chemiluminescence was used for exposure. Quantification was done using ImageJ (NIH).

Microscopy: Florescence and brightfield microscopy were performed using either a Nikon Eclipse E800 microscope equipped with a Retiga digital camera and Northern Eclipse software (version 6.0, Empix) or a Leica DMIRB inverted microscope with a Retiga digital camera and Northern Eclipse software. Image analysis was performed using Northern Eclipse software or Image J (available from NIH).

Statistical analysis: The mean and standard deviation will be obtained for all measured values, and statistical significance between the groups will be determined by a t-test (Microsoft Excel) or 1-way or 2-way ANOVA (SigmaStat) as appropriate. In the event of a significant ANOVA, the appropriate multiple comparisons test was used for post-hoc analysis (Tukey test).

5.3 RESULTS

Differentiation/Proliferation: NAC-treated cells differentiate toward skeletal muscle *in vitro* to a substantially greater extent than both control and DEM-treated cells (* is $p<0.05$ to DEM, # is $p<0.05$ to untreated cells), Figure 5.1A, B, C, D). NAC treatment did not affect proliferation of MDSCs, with the rate remaining the same as non-treated MDSCs, whereas DEM treatment decreased proliferation (Fig 5.1E, * is $p<0.05$ to untreated and NAC groups).

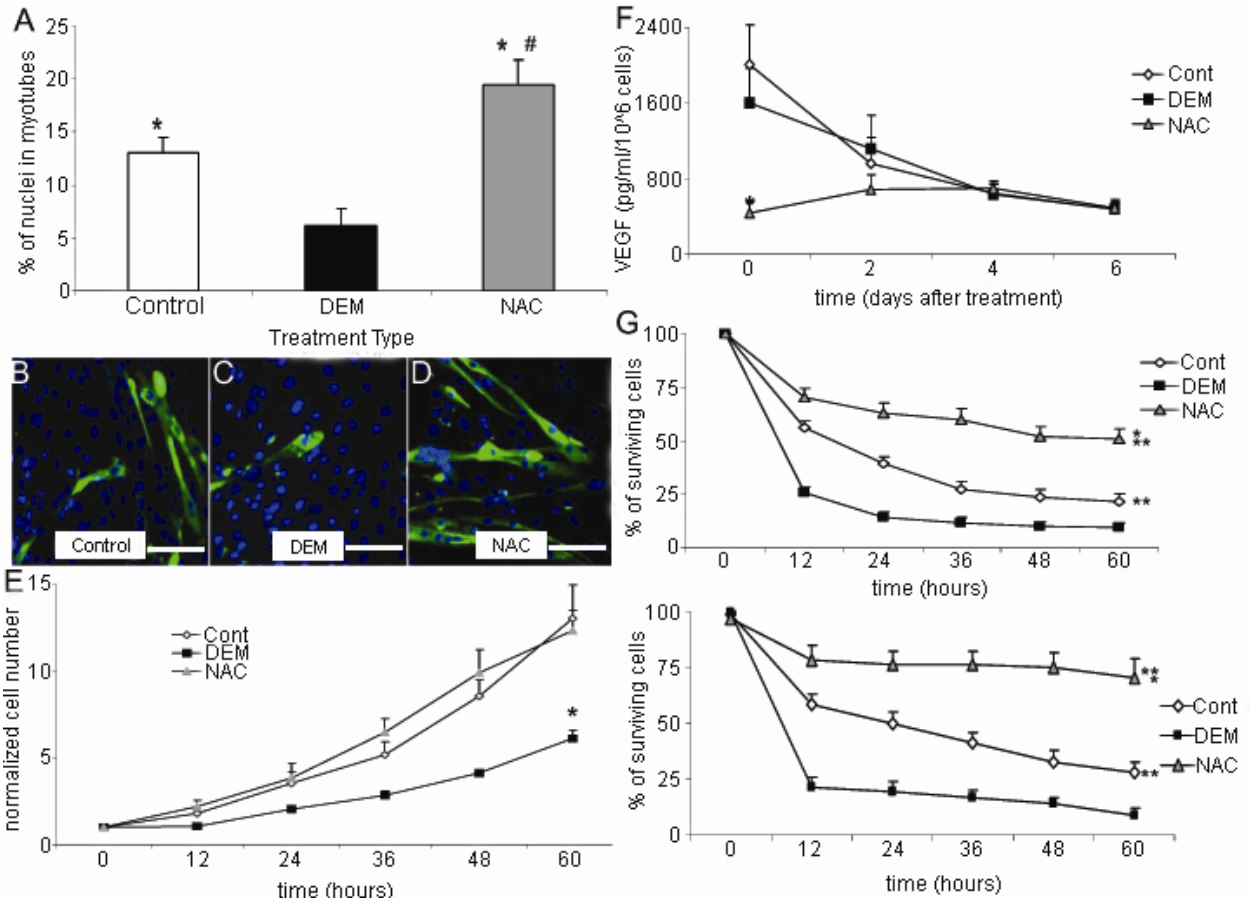

Figure 5.1 ***In vitro*** **effects of antioxidant levels in MDSCs. (A) Differentiation (B) Control (C) DEM (D) NAC (E) Proliferation (F) VEGF secretion (G) Cell survival under oxidative stress (H) Cell survival under inflammatory stress**

