THE ROLE OF STRESS RESISTANCE IN CELL TRANSPLANTATION

EFFICACY FOR MUSCLE REGENERATION

by

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THE ROLE OF STRESS RESISTANCE IN CELL TRANSPLANTATION

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Despite the regenerative capacity of skeletal muscle, chronic myopathy and muscle trauma present significant clinical problems with limited therapeutic options. Myogenic cell therapies are being actively investigated to mitigate muscle degeneration that otherwise progresses to fibrosis and long-term loss of function. The rationale for this strategy is based on augmenting or recapitulating the normal process of muscle regeneration by injecting myogenic progenitor cells into the injured muscle. These exogenous progenitor cells may either fuse with the damaged myofibers or take up residence in an endomysial or satellite cell niche to be activated at some later date. However, engraftment efficiency in myogenic cell transplantation is impaired by rapid cell death, which represents a precipitous loss of cell viability within the first two days following injection, therefore severely limiting engraftment and tissue regeneration.

Upon transplantation, cells experience a host response of rapid inflammation; an environment of oxidative and inflammatory stress that causes a dramatic loss in cell viability. This effect may determine the regeneration capacity irrespective of the cell's multilineage differentiation potential. Our lab has isolated and characterized multiple populations of myogenic progenitors from murine and human skeletal muscle, including the muscle derived stem cell (MDSC) isolated by a modified preplate technique. In our studies of MDSCs, we observed an increase in post-transplantation survival and skeletal muscle regeneration capacity

compared to myoblasts. Furthermore, a strong correlation of improved survival and regeneration with inflammatory and oxidative stress resistance emerged.

In this dissertation, we examined the role stress resistance and survival during myogenic differentiation of MDSCs. By treating the transplanted cells with a membrane permeable reactive oxygen species scavenger, XJB-5-131, prior to transplantation, the survival and myogenic differentiation capacity under stress conditions was significantly improved. Furthermore, we isolated a novel sub-population of muscle derived cells with elevated stress resistance from murine and human skeletal muscle by their enhanced aldehyde dehydrogenase activity. $ALDH^{lo}$ and $ALDH^{hi}$ cells were characterized in terms of their myogenic potential and stress tolerance in vitro and muscle regeneration capacity in vivo. These studies are aimed at understanding the importance of survival and stress tolerance of transplanted cells in skeletal muscle cell therapy and how this tolerance modifies the efficacy of cell therapy.

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NOMENCLATURE AND ABBREVIATIONS

ALDH	Aldehyde dehydrogenase
ALP	Alkaline phosphatase
bFGF	Basic fibroblast growth factor
BMP-4	Bone morphogenic protein-4
CDK	Cyclin dependent kinase
Cy3	Cyanine
DAPI	4',6-diamidino-2-phenylindole
DEAB	Diethylaminobenzaldehyde
DEM	Diethyl maleate
DYS	Dystrophin
DM	Differentiation media (2% serum)
EP	Early preplate myoblast
FACS	Fluorescence activated cell sorting
FITC	Fluoroscien isothiocyanate
FoxO	Forkhead family of transcription factors
GAG	Glycosaminoglycan
GSH	Glutathione (reduced)
hMDC	Human muscle derived cell
MDSC	Muscle derived stem cell
MDC	Muscle derived cell
mdx	Dystrophin null mouse
MHC	Myosin heavy chain
mPTP	Mitochondrial permeability transition pore
MTT	Myoblast transfer therapy
NAC	N-acetylcysteine
NOX 2	Nicotinamide adenine dinucleotide phosphate oxidase 2
NOS	Nitric oxide synthase
PI	Propidium Iodide
P/S	Penicillin/ Streptomycin
Pmf	Proton motive force
PM	Proliferation media (20% serum)
PMN	Polymorphonuclear cell (neutrophil)
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCID	Severe combined immunodeficiency
SOD	Superoxide dismutase
TGF-β3	Transforming growth factor- beta3
TNF-α	Tumor necrosis factor-alpha

PREFACE

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This dissertation is dedicated to my family: to my wife Karyn who soldiered with me through every peak and valley of this process: writing off countless weekends so I could tend to cells in culture or to mice whose timepoints had come. To Middy and Caitlin, two of the most beautiful little girls this side of the Allegheny, who have a special gift for erasing any memory of a long day with a well-timed hug or silly joke told loudly, wide-eyed, and with hands flying.

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1.0   INTRODUCTION

Muscle injury can result from insults that range from mechanical overloading, traumatic injury, metabolic disorders, ischemic events, and congenital dysfunction. Often these insults exceed the body's capacity to regenerate functional muscle. Cell therapies, in which myogenic progenitors are transplanted to the muscle to promote regeneration, remain a promising field of regenerative medicine. The field witnessed dramatic advances during the past two decades in the form of myoblast transfer therapy (MTT) primarily for the treatment of Duchenne Muscular Dystrophy (DMD). DMD is the most common (1/3500 live male births) and severe of the congenital muscular dystrophies[1]. It is an X-linked muscle disease characterized by progressive muscle weakness due to the lack of dystrophin expression at the sarcolemma of muscle fibers[2-6]. The lack of dystrophin in skeletal muscle disrupts the linkage between the subsarcolemmal cytoskeleton and the basal lamina, resulting in muscle fiber necrosis and progressive muscle weakness[7,8].

Although researchers have extensively investigated various approaches to deliver dystrophin in dystrophic muscle (for example, cell and gene therapy), there is still no efficient treatment that alleviates the muscle weakness caused by DMD. Transplantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts capable of fusing with dystrophic muscle fibers and restoring dystrophin[9-33]. Although myoblast transplantation can transiently deliver dystrophin and improve the strength of dystrophic muscle,

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clinical trials of this approach yielded various limitations, including immune rejection, poor cell survival rates, and the limited spread of the injected cells[9-32].

Isolation of muscle cells that can overcome these limitations would enhance the success of myoblast transplantation significantly. In this introduction, I briefly review the immunological issues and transplanted cell phenotypes that affect the efficacy of cell mediated transplantation. However, special attention is given to the transplanted cell's ability to withstand inflammation and the deleterious effects of oxidative and inflammatory stress.

1.1   DUCHENNE MUSCULAR DYSTROPHY: A MODEL OF DEGENERATIVE MUSCLE PATHOLOGY

In addition to its contractile function, skeletal muscle is designed to withstand and adapt to repeated cycles of mechanical loading throughout our lifetime. This loading exposes the tissue to injury that induces regeneration without any functional loss. In fact muscle regeneration in this context often induces functional gain. This normal process of injury and regeneration follows a common pattern of interplay between the injured muscle fibers, resident and infiltrating immune cells, and the satellite cells that are primarily responsible for skeletal muscle regeneration. However pathologic muscle injury from insults such as traumatic injury, metabolic disorders, ischemic events, and genetic dysfunction may not always result in functional regeneration due to the severity of the insult or the compromised ability of the host to regenerate the damaged tissue.

One prominent model of chronic muscle degeneration is Duchenne Muscular Dystrophy, a devastating dystrophinopathy characterized by progressive muscle weakness and wasting due

to familial or spontaneous dystrophin gene mutations[2-6,34]. Dystrophin is a structural protein that concentrates at the myocyte z-line and forms a mechanical link between cytoplasmic actin and the basal lamina via a membrane bound dystrophin associated complex. In this way dystrophin transfers the force of contraction to the basal lamina[7,8,35]. In its absence each contraction exposes the myocyte to membrane tearing, loss of cellular potential, and calcium ion leakage which initiates a cascade of cell necrosis, inflammation and satellite cell activation[36]. Similar acute events of membrane tearing that occur during normal muscle loading should trigger regeneration with no loss of strength or satellite cell reserve. However, in DMD this process is widespread, ongoing and ultimately overwhelms the muscle's native capacity for repair. This disorder ultimately leads to cardiomyopathy, prominent kyphosis, and diaphragmatic and respiratory dysfunction. Current medical intervention is primarily supportive and targets delay of symptomatic progression[37-39]. Death results from respiratory insufficiency, pulmonary infection, and cardiac decompensation in the third decade of life[35,38].

Considerable research effort has been devoted to efficient delivery of dystrophin to dystrophic muscle through various means such as cell and gene therapy, however challenges remain before clinical translation can be realized. While transplantation of normal myoblasts or other myogenic cells into dystrophin-deficient muscle can create a reservoir of normal myoblasts capable of fusing with dystrophic muscle fibers and restoring dystrophin, this approach has important limitations, which include immune rejection, poor cell survival rates, and restricted spreading of the injected cells[9-32].

The concept of loss of muscle progenitor reserve in the context of chronic muscle degeneration continues to motivate research into the efficacy of myogenic cell transplantation to augment or replenish this reserve. That is, the satellite cell reserve is unable regenerate at the

same rate as the ongoing process of degeneration. However, perhaps the pathology of degenerative muscle disorders could be alleviated by replenishing the progenitor cell reservoir by allo- or autogenous transplantation of myoblasts. Furthermore, myoblasts could be genetically modified prior to transplantation to correct genetic disorders such as DMD, perhaps halting the ongoing pathology. Despite the isolation of numerous myogenic cell candidates, this cell therapy has so far proved elusive in DMD. One the most severe limitations is the high rate of cell death (up to 99%) within the first 48hrs of injection[9-32]. Researchers continue to explore explanations for this acute loss and to study methods to increase survival during this critical period[40]. It is encouraging to note however that the subpopulation of cells that do survive this initial period go on to fuse to existing myofibers and appear to trigger myofiber regeneration and promote angiogenesis in a paracrine fashion[41-43].

Acute cell loss on transplantation appears to stem from a complex immune response to several simultaneous events including: the tissue damage of the injection and pro-inflammatory activity of host and even donor myoblasts[44,45]. It may even involve local ischemia due to disruption of capillary hemodynamics commonly seen in ischemia/reperfusion injury which generates numerous reactive oxygen species[46]. The cause of this precipitous loss of transplanted cells is not entirely resolved. The temporal profile of the response suggests activity from the innate immune system including inflammation and the influx of activated neutrophils[47], macrophages[48,49], and natural killer (NK) cells[50,51]. The injected cells are likely exposed to a storm of reactive oxygen species (ROS) and inflammatory cytokines. Recent studies have shown that the cells that do survive and engraft also have increased oxidative and inflammatory stress tolerance[43,52].

1.2 PHYSIOLOGIC MECHANISM OF MUSCLE REPAIR: ROLE OF SATELLITE CELLS AND OTHER MYOGENIC PROGENITORS

The fundamental cellular unit of skeletal muscle is the myofiber, a linear, multi-nucleated cell whose contractile actin-myosin machinery is distributed serially along the length of the fiber. During contraction these actin-myosin networks induce radical dimensional changes of the cell and therefore considerable shear strains in the sarcolemma. In cases of excessive loading or trauma, sarcolemmal tearing allows calcium ion leakage into the cytoplasm which can lead to myofiber necrosis facilitated by calcium dependent proteolysis (e.g. via calpains) and increased mitochondrial ROS production. Both events may trigger a cascade of acute immune cell activation, primarily of macrophages and neutrophils[53-57]. While immune cell activation will initially promote further damage, it is critical for muscle regeneration with a particularly vital role for the macrophage[36,58].

Embedded in the basal lamina of myofibers are satellite cells, myogenic progenitors that are primarily responsible for post-natal skeletal muscle regeneration. Activated satellite cells divide asymmetrically to self-renew and yield a rapidly dividing population of myoblast daughter cells, which migrate to the site of muscle injury. Myoblasts can either fuse with the damaged myofiber or with one another to form a new myofiber. In congenital myopathies such as Duchenne muscular dystrophy the satellite cell reserve may be depleted by chronic activation and oxidative damage from on going cycles of sarcolemmal rupture, necrosis, and immune cell activation[59-61]. Furthermore, large muscle lesions induced by trauma or ischemia (e.g. compartment syndrome) can simply overwhelm the satellite cell reserve such that they were unable to recreate the gross muscle morphology required to restore muscle function.

Studies have shown that the satellite cell is not the only myogenic cell that can be activated to induce skeletal muscle regeneration. Bone marrow derived cells (mesenchymal stem cell or marrow stromal cell) can differentiate into myoblasts or even enter the satellite cell position and undergo myogenesis at a later time[62-64]. A number of multipotent progenitor cells have been isolated from muscle such as muscle derived stem cells[42], side-population cells[65], Sk-DN/Sk-34 cells[66], and CD133+ progenitors[67,68]. There is growing evidence that these cells are intimately associated with or even share a common lineage with blood vessels[69-71]. Indeed prospective isolation of pericytes[72], mesangioblasts[73], and myo-endothelial[74] cells have yielded myogenic differentiation in vitro and in vivo[74-76]. Given their multipotency, their ability to regenerate muscle may stem not only from their myogenic differentiation potential but their ability to promote nerve regeneration and angiogenesis in the context of cell transplantation, thus recapitulating a full complement of functioning muscle[77,78].