VEGF ELISA: Angiogenesis is a key aspect of the repair process after ischemic cardiac injury. To determine if levels of VEGF secretion differed based on antioxidant levels within MDSCs and how long this effect would last, VEGF secretion into cell culture supernatant was measured at 0, 48, 96, and 144 hours after treatment with control PM, DEM, or NAC for 24 hours. Directly after treatment (0 hour), NAC treated MDSCs have significantly lower levels of VEGF secretion than both control and DEM-treated cells (Figure 5.1F, $p<0.05$). However, at 48 hours and all later time points, there is no significant difference between any of the treatment groups (Figure 5.1F).

Cell Survival after Oxidative/Inflammatory Stress: MDSCs have a superior ability to survive oxidative stress compared to myoblasts, and this seems to be related to the higher regeneration capacity in skeletal and cardiac muscle. When the level of antioxidants is decreased with DEM, survival under both oxidative (Figure 1G) and inflammatory stress (Figure 5.1H) was significantly decreased compared to control and NAC-treated MDSCs (** is $p<0.05$ to DEM). In contrast, when the level of antioxidants is increased with NAC treatment, survival under both stress conditions is significantly higher than control and DEM (Figure 5.1G, H, * $p<0.05$ to control).

Assessment of Cardiac Function: NAC cell injected hearts better maintained fractional shortening at both 2 and 6 weeks after implantation, with significant differences (Figure 5.2A, * is $p<0.05$ to PBS, # is $p<0.05$ to DEM, § is $p<0.05$ to untreated cells) compared to DEM and PBS at 6 weeks (NAC: 19.0 ± 1.1%, 6 wk: 19.8 ± 0.7%, untreated cells: 2wk: 18.2 ± 1.5%, 6wk: 16.4 ± 1.0%, DEM: 2wk: 13.8 ± 1.2 %, 6wk: 12.4 ± 1.1%, PBS: 2wk: 14.9 ± 2.1%, 6wk: 13.0 ± 0.7%). As assessed by fractional area change (FAC) using B-mode echocardiography prior to transplantation (pretreatment: 41.4 ± 1.1% FAC, Figure 5.2B) as well as 2 and 6 weeks post-

transplantation, PBS-injected hearts had the most severe reduction in function (2 wk: 15.58 ± 1.3%, 6 wk: 14.69 ± 0.74). DEM-treated hearts (Figure 2B; 2 wk: 19.90 ± 1.7%, 6 wk: 17.7 ± 0.85%) were reduced compared to untreated cells (2 wk: 23.48 ± 1.9%, 6 wk: 22.84 ± 2.18%), and NAC-treated cells (2 wk: 24.80 ± 1.1%, 6 wk: 25.93 ± 1.7%) were improved compared to untreated cells (Figure 2B, * is $p<0.05$ to PBS, # is $p<0.05$ to DEM). End diastolic area also demonstrated the beneficial effect of NAC treatment, with the NAC-treatment group having the lowest area at both 2 and 6 weeks after infarction (Figure 5.2C: NAC, 2wk: 11.3 ± 1.3 mm^2, 6wk: 15.2 ± 1.5 mm^2, untreated cells, 2wk: 13.7 ± 1.1 mm^2, 6wk: 17.3 ± 1.6 mm^2, DEM, 2wk: 14.7 ± 1.0 mm^2, 6wk: 19.4 ± 2.1 mm^2, PBS, 2wk: 17.5 ± 0.6 mm^2, 6wk: 19.3 ± 0.8 mm^2, * is $p<0.05$ to PBS, # is $p<0.05$ to DEM). These results suggest that increasing the antioxidants levels in MDSCs has an effect on the attenuation of functional deterioration of post-infarcted LV.

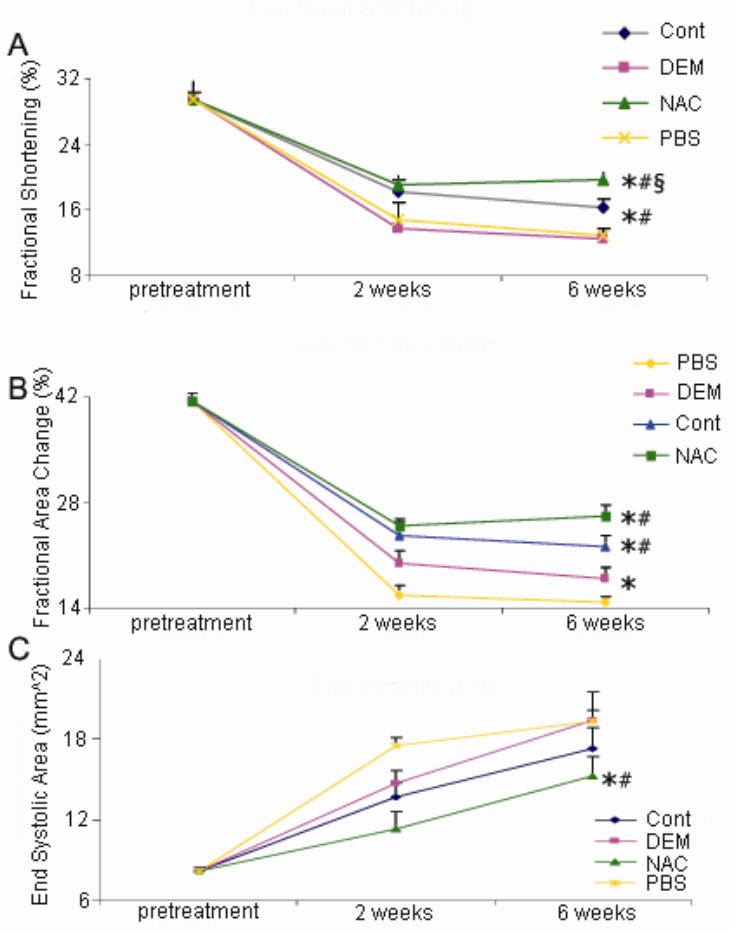

Figure 5.2 Cardiac function is improved with NAC pretreatment. (A) Fractional shortening (B) Fractional area change (C) End systolic area

Neoangiogenesis: NAC-treated cells had significantly higher levels of angiogenesis in the peri-infarct area (17.1 ± 1.4 x 100 CD31+ structures per mm^2, Fig 5.3A,E) compared to untreated cells (11.3 ± 0.7, Fig 5.3C), DEM cells (8.0 ± 0.7, Fig 5.3D), and PBS (7.7 ± 0.6 CD31+ structures per 100 fsMHC+ nuclei, Fig 5.3B). Untreated cells had significantly more blood vessels than DEM cells and PBS, and there was no significant difference between DEM and PBS. These results show that antioxidant levels upon injection can have a sustained impact on angiogenesis.

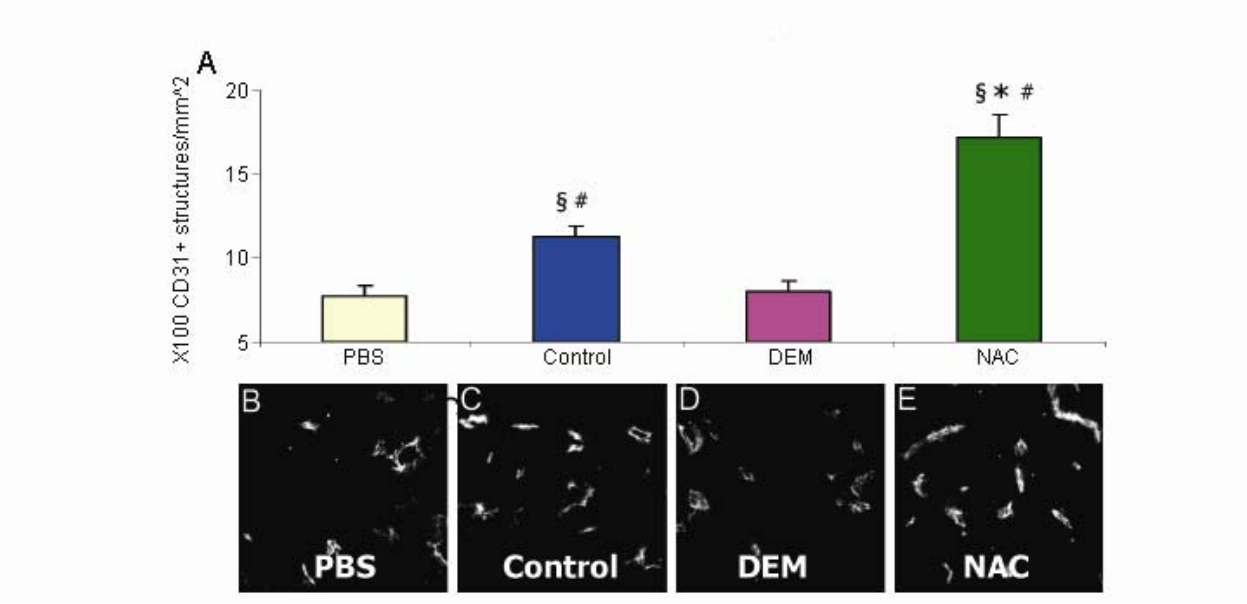

Figure 5.3 Angiogenesis is increased in peri-infarct region after NAC treatment (A) Angiogenesis in peri-infarct region (B,C,D,E) Images of PBS, control, DEM, and NAC treated cells

***In Vivo* Cell Engraftment and Proliferation:** There was no difference in the area of fsMHC+ cells at 6 weeks between the different cell-treated groups (untreated: 8.53 ± 2.7% fsMHC+ cells, DEM: 6.34 ± 1.54%, NAC: 7.55 ± 1.86%, Fig 5.4). Hearts in the NAC-cell treated group had significantly higher levels of ki-67(+) nuclei per high-powered field in the peri-infarct region of the heart than untreated controls, DEM, or saline. The untreated cell injected group had significantly higher numbers of ki-67(+) cells than DEM or saline treatment groups.