1.3   MYOGENIC PROGENITORS FOR CELL THERAPY

Irrespective of their physiologic function, a multitude of myogenic cells have been isolated to date and have been shown to recapitulate myogenesis in vitro by fusing into syncytial and contractile myotubes. More importantly, these cells when transplanted have demonstrated their ability to participate in skeletal and in many cases cardiac muscle regeneration[45]. They do so either by fusion with existing myofibers, generation of new myofibers by fusing with each other, or stimulating regeneration in a paracrine fashion. Therefore we have accumulated a virtual library of cells to study possible cell therapies for muscle degeneration. Which of these cells

offer the greatest ease of isolation, greatest capacity for regeneration, and how they are related to one another are all active of areas of research[45].

Muscle derived stem cells (MDSCs) have been isolated by our lab from skeletal muscle using a modified preplate technique[42,79]. Briefly, this technique involves enzymatically dissociating cells from excised muscle and isolating MDSCs by their slow adherence to collagen coated flasks. Those cells that adhere after two hours in media culture conditions constitute pre-plate 1 (PP1) and non-adherent cells are transferred to a new flask. Successive 24hr preplate cycles segregate pre-plate populations PP2 through PP6. After 10-12 passages of the PP6 population, a long-term proliferating population of MDSCs is established. MDSCs, even when expanded from clonal populations express variable surface markers and cytoplasmic markers of stem cell and myogenic lineage such as CD34, Flk-1, Sca-1, MyoD, and desmin[80,81]. However, MDSCs have been shown to differentiate into musculoskeletal tissues such as bone, cartilage, and skeletal muscle, in addition to endothelial, neural and hematopoietic tissues[42,77,78,80,82-84]. Perhaps more important than MDSC multipotency, however is the increased rate of survival and subsequent engraftment in skeletal muscle when compared to myoblasts.

Although the MDSCs isolated by our laboratory were derived from skeletal muscle, many sources may be considered, given previous studies in which pluripotent bone marrow stem cells[63,65,85-89], blood vessel progenitors[90-94], neural stem cells[95-97], and cells from other connective tissues, including adipose tissue[98,99] and dermis[100], have all shown the capacity to differentiate into myogenic cells. Many of these cell types appear to be able to undergo myogenic lineage in vitro but their participation in the regeneration of skeletal muscle in vivo after injury or disease has been found to be extremely limited (especially the hematopoietic, bone marrow and other circulating precursors)[101,102]. Among all of these different cell types, it appears that blood vessel

progenitors (including mesangioblasts) share a number of features with the MDSCs identified by our group. In particular, they share cell-type marker profiles and have high myogenic potentials in vitro and in vivo; similarities that suggest a possible relationship between these types of cells[45].

1.4   CHALLENGES IN TRANSLATION OF MYOGENIC CELL TRANSPLANTATION

Early clinical trials of myoblast transfer therapy therapies faced difficult challenges, including immune rejection, poor cell spreading (less than 200μm from injection site), as well as rapid death on injection[45]. Many studies have generated novel ideas for ameliorating these obstacles and increasing efficiency of engraftment, however they have yet to translate into a clinically viable solution for DMD treatment.

The first major breakthrough in developing this therapy came with the validation of the *mdx* mouse model of DMD, which ushered in studies of cell transplantation and evidence of engraftment of dystrophin positive fibers in this model[22,103]. What followed were human clinical trials that demonstrated limited engraftment of dystrophin positive myofibers[10-12,23,28,30,104,105]. In Human clinical trials, myoblast transfer therapy proved to be of limited therapeutic value due to low efficiency of engraftment, delivery of dystrophin to the deficient muscle, and poor rates of improvement of muscle function. Mendell et al. attained a biceps muscle engraftment rate of ~10% using cyclosporine immunosuppression, yet muscle function tests were disappointing[28]. Ironically it was found that cyclosporine can induce apoptosis in differentiating myoblasts, despite the survival benefit of immunosuppression[106,107]. Further studies confirmed that the poor

engraftment efficiency arose due to immune rejection[18,31,108], limited cell spreading[109], and a phenomenon of rapid cell death within 48hrs of injection[9,17,18,47,110,111].

1.4.1 Acute immunogenicity of myogenic cell transplantation

Upon injection into the host skeletal muscle, there are a number of simultaneous immune related responses that endanger the survival of the donor cells. Insertion of the needle and injection of fluid in the muscle will induce tearing of multiple groups of myofibers and vascular structures which will initiate an immune reaction associated with myofiber damage and necrosis as well as a clotting cascade, both of which will stimulate a state of inflammation. It is possible that this reaction is similar phenomenon of instant blood-mediated inflammatory reaction (IBMIR) observed in pancreatic islet cell transplantation[112]. IBMIR is essentially a thrombotic and inflammatory reaction observed in islets cells infused into the portal vein, which is induced by tissue factor expression of the islet cells[113]. Rapid leukocyte infiltration, especially that of neutrophils, is thought to incur a rapid loss of cell viability within the first minutes and hours of transplantation[113]. In the case of myogenic cell intramuscular injection, inflammation rapidly alters the environment of the injection site in terms of the redox state, the nature and concentration of pro-inflammatory cytokines, the presence and activity of inflammatory cells, and consequently the phenotypic expression of host and donor cells. This complex interplay of host and donor cells is briefly reviewed below.

The hallmarks of inflammation are increased vascular permeability, activation of inflammatory cells, and change in the intra- and extracellular redox state[114]. Rupturing the sarcolemmal membrane of the myofiber will allow Ca^{++} influx, lysosomal enzyme efflux and

expose neighboring cells including macrophages to other cellular products of necrosis which induce inflammation within seconds or minutes[44].

Neutrophil and macrophage infiltration propagates inflammation by releasing cytotoxic chemicals and pro-inflammatory cytokines (e.g. TNF-α, IL-1, and IL-6). Degranulation of neutrophils releases reactive oxygen species, such as superoxide ($O_2^{\cdot}$) a highly reactive ROS that can be converted to H_2O_2 by superoxide dismutase, which can interact with iron and by a Fenton reaction can be converted to the hydroxyl radical ($OH^{\cdot}$), a far stronger oxidant[115]. This not only causes cellular damage via protein oxidation and lipid peroxidation but oxidizes intracellular non-enzymatic anti-oxidant molecules such as reduced glutathione (GSH) and alters the intracellular redox balance toward a pro-apoptotic state[57]. There are many putative mechanisms by which muscle cells sense changes in their intra- and extra-cellular environment[116,117], however one common signaling pathway upregulated by inflammation is NF-κB nuclear translocation[118,119]. Upregulation of NF-κB has a number of potential effects including upregulation of genes associated with anti-oxidant defenses, quiescence, and apoptosis[120,121]. Its activity in muscle cells remains poorly understood, however recent studies of p65 (a class I member of the NF-κB family) knockout (KO) muscle derived stem cells suggest that p65 KO cells have increased oxidative stress tolerance *in vitro* and yield increased rates of engraftment when injected into the *mdx* mouse gastrocnemius muscle (A. Liu et al., unpublished observation).

Studies of various muscle injuries, including eccentric loading and ischemia/reperfusion injuries, have shown that neutrophil infiltration occurs within an hour of injury and can remain for up to five days[44]. The rapid sequence of events that precedes neutrophil invasion is not fully elucidated, however there is evidence that the muscle itself can promote inflammatory cell

invasion.   Human satellite cells have been shown to release factors that promote macrophage/monocyte endothelial transmigration in vitro[44,122].   Neutrophil invasion is important to healing insofar as they release proteases that help clear muscle debris created by injury, however the cytotoxic species such as superoxide anion released on neutrophil degranulation also cause damage to bystander cells promoting further inflammation.

It is clear that macrophages also rapidly invade the injured muscle, but they have a far more complex role.   They participate in cell lysis using a nitric oxide (NO) dependent mechanism and phagocytose cell debris.   They then take a mediating role in the inflammatory process by promoting further immune cell infiltration (e.g. neutrophils, NK cells), however once necrotic cell debris has been cleared they undergo a phenotypic change that allows them to participate in the healing and regeneration phase of injury[36,48,49].   This change in role is associated with its conversion from an inflammatory phenotype (M1) to that of a healing phenotype (M2)[114]. In fact, macrophage depletion has been shown to greatly inhibit muscle healing[36].

Damage of vascular barriers that is induced on injection of myoblasts is likely to stimulate activity of the clotting system, whose activity is pro-inflammatory through the protease activity of thrombin (factor IIa)[114].   Thrombin binds protease activated receptors (PARs), which are G-protein-coupled receptors expressed on platelets, endothelial and smooth muscle cells. Binding induces several pro-inflammatory events including production of chemokines and expression of leukocyte adhesion molecules necessary for leukocyte binding and diapedesis[114]. However, to my knowledge experiments exploring the utility of interrupting the clotting cascade on myoblast survival have not been pursued.

The timing of cell death immediately following transplantation suggests loss by the innate and non-specific humoral immune system. One basic component of the non-specific humoral immune system is complement activity, in which 20 complement proteins coordinate to form a membrane perforating membrane attack complex (MAC)[123] as well as to activate B-cells[124]. However, several of the complement proteins (C3a, C5a, and C4a) also act to stimulate resident mast cells to release histamine, which causes vasodilatation and increases vascular permeability. C5a is a powerful chemotactic agent for neutrophils, monocytes, eosinophils, and basophils and can also stimulate phospholipase activity, which initiates arachodonic acid production. However, complement depletion has been shown to only marginally improve transplanted myoblast survival[125].

Subsequent detection of foreign cells by macrophages generates numerous chemotactic signals that trigger infiltration of natural killer (NK) cells. NK cells are lymphocytes that recognize and kill cells that do not express a "self" major histocompatibility complex class I (MHC-I) surface protein[126]. They target exogenous cells by secreting membrane perforating enzymes such as perforin and granzyme, which may induce apoptosis or pro-inflammatory necrosis. NK cells often coordinate with CD8^{+} or cytotoxic T-cells to kill foreign cells which has also been a focus of myogenic cell transplantation optimization[108], although these studies are inconclusive and offer conflicting conclusions on the importance and temporal profile of their infiltration[51,127,128].

1.4.2 Limited migration of transplanted myogenic cells

Poor myoblast migration following intramuscular injection is likely a consequence of the connective tissue barriers that exist between muscle fibers. Skeletal muscle is surrounded by

fibrous connective tissue at multiple scales. At the finest scale, myofibers are made within endomysial tissue, groups of which are organized into fascicles by perimysium. At the largest scale, epimysium and a thick muscle fascia encases the entire muscle body. Each scale of connective tissue generates an increasing barrier to myoblast migration. Several studies have suggested that myoblast migration may be mediated by the production of metalloproteinases, particularly MMP-1 and MMP-2[129]. Production of these metalloproteinases could be stimulated by viral transfection or in the case of MMP-9, by simple treatment with basic fibroblast growth factor (bFGF) or tumor necrosis factor (TNF-α). Furthermore soluble fibronectin yielded increases in MMP-1 activity. Interestingly, n-acetylcysteine, which may increase the anti-oxidant capacity of the myoblast, was shown to decrease MMP activity (particularly MMP-2 and MMP-9) and connective tissue transmigration in vitro[129,130]. Poor spreading can also be overcome by increasing the number and density of injection sites. In one monkey trial engraftment was enhanced by injecting 30 million cells per square millimeter[109]. Recent clinical trials in Canada of a similar "high density" injection technique (~100 injections per square cm of muscle surface) with tacrolimus immunosuppression demonstrated the feasibility of long-term dystrophin expression (up to 18 months)[131,132]. While the prospect of receiving "high density" injections to all dystrophic muscles would not seem clinically feasible as a standard treatment, it was well tolerated by the eleven patients enrolled in these trials, which should be considered in light of the severity and progressive nature of DMD.

Intravascular delivery of myogenic cells may offer a more favorable distribution of dystrophin expressing fibers especially in muscles such as the diaphragm. Capillary networks reach all myofibers of a given muscle and cells could be selected to home to areas of inflammation. Several studies have demonstrated the feasibility of this delivery route[67,68]. The

promise of this technique relies on the interaction of VLA-4 in CD133^{+} stem cells with endothelial vascular cell adhesion molecule (VCAM-1) in areas of muscle inflammation[45].