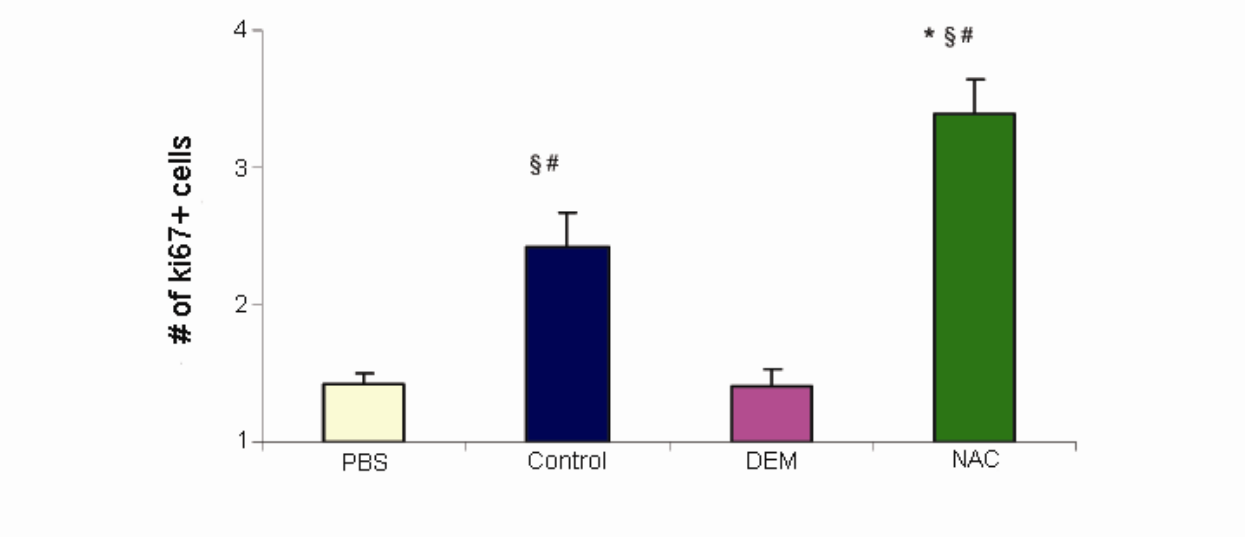

Figure 5.4 Cell proliferation *in vivo* is increased after NAC treatment

Scar Tissue Formation: Cross sections of infarcted hearts were stained with Masson's trichrome, and LV scar tissue ratio was measured. PBS-injected hearts had the highest ratio of scar tissue, followed by DEM-treated cells (PBS: 66 ± 5% collagen, DEM: 53 ± 4%). NAC-treated cells had the lowest amount of scar tissue formation, significantly lower than PBS, DEM-treated cells, and untreated cells (NAC: 25 ± 4%, untreated: 40 ± 4%, Figure 5.5A, B-E). Untreated cells had significantly lower levels of scar tissue than PBS, but the difference was not significant with the DEM-treated cells (P=0.09).

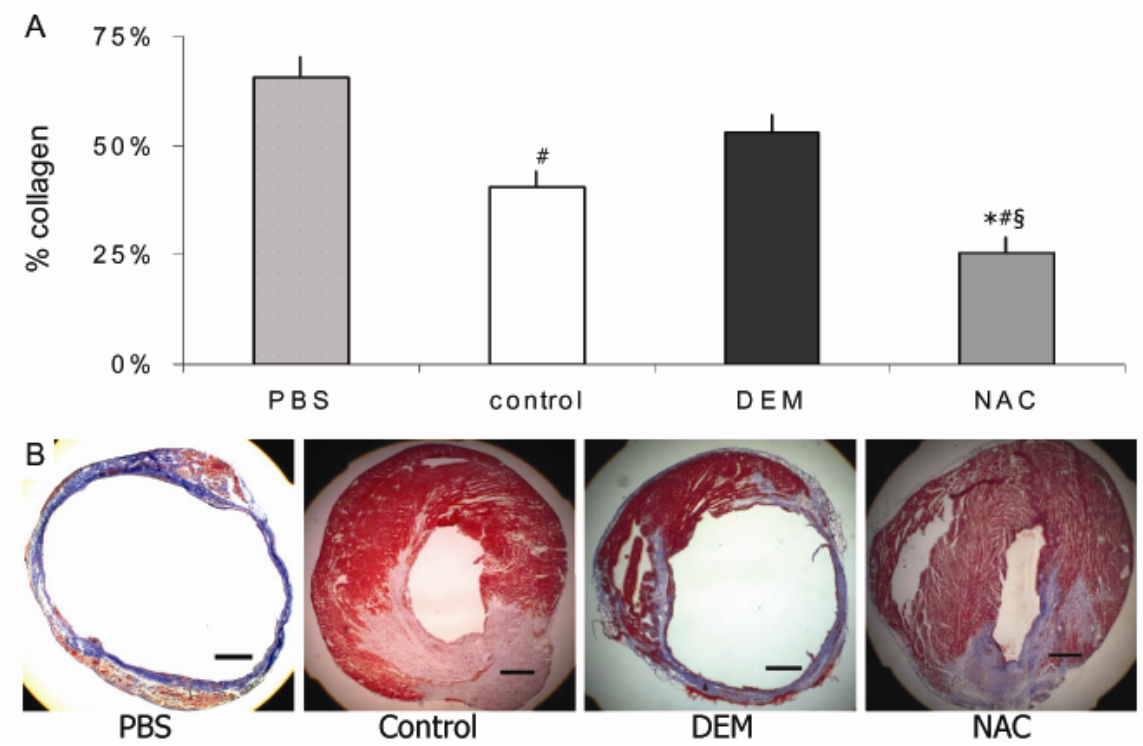

Figure 5.5 Scar tissue formation is decreased after NAC treatment. (A) Scar tissue area (B,C,D,E) Images of PBS, control, DEM, and NAC treated MDSCs

Cell Survival after Inhibition of MEK pathway: After inhibition of the MEK pathway using PD980459 (PD), cell survival under oxidative stress in the NAC treatment group was reduced close to control cell levels (Figure 5.6A). However, the reduction was not reduced to untreated levels, implying that the signal was not completely inhibited or a secondary pathway is involved. Knockdown of ERK signaling using PD was confirmed using western blots (Figure 5.6B,C).

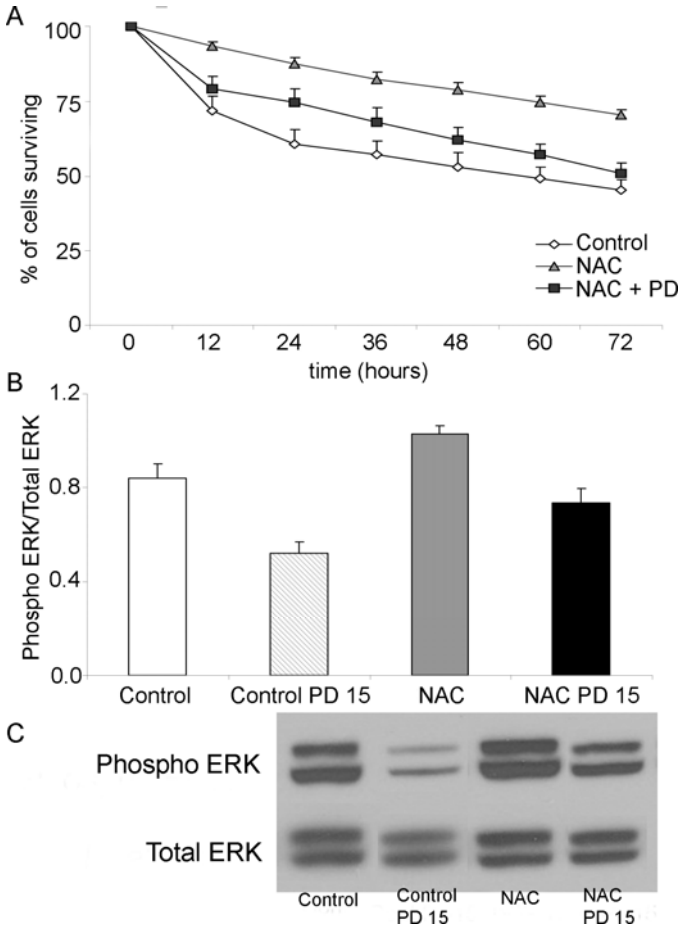

Figure 5.6 Cell survival signaling appears to be dependent on ERK signaling pathway. (A) Cell survival (B) ERK levels (C) Western blots

Regeneration after Injury in Skeletal Muscle: After injection with cardiotoxin and subsequent injection with MDSCs, when examining donor cell engraftment with dystrophin staining, NAC treated cells had significantly more dystrophin+ fibers than the DEM treated cell group (Figure 5.7A, D-F, $p<0.05$), but the difference was not significant compared to the untreated cell group ($P=0.43$). An increase in host regeneration, as assessed by centrally located nuclei, was seen in untreated cells and NAC treated cells above the DEM group and PBS control (Figure 5.7B, * is $p<0.05$ to PBS, # is $p<0.05$ to DEM).