1.4.3   Immune rejection of transplanted myogenic cells

Immune suppression is typically required to induce successful engraftment of allogenic myoblasts. Tacrolimus (or FK506) has often been used to control inflammation and graft rejection because it, like cyclosporine, interacts with calcineurin to inhibit both T-lymphocyte signal transduction and IL-2 transcription[133]. Chronic administration following transplantation appears to be necessary as when it is withdrawn the transplanted myoblasts die in short order[134,135]. The nephrotoxic and carcinogenic side effects of chronic tacrolimus limits the utility of this approach. Immune tolerance can be induced either by non-myeloablative irradiation treatments with anti-CD45RB to anti-CD154[136] or cyclophosphamide and busulfan[137] or central tolerance using whole body irradiation and allogenic bone marrow transplant[136]. However, this central tolerance does not seem to include all muscle neoantigens following myoblast engraftment[138]. It should be noted that some myogenic cells under study have demonstrated immune privilege such as muscle derived stem cells, thus long term allogenic cell engraftment without immune suppression may be possible[42].

1.4.4   Reactive oxygen species and antioxidant capacity of MDSCs

Our lab has focused considerable effort on isolating and characterizing skeletal muscle derived multipotent cells, called muscle derived stem cells[79]. This population of cells have been shown to be heterogeneous in terms of cell surface markers (e.g. CD56, CD34, and Sca-1) has a

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tendency to change during in vitro expansion[42]. Rather they are isolated by a modified preplate technique in which cells that are slow to adhere to a collagen coated flask have been shown to display stem cell markers and behavior[79]. We ascertained that MDSCs were not only multipotent but their regenerative capacity in both skeletal and cardiac muscle consistently exceeded that of early preplate myoblasts. In a sense the failures of MTT paved the way for the discovery of MDSCs as the putative subpopulation of cells that survive the transplantation[15,139].

Reactive oxygen species in addition to reactive nitrogen species (RNS) have received a great deal of attention recently given their pathologic implications in aging, cancer, and inflammatory diseases as well as their important signaling functions[120,140-142]. Clearly the redox environment of the intramuscular injection site is altered by the action of neutrophils and macrophages as they generate numerous cytolytic ROS and RNS species. Neutrophils rely on NADPH oxidase 2 (NOX2) to transfer electrons to oxygen to create the superoxide radical that is itself quite toxic but can be converted to far more reactive hypochlorous and hypobromous acids by the sequential action of superoxide dismutase (SOD) and myeloperoxidase[143,144]. These oxidizing species are extremely efficient in oxidizing thiols and other biomolecules which can shift the redox balance intra- and extracellularly. Similarly, macrophages induce cell-lysis though production of nitric oxide via nitric oxide synthase (NOS2 or iNOS), rather than superoxide production[145].

One intriguing characteristic of MDSCs that appears to correlate quite well with its regeneration capacity is its resistance to oxidative and inflammatory stress. We have found that these cells produce constitutively higher levels of GSH and SOD than their more differentiated myoblast counterparts[52]. They maintain their myogenic differentiation and proliferation capacity as well as their ability to resist apoptosis in the presence of oxidative (H_2O_2) and inflammatory

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challenge (TNF-α)[43,52]. When GSH levels are depleted by diethyl maleate their regenerative capacity in both cardiac and skeletal muscle is reduced[52]. On the other hand when the GSH levels of MDSCs were increased by treatment with n-acetylcysteine, their regenerative capacity in skeletal and cardiac muscle was increased[130]. Recent studies have shown that the cells that survive and engraft also have increased oxidative and inflammatory stress tolerance in vitro[52].

In fact, stress tolerance may be an essential attribute of stemness. This has been found in multiple lineages including neural stem cells[146], hematopoietic stem cells[147], endothelial progenitor cells[148,149], cancer stem cells[150-152], and other circulating progenitors[153]. One may speculate that this is a consequence of a logical evolutionary drive to protect these rare and multipotent cells from senescence and dysfunction through oxidative damage. It also appears to be a signaling mechanism to preserve the progenitor phenotype[154,155]. A major focus of this dissertation is a finding that sorting muscle derived cells on aldehyde dehydrogenase (ALDH) activity yields a subpopulation of ALDH^{hi} cells with not only increased stress tolerance but also increased rates of engraftment when injected in the *mdx* mouse. From the perspective of regenerative medicine this anti-oxidant capacity offers another critical benefit of stem cells for cell therapy and may prove to be a primary determinant of the efficacy of stem cell therapy[52]. ALDH plays an essential role in regulating retinoic acid production, which has been shown to mediate differentiation in a number of tissue specific stem cell populations[156-159]. However, why ALDH production appears to be upregulated in a number of tissue specific stem cells is not entirely clear, however it's role in stem cell maintenance is likely to stimulate further study given its increasingly important role in isolating somatic stem cells.

The cell's normal anti-oxidant defenses include enzymatic and non-enzymatic scavengers such as SOD, GSH, catalase, glutathione peroxidase, thioredoxin, and a peroxiredoxin family of

proteins[160]. These molecules are responsible not only for protecting the cell from oxidative damage but they are also responsible for maintaining a precise intracellular balance of redox potential, given the important signaling role of reactive oxygen species. Augmenting these normal anti-oxidant defenses may improve transplanted cell survival post-transplantation. Exogenous and synthetic SOD has been used to preserve function in isolating islet cell grafts and may prove useful for myogenic cell engraftment as well[161,162]. Thiol based anti-oxidants are also of interest in improving engraftment such as n-acetylcysteine[163-167].

1.4.4.1 Calcium dysregulation and the mitochondria

Following membrane damage through oxidative stress and other mechanisms, excess cytosolic calcium concentrations can activate Ca^{++} dependent proteases such as the calpains[168]. However, Ca^{++} dysregulation can trigger other pro-apoptotic pathways by interactions with muscle mitochondria[169]. Ca^{++} plays a physiologic role in regulating mitochondrial dehydrogenases by increasing NADH levels to fuel the respiratory chain. However, if the mitochondrial matrix becomes overloaded with Ca^{++}, it can trigger the opening of the mitochondrial permeability transition pore (mPTP) which destroys the proton motive force (pmf), including pH and mitochondrial ionic potential. This pore opening is further potentiated by oxidative stress[170-172].

With the loss of pmf, mitochondrial ATPases are driven to reverse their ATP forming reactions such that they consume ATP to pump protons out of the mitochondria. ATP depletion leads to loss of ion homeostasis throughout the cell and ultimately necrotic cell death[169]. A further deleterious consequence of mPTP opening is mitochondrial swelling driven by colloidal osmotic pressure from matrix proteins. This can perforate the outer mitochondrial membrane and release pro-apoptotic proteins such as cytochrome C[169]. However there are drugs that have been shown to inhibit mPTP opening through interactions with the matrix protein cyclophilin-D,

such as cyclosporin-A[173] and sanglifehrin-A[174], which may have some value in improving engraftment efficiency[175,176].

1.4.5 Aldehyde dehydrogenase: A marker for early progenitor status

Aldehyde dehydrogenase represents a family of enzymes that are responsible for the conversion of aliphatic and aromatic aldehydes to their corresponding carboxylic acids. It is also considered a detoxifying enzyme by eliminating reactive exogenous and endogenous aldehydes[177]. It has a vital role in embryological development by its conversion of retinaldehyde to retinoic acid, which mediates numerous developmental processes including somite migration, limb patterning, and tissue differentiation[178]. Retinoic acid induces differentiation by binding RARs (retinoic acid receptors), RXR (retinoid X receptor) associated transcription factors, and nuclear (RAREs) (retinoic acid response elements). Initial interest in the ALDH as a marker of hematopoietic stem cells (HSCs) began with an observation that HSCs with elevated ALDH are spared from cyclophosphamide ablation and subsequent elucidation of the role of ALDH in detoxifying this alkylating agent[179].

Elevated expression of ALDH has been shown to be a marker of early progenitor status in hematopeitic[180,181], mesenchymal[182], endothelial[183], neural[184,185], and recently skeletal muscle[186] populations. ALDH expression is associated with retinoic acid metabolism, a mediator of stem cell maintenance and differentiation, and confers oxazaphosphorine chemotherapy resistance in metastasis competent cancer stem cells[151,187,188]. Using elevated ALDH activity as a marker to isolate somatic stem cells has particular utility in light of the commercial availability of a boron-dipyrromethene (BODIPY) labeled amino acetaldehyde (BAAA) that fluoresces when oxidized by the cytosolic isoform of ALDH (ALDH1A1). BAAA diffuses freely into the

cytoplasm and is converted to BIODIPY-aminoacetate by ALDH[189]. Cells with elevated ALDH activity become fluorescent when exposed to boron-dipyrromethene (BODIPY) labeled amino acetaldehyde (BAAA) and can be isolated using fluorescence activated cell sorting (FACS). Furthermore, given the role of ALDH in embryological development and organism-wide tissue differentiation processes, its use as a marker to isolate early progenitor cells has sparked a surge of research activity in the somatic stem cell and cancer stem cell fields.

Many of the early progenitors, including somatic and carcinoma stem cells, that may be isolated by elevated ALDH activity have also be shown to have increased anti-oxidant capacity. In hematopoietic stem cells which can be isolated by multiple markers such as CD133, CD34, and ALDH, increased stress oxidative resistance is conferred by increased expression of the FoxO (Forkhead O) transcription factors[190]. The FoxO family of transcription factors play mediating roles in cell cycle arrest, oxidative stress resistance and apoptosis[191]. They are downstream targets of the PI3K-AKT pathway and regulators of the oxidative stress response, which includes induction of MnSOD, catalase, and GADD45[192-194]. Similar properties of increased expression of free radical scavenging systems and decreased levels of reactive oxygen species is observed in epithelial breast cancer stem cells which confers increased resistance to ionizing radiation therapy[195]. Similar properties were found in non-tumorigenic mammary epithelial stem cells. Breast cancer stem cells with increased malignancy and metastasis competence have been found to have increased ALDH activity[150]. In umbilical cord blood cells which may isolated ALDH activity have also been shown to give rise to endothelial progenitors with elevated MnSOD levels that significantly increases their oxidative stress resistance and therefore utility in treating ischemic tissue[148,196].

In adult tissues, the role catalytic and metabolic role of the ALDH family of enzymes is in an active area of research. Its enzymatic activity in the conversion of aldehydes to their corresponding carboxylic acids proceeds by the following reaction:

$$R\text{-}CHO \xrightarrow[\text{ALDH}]{NAD(P) \quad NAD(P)H} R\text{-}COOH$$
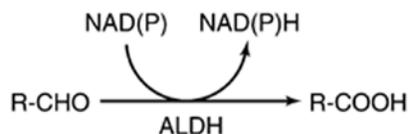
Figure 1. General reaction of aldehyde dehydrogenase[197]

It is the cytosolic form of ALDH, ALDH1A1, which has been the focus of many studies aimed at isolating somatic and carcinoma stem cells. Although it's activity in oxazaphosphorine chemotherapy resistance is well documented, it is not at all clear whether it directly participates in the oxidative stress resistance of these cells[198]. While our studies suggest that ALDH may be simply a marker for early progenitor status, it is also possible that it replenishes NADPH levels by conversion of endogenous aldehydes, which would provide a necessary substrate for reduced glutathione recycling (GSSG → 2 GSH) via the multiple NADPH dependent enzymes such as glutathione reductase[199].

Vauchez et al. recently identified an ALDH$^+$ myogenic progenitor population in skeletal muscle, which occupies an endomysial niche similar to that of the satellite cell[186]. The CD34$^-$ subpopulation of these cells were found to be myogenic both in vitro and in vivo. Another important result from this paper was the robust engraftment of these ALDH$^+$CD34$^-$ cells in the skeletal muscle of SCID mice. The co-authors estimated that the engrafted cell population at 4 weeks post-transplantation was roughly 2-5 times the number of cells that were injected, as estimated the number of donor nuclei counted in 8-10 serial cryosections. Perhaps more importantly however was that the transplanted cells not only fused with existing myofibers but many of some of these cells took up residence in an endomysial niche. This suggests that these

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cells may not only be used to induce immediate skeletal muscle repair but also to replenish the satellite cell reservoir. Although these results were quite similar to our study on ALDH[hi] muscle derived cells, our study focuses on the potential of these cells for myogenic therapy in mitigating the phenomenon of rapid cell death, particularly on the cells' ability to withstand oxidative and inflammatory stress.