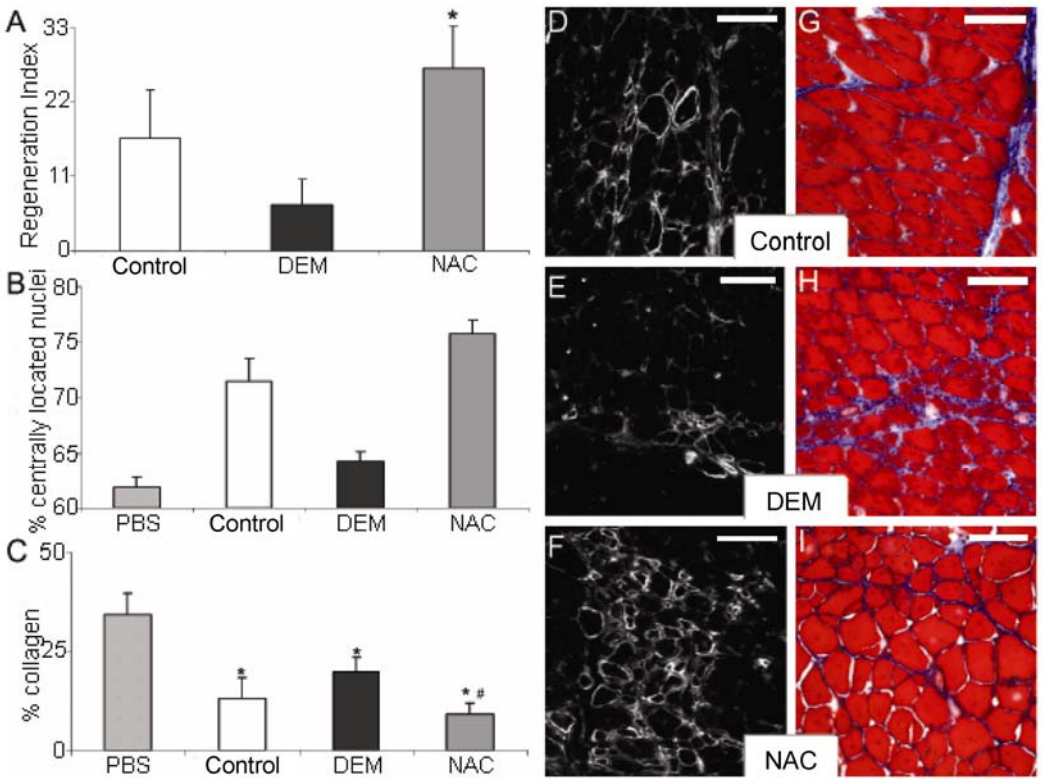

Figure 5.7 Effect of antioxidant levels in MDSCs on skeletal muscle injury. (A) Regeneration (B) Host regeneration (C) Scar tissue fraction (D,E,F) Images of dystrophin in control, DEM, and NAC treatment groups (G,H,I) Images of scar tissue formation in control, DEM, and NAC MDSC groups

Scar Tissue Formation in Skeletal Muscle: The DEM treated cell group has significantly higher levels of scar tissue formation after injury in skeletal muscle than NAC treated cells ($p<0.05$, Fig 5.7 C, G-I). NAC treated and untreated cells have significantly lower levels of scar tissue than PBS injected controls ($p<0.05$). These results are similar to those seen in the infarction model.

Myoblast Proliferation and Cell Survival: NAC treatment did not affect proliferation of MDSCs, with the rate remaining the same as non-treated MDSCs, whereas DEM treatment decreased proliferation at later time points (Fig 5.8A, $p<0.05$). When the level of antioxidants is

increased with NAC treatment, survival under oxidative stress is significantly higher than control and DEM at later time points (Figure 5.8B, $p<0.05$).