1.5   SPECIFIC AIMS OF DISSERTATION

Myogenic cell therapies have been limited by rapid and precipitous cell death within days of transplantation[41,200,201]. Several mechanisms for this loss have been proposed including inflammatory and oxidative stress at the injection site[18,41,127,202], hypoxia/ischemia[203], and anoikis (apoptosis induced by loss of extracellular matrix (ECM) or adjacent cell contact)[204,205]. In fact most cell therapies suffer from poor post-transplantation survival[206-209]. Increased engraftment is observed following inhibition of LFA-1[18] and IL-1[41], implicating inflammation and the innate immune response. However direct interference with immune cell signaling and recruitment is problematic because the actions of pro-inflammatory cells such as the macrophage are essential for subsequent muscle healing[36,48,49,114]. Instead enhancing the rate of survival of these post-transplantation events by scavenging the numerous cytolytic reactive oxygen species produced by neutrophils and macrophages may improve engraftment efficiency without hindering subsequent regeneration[210]. In this study, we specifically address transplanted cell survival as it relates to inflammation and oxidative stress. Recently, we observed a correlation of increased inflammatory and oxidative stress resistance in vitro in myogenic cells with increased regeneration capacity in vivo[52,74]. When the antioxidant defenses of the transplanted cells were

enhanced or depleted we observed a corresponding increase or decrease in regeneration of the treated tissue[52,130]. These observations led us to hypothesize that *myogenic cells with enhanced capacity for stress resistance, a putative characteristic of stem and early progenitor cells, will induce increased skeletal muscle regeneration compared to myogenic cells with less stress resistance capacity*.

To test this hypothesis, I proposed three specific aims. The first sought to differentiate the effects of myogenic capacity from stress resistance on muscle regeneration capacity in vivo. A sub-aim of this aim was to seek to enhance the regenerative capacity of myogenic cells using a mitochondrial targeted ROS scavenger, XJB-5-131. The second aim sought to segregate murine myoblasts and MDSCs in ALDH^{hi} and ALDH^{lo} sub-populations to determine whether the anti-oxidant and regenerative capacity of ALDH^{hi} cells are enhanced compared to ALDH^{lo} and unsorted preplate populations. The third aim was designed to characterize a human muscle derived cell population isolated by their enhanced ALDH activity in terms of stress resistance in vitro and regeneration capacity in vivo.

1.5.1 Specific Aim 1: To elucidate the role of survival in myogenic differentiation capacity in vitro and in muscle regeneration capacity in vivo

Hypothesis: While myogenic capacity is a prerequisite for effective cell therapy, enhanced cell survival in oxidative and inflammatory stress is the determinant of regeneration capacity. As such, pretreatment of myogenic cells with an ROS scavenger, XJB-5-131, will improve post-transplantation survival and muscle regeneration capacity. We have demonstrated that MDSCs are more resistant to oxidative and inflammatory stress relative to more differentiated muscle cells, including myoblasts, which we believe contributes to the enhanced capacity of MDSCs to

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regenerate injured musculoskeletal tissues. However, the reduced ability of early preplate (EP) myoblasts to survive and differentiate in vitro and in vivo may be due to their increased sensitivity to stress. We will measure the survival, proliferation and differentiation potential of early preplate cells after exposure to inflammatory cytokines (TNF-α) and hydrogen peroxide (H_2O_2) in differentiation media. These experiments are designed to reveal if the decrease capacity of myoblasts to differentiate is due to decreased resistance to stress. While improved anti-oxidant defense appears to improve post-transplantation survival in MDSCs, we will determine if the myoblast regeneration capacity can be improved simply by improving its anti-oxidant capacity. XJB-5-131 is a membrane permeable, mitochondrial targeted ROS scavenger with low toxicity and will be used to increase the anti-oxidant levels of both myoblasts and MDSCs.

1.5.2   Specific Aim 2: To determine the muscle regeneration capacity of a sub-population of murine preplate cells with elevated stress resistance

Hypothesis: While the preplate technique has been shown to isolate murine myoblasts and muscle derived stem cells, the muscle regeneration capacity of these cells may be enhanced by isolating sub-fractions with elevated ALDH activity, a marker of early progenitor status. ALDH is a modulator of the embryonic morphogen retinoic acid and is highly expressed in several tissue specific progenitors and adult stem cells. ALDH^{hi} and ALDH^{lo} sub-populations of muscle derived cells were isolated in order to compare their proliferation, differentiation, and regeneration capacities in conditions of inflammatory and oxidative stress.

1.5.3 Specific Aim 3: To determine the stress resistance and myogenic capacity of cultured human muscle derived cells with elevated aldehyde dehydrogenase (ALDH) activity

Hypothesis: Human muscle derived cells with elevated ALDH activity (ALDH^{hi}) will demonstrate enhanced stress resistance and regeneration capacity when compared to ALDH^{lo} and unsorted muscle derived cells. Vauchez et al. recently detected the presence of endomysial cells with elevated ALDH activity, which were isolated and shown to undergo myogenic differentiation in vitro and in vivo. We will isolate ALDH^{hi} subpopulation of cultured human muscle derived cells (hMDCs) and determine their stress resistance and differentiation capacity in vitro and in vivo.

1.5.4 Tissue specimen acquisition and animal use

1.5.4.1 Human Subjects

Tissue from RIA procedures were obtained through the honest broker system and University of Pittsburgh IRB #PRO0305079. Skeletal muscle was obtained from surgical tissue specimens from orthopedic procedures that would otherwise be discarded. The excised tissue was placed in phosphate buffered saline solution and transferred to the host broker who de-identified and transported it to the Bridgeside Point II Research Building, where it was given to investigators at the Stem Cell Research Center.

1.5.4.2 Vertebrate Animals

The use of animals required for these studies was approved by the IACUC of University of Pittsburgh prior to the experiments. All animals were cared for by the Division of Laboratory

Animal Resources at the University of Pittsburgh Biomedical Science Tower, S 1040. The University of Pittsburgh adheres to the NIH Guide for Care and Use of Laboratory Animals. Institutional guidelines for the use and care of animals in research are assured by the Animal Welfare Assurances of the University of Pittsburgh. The investigators adhere to the instructions described in the Guide for the Care and Use of Laboratory Animals (NIH Publications 85-23) as promulgated by the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Sciences, the National Academy of Sciences, and the National Research Council. All of the animals were euthanized by way of compressed CO_2 (70%) gas until unconscious than CO_2 were increased to 100% until death, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

2.0   SPECIFIC AIM 1: ROLE OF CELL SURVIVAL IN MYOGENIC CAPACITY

2.1   BACKGROUND

2.1.1   Myogenic cell transplantation is limited by poor cell survival

The rationale for myogenic cell transplantation is to augment or recapitulate the normal mechanism of skeletal muscle regeneration by transplanting myogenic progenitors into the injured muscle. Cells with myogenic potential have been isolated from skeletal muscle[42,65,66], bone marrow[62-64], circulating blood[67,68], and blood vessels[69-71]. Myogenic progenitors isolated from skeletal muscle appear to be physically associated or share a common lineage with blood vessels[72-76]. While their contribution to normal muscle maintenance and regeneration appears to be quite small[211], when expanded exogenously and transplanted they have been shown to promote regeneration either by regeneration of muscle fibers or paracrine mediated repair processes. However, limitations of myogenic cell transplantation remain including: rapid cell death, limited cell migration, and immune rejection[9-32].

Myogenic cell transplantation therapies are limited primarily by high mortality rate (up to 99%) of the injected cells within the first few days of transplantation[9,16,47,200], which is of the same timeframe as rapid activation and infiltration of polymorphonuclear (PMN) cells. The degranulation of PMNs and other inflammatory leukocytes creates a local environment of

inflammatory and oxidative stress that is deleterious to transplanted cell survival[18,41,47,162,207,212-217]. Qu et al. observed increased concentrations of pro-inflammatory cytokines such as IL-1α, IL-1β, and TNF-α at the site of myoblast injection[41]. Ischemia and hypoxia of the implanted cells is another likely source of cell death that has been shown to induce intracellular oxidative stress[203, 218].

The process of inflammation yields a condition of oxidative stress in affected tissue[114]. This occurs by both the release of oxidizing species by inflammatory cells such as neutrophils and macrophages and by receptor mediated cytokine signaling that modifies the intracellular redox environment. Following a muscle injury an increased accumulation of neutrophils may be observed at the injury site within 1hr and remain for up to 5d[44]. The role of neutrophils appears to be primarily cytotoxic and cytolytic to prepare the injured site for debris phagocytosis. This is immediately followed by the accumulation of macrophages in their type I, or pro-inflammatory state, which by their secretion of numerous cytokines, growth factors, and oxidizing species, promotes satellite cell activation[219]. During the oxidative burst, neutrophils generate ROSs by reducing O_2 to superoxide anion via NADPH oxidase 2 (NOX2), which is converted to hydrogen peroxide by SOD and may be subsequently converted by myeloperoxidase to far more reactive hypochlorous and hypobromous acids[143,144]. Similarly, macrophages may induce cell-lysis though production of nitric oxide via nitric oxide synthase (NOS2 or iNOS)[115,145]. These species may cause oxidative damage via protein oxidation and lipid peroxidation thus modifying protein and lipid structural, chemical, or signaling functions[57,116-119].

The role of hydrogen peroxide as an effector, mediator, and second messenger in the process of inflammation is the subject of much ongoing research. It not only acts as a cytotoxic reactive oxidizing species, but can also potentiate or amplify the TNF-α activation of NF-kB[220].

It also is generated intracellularly by mitochondria following TNF-α binding to TNF-R1[221,222]. In vivo measurements of hydrogen peroxide during inflammation (5-15μM)[223] are lower than that needed to disturb proliferation and differentiation in vitro (25-500μM)[52,224]. However, it should be kept in mind that cells in vivo are exposed to H_2O_2 in concert with other oxidizing species and pro-inflammatory cytokines. Furthermore the serum rich media in which hydrogen peroxide is diluted will reduce much of the H_2O_2 via serum protein and lipid peroxidation. Therefore elevated concentrations of H_2O_2 were required to measurably perturb cell behavior such as proliferation and myogenic differentiation.

2.1.2   Increased stress resistance is associated with enhanced regeneration capacity

Oxidizing species such as ROS are linked not only to transplanted cell death but also altered signaling through MAPK, NF-kB, and Forkhead box O (FOXO) transcription factors, which can affect phenotype and cell activity such as proliferation, differentiation, and apoptosis[118,155,225,226]. The cell maintains a complex system of enzymatic and non-enzymatic mechanisms in order to regulate this redox state, including GSH, glutathione peroxidase (GPX), glutathione reductase (GR), catalase, SOD, and others which are depicted in Figure 2[120]. The primary scavenger of oxidants in the cell is GSH, a cysteine containing tripeptide whose thiol group may donate its electron and form a disulfide with another GSH molecule (GSSG). Intracellular GSH concentrations are in the millimolar range, orders of magnitude greater than any other cellular anti-oxidant. In fact, a simple measure of the cell's redox state is the ratio of GSH to GSSG concentration.
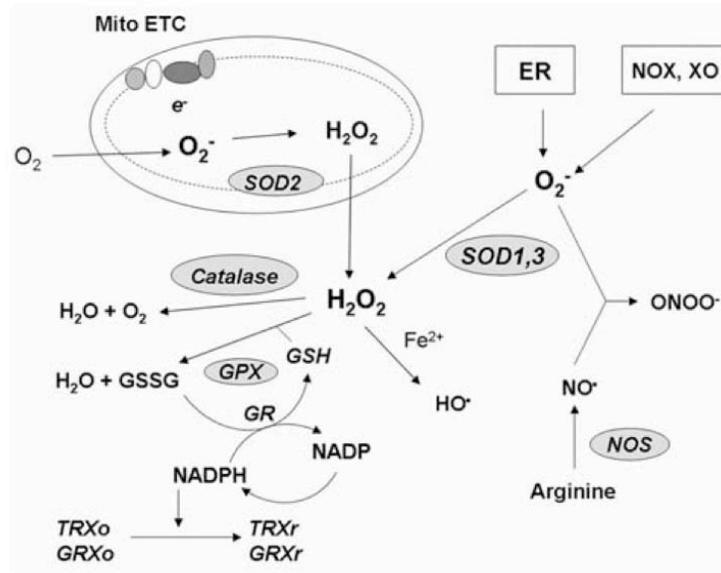
Figure 2. Schematic of major intracellular antioxidants

The maintenance of stem cell reserve is critical to repair damaged tissue or replace senescent cells over the course of our lifetime, especially in those tissues such as skeletal muscle that are exposed to frequent cycles of stress and damage requiring nearly constant repair and regeneration. It follows that these tissue specific stem cells should have a mechanism to protect themselves from cycles of inflammatory and oxidative stress. Oxidative stress is defined as the injurious effects such as lipid, protein, or DNA oxidation of oxidizing molecules that exceeds the cell's ability to neutralize or eliminate them. Whereas inflammatory stress is the exposure to or generation of pro-inflammatory cytokines that may activate the various signaling pathways of NF-kB as well alter the redox status of the cell. Protection from these stresses may be extracellular, in the form an isolated or immune privileged niche or intracellular in the form of enhanced mechanisms to reduce or eliminate intracellular oxidizing species. Some studies have also shown increased expression of anti-oxidant enzymes in stem and progenitor cells[154,227,228]. In our studies of the MDSCs, we have found increased levels of SOD and GSH over that of the

myoblast, conferring a constitutive readiness to mitigate oxidative stress. This, we believe, is a critical aspect of increased regeneration capacity in cardiac and skeletal muscle of MDSCs[52].

Urish et al. observed that MDSCs have a survival and differentiation advantage over myoblasts when exposed to oxidative (H_2O_2) and inflammatory stress (TNF-α) in vitro[52]. In the same paper, co-authors observed that increased stress tolerance correlated with increased intracellular concentrations of the anti-oxidants GSH and SOD and with decreased levels of intracellular reactive oxygen species. To assess the impact of this increased antioxidant capacity on the in vivo regeneration capacity, GSH levels of the MDSC were depleted to that of myoblasts using diethyl maleate (DEM) prior to skeletal muscle transplantation[229]. Indeed, the regeneration index (RI, defined as the maximum number of dystrophin positive myofibers per 100,000 of injected cells in a single cryosection) of the DEM treated MDSCs in skeletal muscle was significantly less than untreated MDSCs but was not significantly greater than the myoblast group[52]. In recent work by Drowley et al., MDSCs treated with n-acetylcysteine, a GSH precursor substrate and anti-oxidant, prior to myocardial transplantation induced greater functional recovery following coronary infarction than untreated MDSCs[130]. Although this increased functional recovery is thought to be due to paracrine action and promotion of angiogenesis rather than by cardiomyocyte differentiation of MDSCs, the improved functional outcome suggests improved survival and subsequent action of the transplanted MDSCs.

2.1.3   Targeting mitochondrial reactive oxygen species to improve myogenic cells survival

While inflammatory events yield extracellular ROS, oxidative damage of the transplanted cells as well as cytokine mediated signaling also alter the intracellular redox status. During exposure to oxidative stress and hypoxia, reactive oxygen species are generated within the cell, primarily

by mitochondria, which can affect pathways of differentiation, proliferation, quiescence, senescence, and apoptosis[230]. The mitochondrion is particularly suited to generate ROS as it is constitutively shuttling electrons from molecular oxygen through the complexes of the electron transport chain. During this process, electrons may leak from complex III and complex I to produce superoxide radicals, which may peroxidize mitochondrial phospholipids such as cardiolipin[231,232]. This may in turn release cytochrome C and allow mitochondrial transition pores to form, both of which may induce apoptosis[233-235].

XJB-5-131 (XJB) is a mitochondrial targeted anti-oxidant with two moieties: a ROS scavenging payload and a mitochondrial targeting fragment of the antibiotic gramicidin S[236,237]. The ROS scavenging is performed by a stable nitroxide radical, 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL). Upon accepting an electron the nitroxide is converted to a hydroxylamine, which can also accept an electron to be converted to another nitroxide, undergoing a form of redox recycling[238,239]. XJB has proven to be cytoprotective in disease states associated with oxidative stress, such as hemorrhagic shock, and mitigates apoptotic cell death in vitro[240,241]. Its non-toxicity and ROS scavenging activity makes XJB an excellent candidate to study the role of intracellular oxidative stress in transplanted cell death.

2.2   RATIONALE

In the pursuit of cell-mediated therapies, primitive progenitor or stem cells are sought on the premise that differentiation into various lineages native to the targeted tissue or organ is necessary to promote regeneration. However, our experience with myoblasts and MDSCs suggests that tissue specific differentiation potential is not sufficient. In fact, it may not even be

necessary. For example, in the case of MDSC transplantation following cardiac injury, MDSCs do not differentiate into cardiomyocytes however improved rates of angiogenesis and myocardial function are observed compared to myoblast transplantation[43,242]. Clearly MDSCs can play a role in myocardial regeneration, however this indirect role suggests that multipotency is not the determinant of regeneration. Perhaps it is the case that myoblasts could also promote similar levels of regeneration if they had similar post-transplantation survival rates as the MDSCs.

Furthermore we demonstrated that when the stress defenses of MDSCs were impaired by treatment with DEM their capacity to improve post-infarction cardiac function was lost[52]. These two results suggest that *the improved regenerative capacity of the MDSC may be conferred by its improved post-injection survival rather than its multipotency*. We have shown previously that the increased regeneration index of MDSCs over that of myoblasts in skeletal muscle is accompanied by increased initial survival at 24hrs post-injection[42]. In this specific aim, we seek to determine whether the increased myogenic differentiation capacity is a result of its increased stress tolerance. This has important implications for how we interpret in vitro differentiation capacity assays such as the myogenic differentiation index (MDI) or fusion index metric in vitro and regeneration capacity in vivo. That is, the differentiation assay results may be confounded by different rates of cell survival in media conditions used to induce myogenic differentiation. We hypothesize that *myoblasts are not in fact less myogenic, in the strict sense of myogenic differentiation potential, than MDSCs but are less capable of surviving in stress conditions relevant to the inflammatory and oxidative stress of the transplantation process*.

In vivo studies of murine MDSCs and early preplate cells (myoblasts) have shown a correlation between stress resistance and skeletal regeneration capacity[52]. The argument for the link between regeneration capacity and stress resistance was further strengthened by the use of

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diethyl maleate (DEM) in reducing the GSH levels of MDSCs, which effectively reduced the muscle regeneration capacity of MDSCs to that of myoblasts. However, in the current study no corresponding anti-oxidant gain of function studies were performed in skeletal muscle. N-acetylcysteine (NAC), a cysteine containing antioxidant and GSH precursor, however was recently used to pretreat MDSCs transplanted in infarcted myocardium and shown to improve contractile function however no improvement in engraftment was observed[130]. I proposed the use of XJB, a powerful anti-oxidant with high membrane permeability and low toxicity, to perform a corresponding gain of anti-oxidant function study in skeletal muscle.

2.3   EXPERIMENTAL DESIGN

Myogenic differentiation capacity was quantified in vitro by growing cells to 2D confluence in a flat bottom flask and exposing the colony to low serum media (2% horse serum in DMEM). After 4d cells were fixed and were characterized with immunohistochemical markers of myogenic differentiation such as myosin heavy chain. Previous reports suggest that MDSCs demonstrate enhanced differentiation capacity in these serum starved conditions[52]. We will determine if this differentiation assay may cause the loss of differentiation competent cells thus artificially deflating the MDI of myoblast populations (early preplate cells). Equally important however is the differentiation capacity of these cells in stress conditions like those seen in tissue following transplantation, such as oxidative and inflammatory stress. Stress resistance of MDSCs and myoblasts in myogenic differentiation conditions were investigated in terms of survival, proliferation, and myogenic differentiation. Stress conditions were modeled in vitro using hydrogen peroxide and TNF-α. Proliferation and survival of myoblasts and MDSCs plated in a

24well plates were quantified using a live-cell imaging microscope[243] in differentiation media. Well allocation and experimental flow is represented Figure 3 below.

Stress resistance of MDSCs and myoblasts when treated with the ROS scavenger XJB was also assessed in vitro. Myogenic differentiation and survival capacity was quantified in conditions of oxidative (H_2O_2) stress conditions as indicated the schematic illustrated in Figure 3 below.
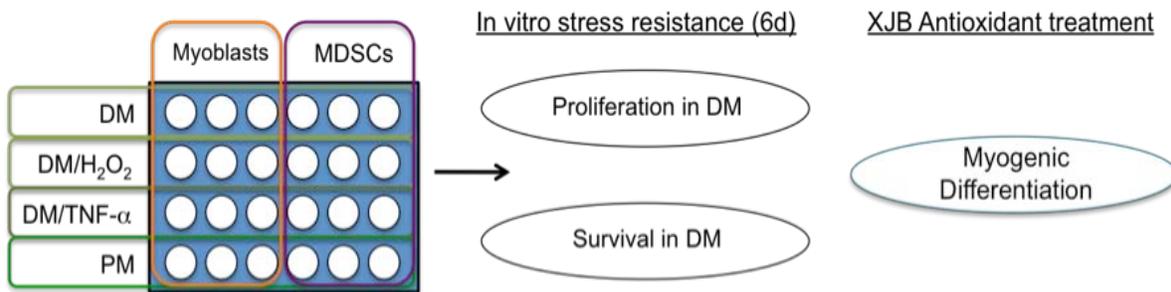
Figure 3. Experimental schematic of MDSC and myoblast survival during myogenic differentiation in vitro Proliferation rate and survival was quantified in conditions of low serum media (DM) and oxidative and inflammatory stress. Myogenic differentiation under these conditions was quantified using the myogenic differentiation index (MDI) metric. Myogenic differentiation and cell survival in conditions of oxidative and inflammatory stress was quantified in MDSCs pre-treated with the XJB anti-oxidant.

The effect of XJB on the regenerative index MDSCs and myoblasts in the skeletal muscle was assessed in the *mdx* mouse (female. Cells were expanded in vitro, trypsinized, and washed in cold PBS (4°C). MDSCs and myoblasts (2×10^5 in 20μL of cold PBS) were injected intramuscularly in to the belly of the gastrocnemius muscle as described previously and shown in the schematic in Figure 4 below. At 14 days the muscle was harvested and cryosectioned for immunohistochemical analysis.
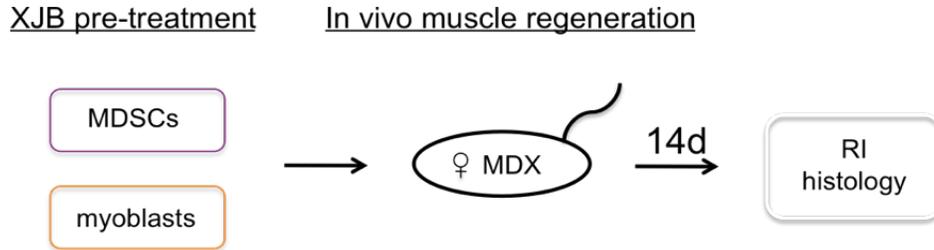
Figure 4. Experimental schematic to examine the regenerative capacity of anti-oxidant (XJB) treated muscle derived cells

MDSCs and myoblasts (2 x 10^5 in 20μL of cold PBS) were injected intramuscularly in to the belly of the gastrocnemius muscle. At 14 days the muscle was harvested and cryosectioned for immunohistochemical analysis.

2.4   METHODS

2.4.1   Murine MDSC and myoblast isolation via the preplate technique

Murine myoblasts and MDSCs were isolated from the skeletal muscle of 3-wk-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) as previously described using a modified preplate technique[42,79]. Muscle extracted from hindlimbs were minced into a slurry and enzymatically dissociated at 37°C in 0.2% collagenase-type XI (Sigma-Aldrich) for 1hr. The cells were then incubated in dispase (2.4units/ml HBSS, GIBCO, Invitrogen) for 45min then for 30min in trypsin-EDTA (0.1% in HBSS, GIBCO, Invitrogen). The dissociated cells were filtered, centrifuged and resuspended in proliferation medium (PM) to initiate the preplate process. PM consists of 10% horse serum, 10% FBS, 0.5% chick embryo extract, and 1% penicillin–streptomycin (all reagents from GIBCO).

The preplate technique segregates muscle derived cell populations by how quickly they adhere to collagen-coated plates as described elsewhere[41,79]. Briefly, cells are plated on collagen coated plates for a predetermined amount of time (PP1: 2hrs, PP2-PP6: 24hrs) at which point

non-adherent cells are collected, centrifuged and replated into the next preplate cycle. Each preplate population has been previously characterized. The PP1 population is populated predominantly with fibroblasts, whereas each subsequent preplate cycle is enriched with myogenic, desmin-positive cells [27,244,245]. MDSCs are isolated from long-term proliferating colonies of PP6.