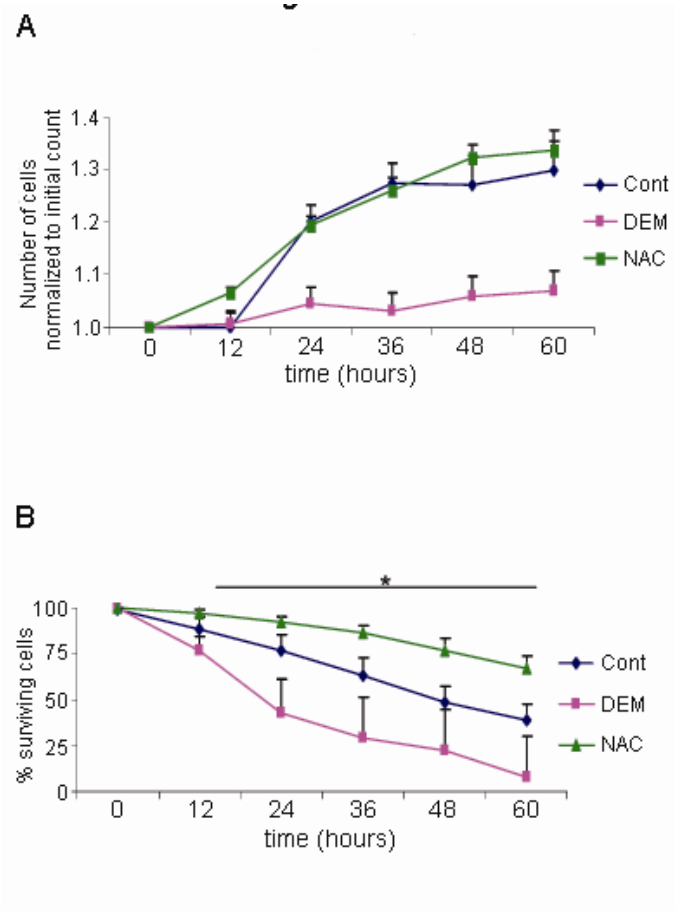

Figure 5.8 Effects of antioxidant levels on myoblasts. (A) Proliferation (B) Cell survival

5.4 DISCUSSION

Many different cell types are under evaluation as treatments for cardiac repair, and though all are beneficial to some extent, methods to further improve cellular therapy are still needed. Cell survival after implantation is critical, as there is inflammation and oxidative stress in the *in vivo* environment after injury, and typically few donor cells survive. It is hypothesized that this low level of cell survival limits functional repair, and so a treatment to increase cell survival after transplantation could have significant therapeutic benefits in clinical application.

Stem cells are defined by their multipotency, ability to proliferate long-term, and their self-renewal capacity. Some groups have demonstrated that stem cell populations have increased resistance to stress when compared to differentiated cells [114], and others have shown that stem cells have higher basal levels of antioxidants [16, 116]. In this paper we show that antioxidants play a critical role in protecting the cell from both inflammatory and oxidative stress, and when antioxidant levels are increased in cells, there is a corresponding increase in regenerative capacity for cardiac repair. This provides further support to the conclusion that there is another definition for stem cells that includes an ability to survive stress, which appears to be connected to increased antioxidant levels.

Treatment with antioxidants, particularly NAC since it has been used clinically for other purposes for many years, is an attractive option to improve cell survival. It is simple, inexpensive, and does not cause any permanent transformation of the cell that could cause problems in the future. We have shown that the level of antioxidants is important for cell survival under oxidative and inflammatory stresses both *in vitro* and *in vivo*, and increases *in vitro* differentiation of the cells, likely as a method to protect against apoptosis [131]. Most significant is the ability of NAC-treated cells to maintain increased cardiac function after MI

significantly better than both untreated and DEM-treated cells. Though we have previously shown that VEGF is critical for cardiac repair, NAC-treated cells were able to reduce harmful remodeling without secreting high levels immediately after transplantation, implying that cell survival is also of key importance [132, 133]. With increased survival, there is likely increased angiogenic factor secretion, as more cells will be alive to have a paracrine effect on the host myocardium. At six weeks post-transplantation, the NAC treatment group had significantly more blood vessels in the peri-infarct area than DEM, untreated cells, or saline. NAC treated cells also significantly decreased scar tissue formation over untreated cells and DEM treated cells, and untreated cells had significantly lower levels of scar than DEM treated cells. These results highlight the influence of cellular antioxidant levels in cardiac function, angiogenesis, and scar tissue formation after injury.

The mitogen activated protein kinase (MAPK) family of protein kinases, including extracellular signal-regulated kinases (ERK) pathway influences a wide variety of cellular mechanisms, including survival and proliferation [134-136]. Blocking the ERK 1/2 signaling pathway decreases cell viability in myogenic stem cells, and in cardiomyocytes ERK 1/2 signaling protects against induced apoptosis [134, 135]. It has previously been shown that NAC activates the ERK pathway and inhibits activation of downstream signals, including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [137-141]. We have confirmed that NAC treatment of MDSCs does activate the ERK pathway, and inhibition of the pathway decreases NAC-mediated cell survival. ERK pathway inhibition did not decrease NAC-treated cell survival to control levels, however, which implies a compensatory mechanism or signaling through another pathway.

NAC treatment has been used for ischemia reperfusion injury in animal models with encouraging results [128], and with the addition of cell transplantation, the therapy is further improved. This paper demonstrates that in a mouse model of myocardial infarction, NAC treatment prior to cell transplantation increased angiogenesis, decreased scar tissue formation, and decreased negative remodeling to maintain cardiac function. Antioxidant treatment is a potential method to increase efficacy of cell transplantation.

5.5 ACKNOWLEDGEMENTS

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6.0 CONCLUSIONS

Cell transplantation, particularly using stem cells, is an innovative therapy for cardiac repair after a myocardial infarction (MI). The gold standard of cell therapy is autologous transplantation to circumvent the need for and adverse effects of life-long immunosuppression. MDSCs are an excellent choice for cell therapy, as they exhibit stem cell characteristics, are easy to isolate, and can be used for autologous therapy. In addition, they have been shown to be superior to more differentiated cells (i.e. myoblasts) for the repair of bone, cartilage, skeletal and cardiac muscle, and nerve [34, 35, 41, 42, 46]. Most importantly, MDSC cell therapy after MI results in significant improvement of cardiac function and angiogenesis and decreased scar formation.