Myoblasts were isolated from rapidly adhering cell fractions of the early preplate (EP) cycles (PP2-PP4). "EPs" is an abbreviation for myoblasts isolated in this fashion. MDSCs were isolated from the slowly adhering cell fraction of the late preplate cells (PP5-PP6). All cells were cultured and expanded in PM as described previously[79].

2.4.2   Survival and proliferation of myoblasts and MDSCs in DM

Time-lapse live cell microscopy was employed to monitor the rate of proliferation and survival in differentiation media (DM, 2% horse serum in DMEM) under conditions of oxidative (H_2O_2, 250μM) and inflammatory stress (TNF-α, 2.5ng/ml)[246,247]. Cells were plated in collagen-coated 24-well plates at an initial density of 1000 cells/cm^2 in proliferation medium for 6hrs or until adherence to the collagen coated flask bottom. Each experimental group was represented in triplicate (three wells) and three areas were imaged in each well (n=9). 30min prior to placing the plate on live cell imaging incubator, the proliferation medium was exchanged with differentiation media under conditions of oxidative (250μM, H_2O_2) and inflammatory stress (2.5ng/ml, TNF-α), except in the case of one row of un treated PM which will serve as a control for proliferation and survival. All wells were treated with 10μg/ml of propidium iodide whose intracellular accumulation, DNA intercalation, and fluorescence indicates cell death.

Proliferation was quantified by counting cells manually and survival was quantified by excluding cells that exhibit propidium iodide fluorescence in time-lapsed images as described previously (30min intervals in three 10x microscope fields over 48 hr)[52].

2.4.3   Myogenic differentiation assays

The 24well plates were transferred to a (non-imaging) incubator for an additional 48hrs to allow for myogenic differentiation and fusion. The cells were fixed in cold methanol (-20°C) and stained for myogenic differentiation. Differentiation of myogenic cells into myosin heavy chain (fast) expressing myotubes were quantified using the MDI metric described previously. Briefly, samples were blocked with 5% horse serum and incubated with monoclonal antibodies for MHC (Sigma; 1:300) followed by Cy3-conjugated anti-mouse antibodies. Nuclei were stained blue by DAPI.   At least 200 cell nuclei were counted for each myogenic differentiation index measurement.

2.4.4   In vitro proliferation capacity

Time-lapse live cell microscopy was used to monitor the rate of proliferation of XJB treated cells under conditions of oxidative and inflammatory stress[246,247]. Cells were plated in collagen-coated 24well plates at an initial density of 1000 cells/cm^2 in XJB treated (500nM) proliferation medium and incubated under normal conditions for 24hrs.   Stress conditions of oxidative (250µM, H_2O_2) and inflammatory stress (2.5ng/ml, TNF-α) in proliferation media were introduced to each well 30min prior to placing the plate on live cell imaging incubator.

Proliferation was quantified at 15min intervals in three 10x microscope fields over a 48 hr time period.

2.4.5   In vitro myogenic differentiation capacity

Cells were plated at 1000 cells/cm^2 in 24-well plates for 2d in PM or until near confluence. During the last 24hrs of incubation PM was exchanged with XJB treated (500nM) PM for the antioxidant pretreatment prior to stress exposure. XJB treated PM was then exchanged with differentiation media treated to simulate oxidative stress (25μM, 100μM, 250μM, and 500μM, H_2O_2) and inflammatory stress (1ng/ml, 2.5ng/ml, and 5ng/ml, TNF-α). These media conditions were renewed daily in a similar protocol to previous experiments[52]. After 4d the cells were fixed in cold methanol (-20°C), and the MDI were quantified as described previously. Briefly, samples were blocked with 5% HS and incubated with monoclonal antibodies for MHC followed by Cy3-conjugated anti-mouse antibodies. Nuclei were stained blue by 4, 6-diamidino-2-phenylindole (DAPI). The myogenic differentiation index (MDI) was quantified by dividing the number of nuclei in MHC positive cells by the total number of nuclei. At least 200 cell nuclei were counted for each MDI measurement.

2.4.6   Skeletal muscle regeneration capacity

XJB treated MDSCs and myoblasts were injected into the gastrocnemius muscles of female *mdx* mice(C57BL/10ScSn-DMD^{mdx}/J, The Jackson Laboratory), aged 6–8 weeks (experimental groups include untreated, XJB-treated, NAC-treated, and PBS controls). The mdx mouse is a model of Duchenne muscular dystrophy, derived from a C57BL strain homozygous for a

spontaneous X-linked mutation of the dystrophin gene and lacks dystrophin protein expression[248]. Cells cultured in collagen coated flasks were trypsinized, counted, washed in cold phosphate buffered saline (PBS), and resuspended in a cold PBS suspension of FluoSpheres, 2 x 10^6 cells in 20ml of PBS, prior to injection. At 14d post-injection, the gastrocnemius muscles were excised and frozen in 2-methyl-butane, cryosectioned (8μm sample thickness), and mounted on glass slides. Cryosectioned muscles were fixed in 5% formalin for 5 min and blocked in 5% horse serum in phosphate-buffered saline (PBS) for 1 h at room temperature (22°C). Skeletal muscle sections were stained for dystrophin (1:300, Dys, Abcam) using protocols previously described in order quantify the regeneration index of the injection muscle[249]. Fluorescence and bright field microscopy were performed using Nikon Eclipse E800 microscope equipped with a Retiga Exi digital camera (QImaging). The animal research protocol was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and University of Pittsburgh (0902596).

2.4.7 Statistical Analysis

Data are expressed as a mean with its standard deviation. Direct comparisons between two cell populations were made using an unpaired, two-tailed Student's *t* test, where statistical significance was determined at a level $p < 0.05$. Comparisons of multiple groups were completed using one-way ANOVA followed by *Tukey* post-hoc comparisons.

2.5   RESULTS

2.5.1   Proliferation and survival of myoblasts and MDSCs in differentiation media

MDSCs exhibit increased oxidative and inflammatory stress resistance in vitro compared to myoblasts as illustrated in Figure 5 below. Significant differences in normalized cell number were observed in MDSCs as a function of time over that of myoblasts in conditions of oxidative (H_2O_2, 250μM) and inflammatory stress (TNF-α, 2.5ng/ml).   This result confirms that the MDSC and myoblast populations exhibited differences in oxidative and inflammatory stress resistance[52]. We then proceeded to study if similar differences in proliferative capacity would be observed in low serum differentiation media conditions.
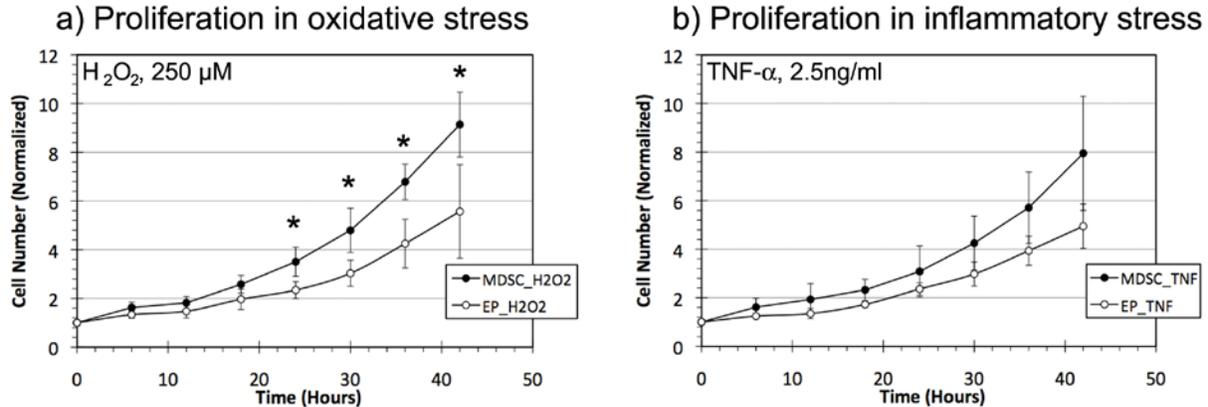
Figure 5. Increased rate of proliferation of MDSCs in oxidative and inflammatory stress

(a) An increased rate of proliferation of MDSCs was observed when cultured in proliferation media (PM) treated with H_2O_2 (250μM) compared to early preplate myoblasts (EPs). (* indicates p<0.05) (b)   A trend of increased proliferation rate of MDSCs in proliferation media treaded with TNF-α (2.5ng/ml) was observed however this difference was not statistically significant.

Proliferation rates of myoblasts and MDSCs in low serum (DM) and oxidative stress conditions were measured using time-lapse microscopy. While no significant difference in MDSC proliferation was observed when grown in DM compared to PM, significant differences in proliferation were observed in myoblasts when grown in DM compared to PM as illustrated in Figure 6a and b. It should be noted that no difference in MDSC and myoblast proliferation was observed when these two cell types were cultured in normal PM conditions, however significant differences in proliferative capacity when they were cultured in low serum DM conditions. Figure 6c and d illustrates that the increased proliferation rate of MDSCs in DM compared to myoblasts.
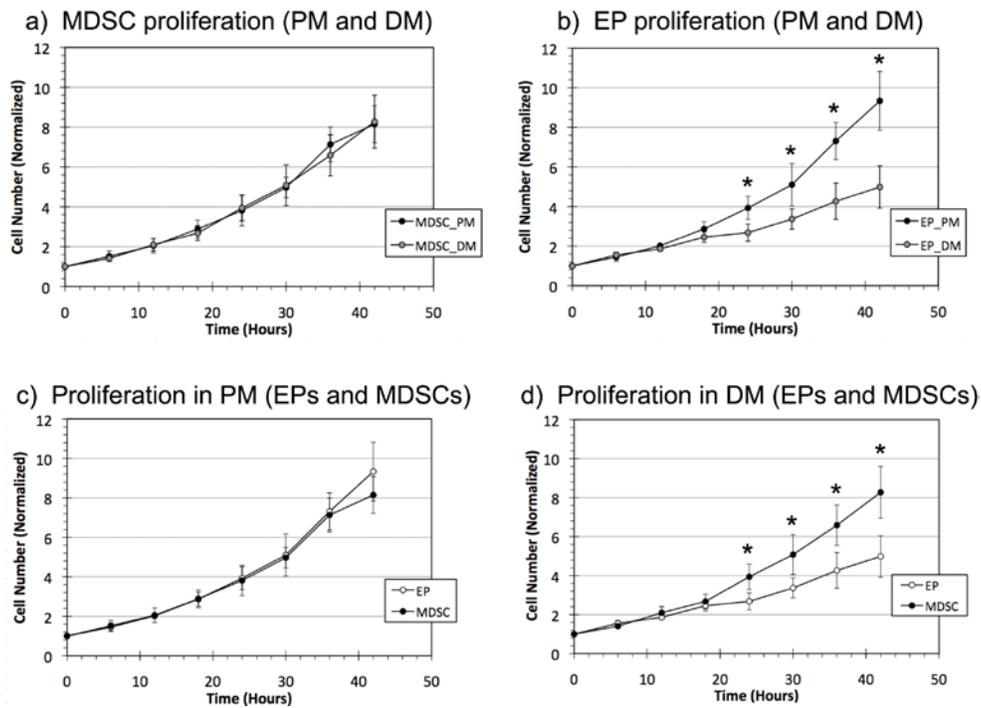
Figure 6. Loss of proliferation capacity in myoblasts in low serum and stress conditions
(a) No decrease in MDSC proliferation rate was observed in the first 48hrs of low serum (2% horse serum) differentiation media (DM) exposure compared to MDSCs grown in serum rich (10% fetal bovine serum + 10% horse serum) proliferation media (PM). (b) However a significant decrease in proliferation rate was observed in early preplate myoblasts (EPs) in DM compared to myoblasts grown in PM. (* indicates $p < 0.05$) (c,d) While no significant difference in myoblast or MDSC proliferation was observed in PM conditions, a significant difference in proliferation rate in DM was observed.

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The differences in proliferation capacity of myoblasts and MDSCs are a combination of differential survival and proliferation in low serum conditions. To better understand the role of survival in this case, cell viability of myoblasts and MDSCs was quantified during DM exposure (2, 3, 4, and 6 days) by propidium iodide fluorescence detection using flow cytometry. Figure 7 illustrates an increase in MDSC viability compared to myoblasts in DM conditions suggesting that survival plays an important role in the difference in proliferation.
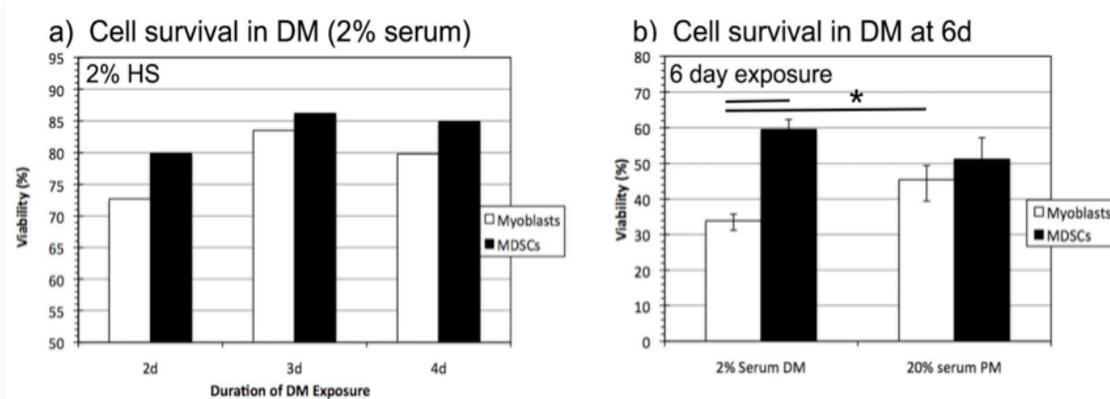
Figure 7. Survival of myoblasts and MDSCs in low serum media

To clarify the role of survival in the proliferation capacity of MDSCs compared to myoblasts the rate of survival at different timepoints (2-6d) in differentiation media was quantified. a) Cells were grown in 24 well plates for the 2, 3, and 4d were trypsinized and incubated for 10minutes in propidium iodide. Cell viability was quantified by flow cytometry. d) This experiment was repeated to a 6d timepoint in triplicate, significant decreases in myoblast was observed when cultured in DM for 6days compared to MDSCs in DM as well as myoblasts in PM.

An additional loss in proliferative capacity of myoblasts in DM was incurred by the addition of oxidative stress conditions (H_2O_2, 250µM), however not in inflammatory stress conditions (TNF-α, 2.5ng/ml). Figure 8 illustrates the loss in proliferative capacity of MDSCs and myoblasts under oxidative stress conditions. Proliferation of myoblasts was essentially arrested by the addition of H_2O_2. Figure 9 on the other hand suggests that TNF-α has no additional impact on the proliferative capacity of myoblasts in DM conditions.
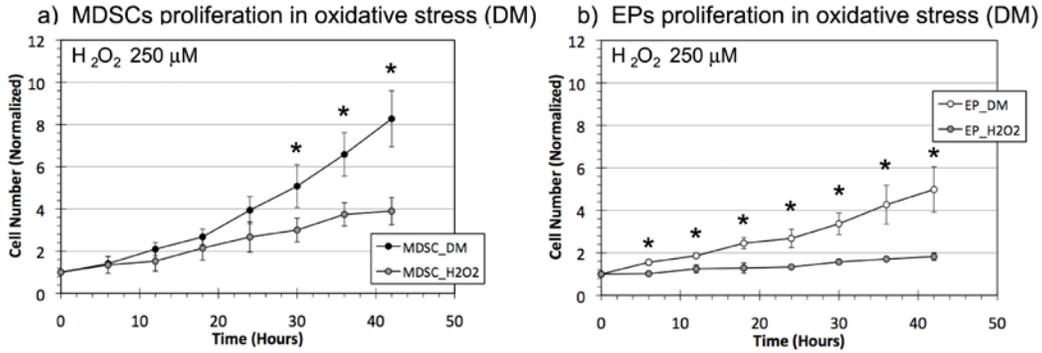
Figure 8. Additional loss in proliferative capacity of myoblasts in DM under oxidative stress conditions
(a-b) Oxidative stress challenge under DM conditions exacerbated the loss in proliferation rate under DM conditions for both MDSCs and myoblasts (EPs). (* indicates p<0.05)
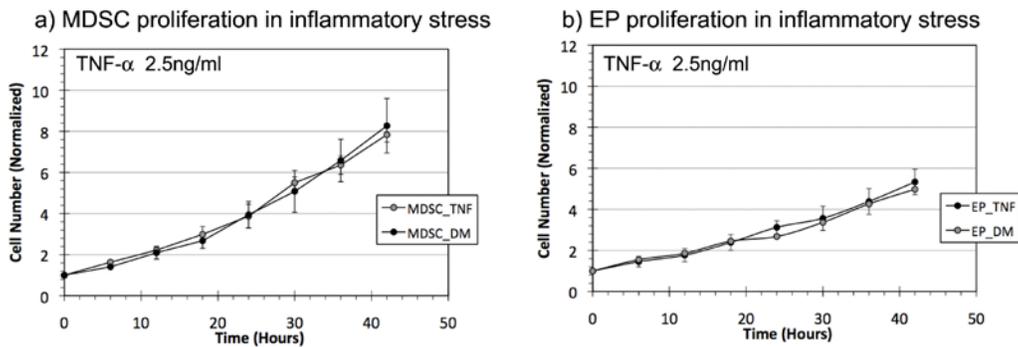
Figure 9. No additional loss in proliferative capacity of myoblasts in conditions of inflammatory stress
(a-b) In contrast to oxidative stress challenge under DM conditions, no additional loss in proliferative capacity was observed in inflammatory stress (TNF-α, 2.5 ng/ml).

2.5.2 Improved differentiation capacity of XJB treated cells in oxidative stress

We observed significant increases (p<0.05) in myogenic differentiation capacity of MDSCs in oxidative stress when the cells were pretreated XJB prior to hydrogen peroxide exposure. Figure 10 illustrates significant increases in differentiation capacity of MDSCs pretreated with 500nM XJB prior to being exposed to low serum DM and oxidative stress (H_2O_2) conditions.
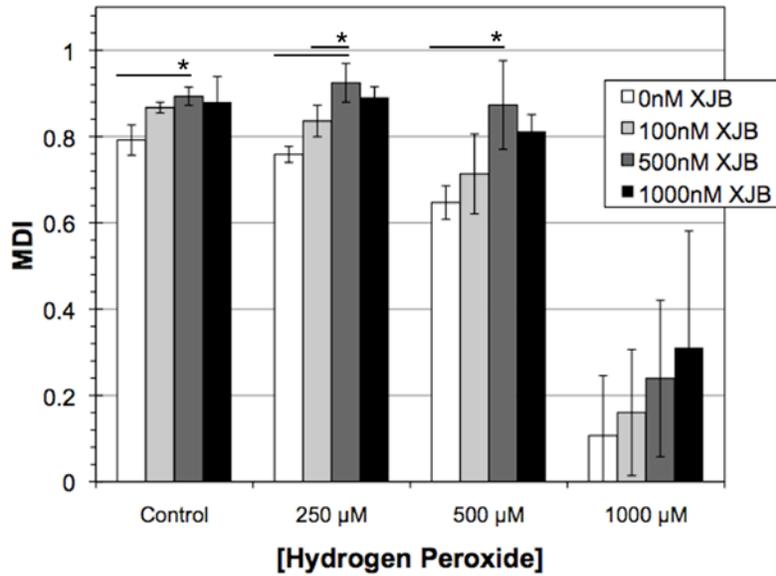
Figure 10. Improved myogenic differentiation capacity of MDSC pretreated with antioxidant XJB
Pre-treatment of MDSCs improved the myogenic differentiation efficiency under conditions of oxidative stress (H_2O_2). Improvements in differentiation were observed up to a concentration of 500nM, which served as the XJB pre-treatment concentration for the remainder of the experiments. (* indicates $p< 0.05$).

Increased myogenic capacity of XJB treated cells in low serum DM and oxidative stress conditions was found to correlate with MDSC survival as well, as illustrated in Figure 11 below. Experimental conditions in this case were nearly identical to those represented in Figure 10, however in addition cell survival in each case was quantified and normalized by cell numbers in MDSCs in DM conditions without XJB pre-treatment or oxidative stress challenge. A strong correlation of differentiation capacity with cell survival was observed (Figure 11d). That is, not only did fewer cells survive DM and oxidative stress conditions but differentiation capacity of those surviving was also impaired.
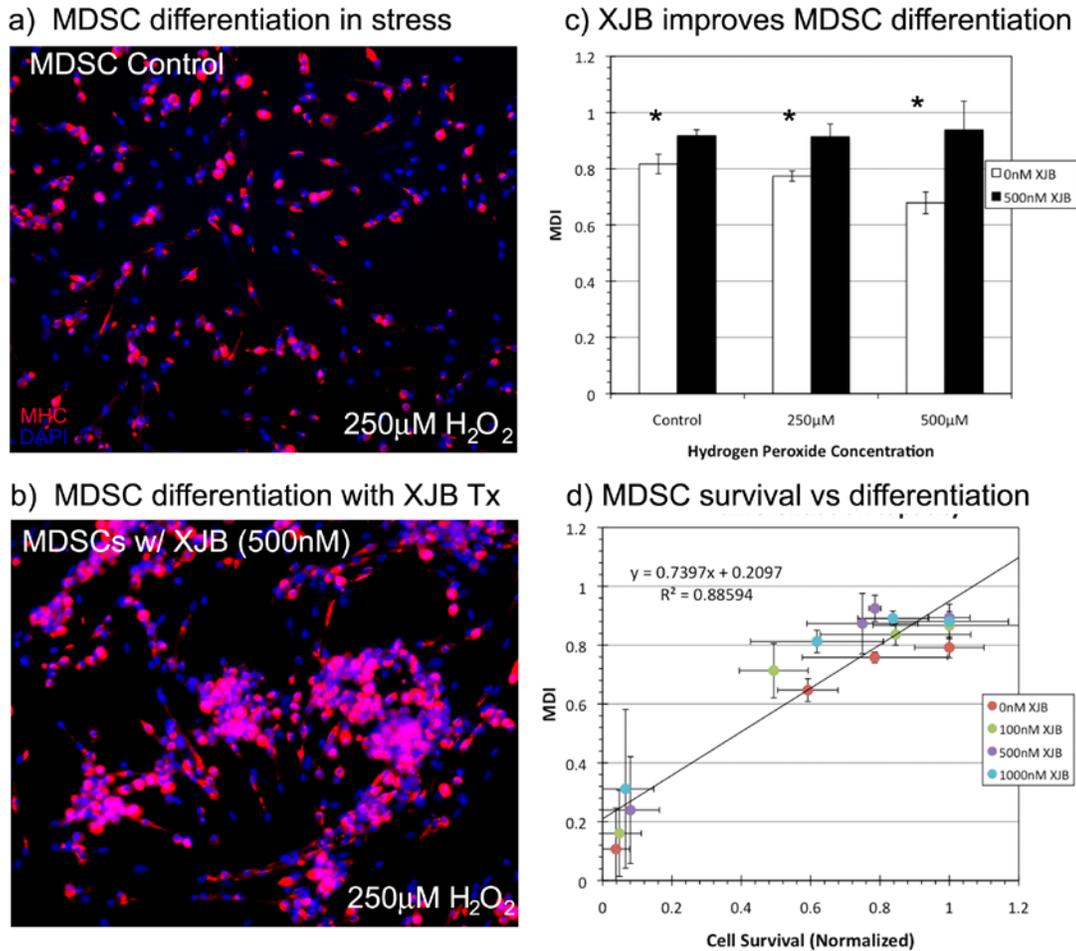
Figure 11. Correlation of myogenic differentiation of MDSCs with survival

(a-b) Immunohistology of myosin heavy chain (MHC-red) expressing MDSCs in differentiation media and oxidative stress (H_2O_2) conditions for 4d. Nuclei are stained blue via DAPI. Increased rates of MHC$^+$ cell differentiation was observed when MDSCs were pre-treated with XJB (500nM) prior to differentiation media conditions. (c) The rate of myogenic differentiation was quantified using the myogenic differentiation metric (MDI). XJB pre-treatment improves myogenic differentiation capacity of MDSCs in oxidative stress (250-500µM). (* indicates $p < 0.05$). (d) The differentiation capacity of MDSCs correlated positively with measurements of cell survival in DM conditions (r^2=0.88594).

2.5.3 Skeletal muscle regeneration capacity of XJB treated cells

MDSCs and myoblasts treated with XJB (500nM) prior to being injected in to the gastrocnemius muscle of female *mdx* mice demonstrated a trend of increased regeneration index over that of

untreated cells and DEM treated MDSCs. The skeletal muscle regeneration index is plotted in

Figure 12. Differences in the regeneration index however did not rise to the level of significance

in either cell type however the greatest mean improvement in RI was observed in myoblasts.