Previous research has shown that the sex of MDSCs can impact osteogenesis, chondrogenesis, and skeletal muscle repair [58, 59, 83]. However, the potential role of MDSC sex in cardiac repair has not been elucidated. In chapter 2 we have demonstrated that transplantation of both male and female murine MDSCs attenuates functional deterioration after MI, increases angiogenesis, and decreases scar tissue formation compared to control (PBS-injected) groups. Male and female MDSCs exhibited similar survival rates under oxidative and inflammatory stresses both *in vivo* and *in vitro* and secrete comparable levels of VEGF. Based on these observations, we found that cardiac repair using MDSCs does not appear to be influenced by the sex of the cells. MDSCs seem to enact repair through paracrine mechanisms

and increasing survival and repair of the host cells rather than by differentiating into cardiomyocytes [8, 57].

Age is another intrinsic characteristic of stem cells that is likely to play an important role in autologous transplantation, as the majority of patients requiring cell therapy for MI are of an advanced age. In chapter 3, we have shown no age-related decreases in the transplantation capacity of MDSC in regards to host regeneration, engraftment, and angiogenesis. However, here we provide evidence that the age of the host does impact regeneration potential; as age increases, engraftment and host regeneration potential decreases. Understanding the influence of inherent stem cell characteristics such as cell sex and age on transplantation outcomes in cardiac repair could provide a window to improving cell therapy.

Interaction of MDSCs with environmental cues at the site of injury plays an important role in their regeneration capacity. After MI, donor cell survival after transplantation is a major limiting factor for cardiac repair [2]. Based on current research, it appears that stem cells used for cardiac therapy do not differentiate in significant numbers down the cardiac lineage, and instead aid the endogenous repair through paracrine mechanisms. We examined two possible mechanisms to further elucidate how MDSCs improve cardiac function and enact repair: increasing cell survival post-transplantation and increasing paracrine factor secretion.

As previously mentioned, VEGF secretion is important for cardiac repair [8, 57], and methods to increase the secretion levels of the implanted cells could improve regeneration. In chapter 4, mechanical stretch of MDSCs prior to injection was investigated as a means to augment normal MDSC-mediated repair by stimulating the secretion of paracrine factors. We found that *in vitro* mechanical stimulation can temporarily increase the level of VEGF secretion and proliferation in MDSCs while differentiation and cell survival remained the same. The *in*

vivo experiments reported here show that mechanical stimulation-induced angiogenesis plays a significant role in improving cardiac function after injury and that the release of VEGF is an essential factor in mechanical activation. These results further support the previous results showing that VEGF secretion is critical for cardiac repair and demonstrates that even a short-term increase in VEGF secretion by MDSCs can have a significant impact on cardiac function.

Poor cell survival after injection at the site of MI most likely limits functional recovery, and so a treatment to increase cell survival after transplantation could have significant therapeutic benefits in clinical application. Stem cells have been shown to be superior to more differentiated cells for repair, and this may be due to their higher capacity to resist stress [16, 114, 116]. In chapter 5 we provide evidence that antioxidants play a critical role in protecting the cell from both inflammatory and oxidative stress *in vitro* and increasing antioxidant levels increases the ability of the MDSCs to differentiate. Pretreatment of MDSCs to increase antioxidant levels results in improved cardiac function after MI, decreased scar tissue formation, and increased angiogenesis. These results demonstrate that a temporary increase in antioxidant levels in MDSCs can significantly improve cell transplantation-mediated repair in a murine model of MI. Overall, the research reported here can make a significant contribution to improving MDSC-mediated therapies for cardiac repair.

While the work presented here, both mechanical preconditioning and pretreatment of MDSCs with antioxidants, provided encouraging results that improved cardiac function, it would be logical to continue the research by combining the two therapies to further improve cardiac repair. We could evaluate the efficacy of pretreating cells with both mechanical stretch and antioxidant treatment in various combinations, which has the potential to further enhance the survival of the injected cells and improve repair after a myocardial infarction. Another

alternative would be to investigate whether intraperitoneal injection or oral treatment with NAC in combination with MDSC injection could provide similar results. In addition, in order to fully validate the feasibility and beneficial effects of MDSCs for clinical translation of cardiac repair, the work needs to be repeated with human cells, as murine MDSCs were used for all the work shown here.

Continued research into cellular therapies for cardiac repair is imperative, as the heart is one of the least regenerative organs and current treatment methods are insufficient. In clinical translation, it is imperative to understand inherent differences in MDSCs and their influence on endogenous cells prior to therapy as these factors could influence the outcome. In addition, examination of safe and effective methods to improve cell transplantation for all types of repair would move the field of regenerative medicine forward and aid in bringing efficacious therapies to patients.

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