This improvement was just shy of the level of statistical significance (p=0.07).
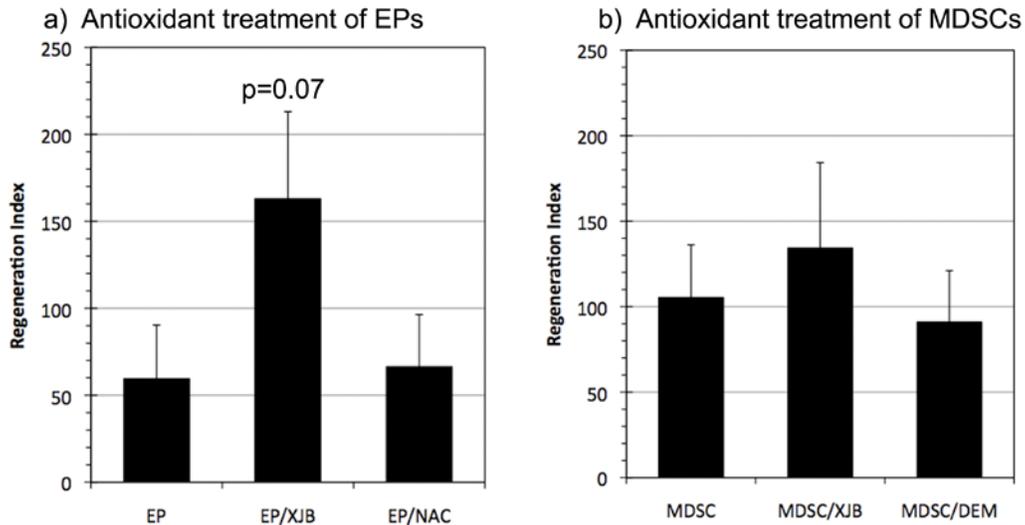
Figure 12. Skeletal muscle regenerative capacity of myoblasts and MSDCs with antioxidant pretreatment
(a) Early preplate myoblasts (EPs) pretreated with XJB anti-oxidant (500nM) prior to intramuscular injection into the *mdx* mouse gastrocnemius induced greater muscle regeneration than untreated and NAC (n-acetylcysteine, 10mM) pretreated myoblasts, although this increase was not found to be statistically significant. (b) A similar trend of increased skeletal muscle regeneration was observed in XJB pre-treated MDSCs. However, again, this increase was not statistically significant.

2.6   DISCUSSION

Myoblast differentiation is typically induced in medium with a low concentration of mitogens,

such as low serum or serum-free differentiation medium[250-252]. On exposure to low serum

conditions myoblasts rapidly and permanently withdraw from the cell cycle and initiate

myogenic differentiation and fusion programs. Wang and Walsh studied the behavior or C2C12

myoblasts under low serum conditions[253]. They observed that of those C2C12 cells that underwent cell cycle arrest, a large proportion of these cells proceeded to apoptose (20-30%) in low serum conditions. This process of cell death began at 24hrs and peaked at 48hrs[253]. Of those cells that successfully underwent differentiation and fusion these cells appeared to be quite apoptosis resistant, surviving up to 2 weeks in low serum conditions. This suggests that myoblasts undergoing differentiation proceed through a time window of vulnerability however once differentiated the cells resist apoptosis. Shen et al. confirmed this result by further mapping pro-apoptotic gene upregulation in low serum conditions[254]. This apoptosis resistant state at the time was correlated with the expression of cyclin dependent kinase (CDK) inhibitors p21 and p16, retinoblastoma (Rb) protein, a tumor suppressor with anti-proliferative activity, and Akt, a serine threonine kinase with anti-apoptotic properties[255-257]. However, apoptosis during myogenic differentiation may also be mitigated by suppression of MyoD one the master regulators of myogenic differentiation, which appears to upregulate anti-apoptotic genes, to repress pro-apoptotic genes and to improve myoblast survival[258].

Mammalian muscle differentiation proceeds though a sequential expression of muscle regulatory factors including Myf5, MyoD, myogenin, and Mrf4, which control expression of differentiation specific genes such as myosin heavy chain and creatine kinase[102,259]. Myogenin in particular appears to mark the transition from a proliferative myoblast to a fully differentiated and post-mitotic myocyte[260]. At this point expression of p21 and Rb appears to induce this loss of proliferative capacity and prevent cell cycle reentry[255,256]. However in the absence of p21 expression significantly increased rates of apoptosis were observed[253]. During primary myoblast differentiation, expression of S phase proteins such as the cyclins D1 and E, auroraB, survivin, anilin and Emi1/FBXO5 are swiftly downregulated indicating that differentiation induces a

decidedly post-mitotic state[261]. Furthermore these proteins were shown to be re-expressed indicating cell cycle reentry when the myocytes were forced to de-differentiate by suppressing Rb and ARF[261].

Studies by Walsh and Shen and others suggest that myoblasts in low serum conditions will either apoptose or exit the cell cycle to undergo differentiation and fusion, however the present study suggests that MDSCs do not reach such a dichotomous checkpoint in these conditions[253,254]. The present study demonstrated that MDSCs do not undergo a dramatic shift in their proliferative capacity when cultured for 48hrs in low serum media. This is in contrast to similar studies performed on C2C12 and L6 rat myoblasts. That their proliferative capacity in low serum conditions is significantly greater than early preplate myoblasts suggest they may not be subject to the same checkpoints that force myoblasts to exit the cell cycle. MDSCs are also able to withstand oxidative stress in theses low-serum conditions at greater rates than myoblasts in terms of their proliferation and myogenic differentiation capacity. The increased proliferative capacity of MDSCs compared to myoblasts in low serum conditions that also drive differentiation may confer their increased myogenic potential[42,52,79]. This increased oxidative stress resistance capacity of MDSCs in low-serum conditions compared to myoblasts further supports our earlier work that increased cell competence in conditions of oxidative stress encountered in vivo following intramuscular transplantation likely drives the increased muscle regenerative capacity of MDSCs[52].

Previously we observed increased stress resistance in MDSCs that was conferred by increased intracellular anti-oxidant levels such as reduced glutathione and super-oxide dismutase compared to myoblasts[52]. We proceeded to study whether increased stress resistance and muscle regeneration could be induced by treating MDSCs with an exogenous anti-oxidant such as n-

acetylcysteine or XJB[130]. In a previous study by Drowley et al., we observed an increase in regenerative capacity following NAC treatment of MDSCs and in the current study we observed non-significant trends of increased regenerative capacity in XJB treated MDSCs as well as myoblasts[130]. That the improvements in skeletal muscle regeneration appeared to be greater in myoblasts suggests that there may be diminishing returns in increasing anti-oxidant capacity. Perhaps once a certain anti-oxidant level is reached further intracellular reduction state is not protective but may even begin to affect the normal ROS signaling of the cell.

3.0 SPECIFIC AIM 2: TO DETERMINE THE MUSCLE REGENERATION CAPACITY OF A SUB-POPULATION OF MURINE PREPLATE CELLS WITH ELEVATED STRESS RESISTANCE

3.1 BACKGROUND

3.1.1 Muscle derived stem cell isolation via a modified preplate technique

Numerous techniques of myoblast isolation from skeletal muscle biopsies have been identified, however one of the simplest is the preplate technique[79,244,245]. The technique utilizes the observation that myoblasts do not adhere to collagen coated tissue culture flasks as rapidly as the numerous fibroblasts and endothelial cells that are found in whole muscle digests. Therefore less rapidly adhering myoblasts could be segregated by collecting non-adhered cells after a few hours of culture. Our group found that this technique could be extended to six days or more to isolate multiple fractions of myogenic cells including the muscle derived stem cell[42]. MDSCs have been shown to differentiate into musculoskeletal tissues such as bone, cartilage, and skeletal muscle, in addition to endothelial, neural and hematopoietic tissues[42,77,78,80,82-84]. Perhaps more important than MDSC multipotency, however is the increased rate of survival and engraftment in skeletal muscle when compared to myoblasts.

3.1.2 Elevated oxidative stress resistance of MDSCs

We have shown that muscle derived stem cells (MDSCs) induce greater skeletal muscle regeneration than myoblasts largely due to their increased oxidative stress resistance[41-43,52]. It is the oxidative and inflammatory environment that the cell population encounters following transplantation that causes a precipitous cell loss and is the greatest limitation in myogenic cell therapy[9,16,47,202]. These conditions can be partially overcome by injecting a myogenic cell population with increased oxidative stress resistance or anti-oxidant capacity, an attribute that we have characterized in muscle derived stem cells. When we decreased their GSH anti-oxidant levels using diethyl maleate, we observed a commensurate decrease in engraftment and skeletal muscle regeneration[52]. On the other hand when we increased the GSH levels of MDSCs using n-acetylcysteine we observed an increase in regeneration capacity[262]. We therefore hypothesized that muscle regenerative capacity may be due primarily to the transplanted cells capacity for oxidative and inflammatory stress resistance rather than tissue specific differentiation capacity. In fact, some groups have suggested that increased stress resistance may be a primary characteristic of stemness[154,263-265].

Numerous myogenic progenitors have been isolated for cell therapy such as myoblasts[245], muscle derived stem cells[42], side-population cells[65], Sk-DN/Sk-34 cells[66], and CD133^{+} progenitors[67,68]. Prospective isolation of perivascular cells such as pericytes[72], mesangioblasts[73], endothelial and myo-endothelial[74] cells based on surface markers such as CD144, CD146, CD56, and CD34 have also yielded myogenic differentiation in vitro and in vivo[69,74-76]. However we are currently focused on the isolation of stem cells based on their behavior and survival characteristics, specifically on elevated oxidative and inflammatory stress resistance[52]. It is this behavior that we believe may be the primary determinant of myogenic cell

therapy. In the current study, we sought to isolate myogenic progenitors with enhanced stress resistance by their elevated expression of aldehyde dehydrogenase, an intracellular marker increasingly being used to isolate primitive progenitors from multiple tissues.

3.1.3 Aldehyde dehydrogenase as a marker for early progenitor status and enhanced stress resistance

Recently elevated aldehyde dehydrogenase has been shown to be a marker of early progenitor status in hematopeitic[180,181], mesenchymal[182], endothelial[183], neural[184,185], and recently skeletal muscle[186] populations, as well as marker of cancer stem cells or metastasis competent tumor cells[152,266-269]. Many of these somatic and cancer stem cells have also be shown to have increased anti-oxidant capacity[79,226]. For example, hematopoietic stem cells may upregulate FoxO (Forkhead O) transcription factors, downstream targets of the PI3K-AKT pathway and regulators of the oxidative stress response, which include induction of MnSOD, catalase, and GADD45[190-194]. Similar properties of increased expression of free radical scavenging systems and decreased levels of reactive oxygen species is observed in epithelial breast cancer stem cells[195,266,270]. Endothelial progenitors isolated from umbilical cord blood have been shown to have elevated MnSOD levels that significantly increase their oxidative stress resistance and therefore in treating tissue ischemia[148,196].

In the current study, ALDH^{hi} and ALDH^{lo} subpopulations of skeletal muscle derived cells were isolated from cultured murine and human skeletal muscle by fluorescence activated cell sorting (FACS). The stress resistance and muscle regeneration capacity of these ALDH sorted subpopulations were examined in vitro and in vivo. We hypothesized that *increased ALDH activity may identify myogenic progenitors with increased oxidative and inflammatory stress*

resistance. As discussed previously, we believe this *increased stress resistance may confer an increased engraftment and muscle regeneration capacity over ALDH^{lo} and unsorted muscle derived populations.* Thus, ALDH may be used as a marker of myogenic cells of increased stress resistance capacity and therefore an excellent candidate for myogenic cell therapies.

3.2   RATIONALE

We sought to isolate myogenic progenitors with elevated anti-oxidant capacity or stress resistance based on a novel marker of early progenitor status: elevated aldehyde dehydrogenase. ALDH^{hi} and ALDH^{lo} populations were isolated from murine preplate cells: myoblasts and MSDCs. We hypothesized that increased ALDH activity may identify myogenic progenitors with increased stress resistance thus conferring an increased engraftment and muscle regeneration capacity in vivo over the ALDH^{lo} and unsorted preplate populations.

3.3   EXPERIMENTAL DESIGN

ALDH^{hi} and ALDH^{lo} sub-populations myoblasts and MDSCs were isolated using fluorescence activated cell sorting (FACS). The stress resistance of ALDH sorted cells were then characterized in terms of their proliferation and differentiation potential in conditions of oxidative (H_2O_2) and inflammatory stress (TNF-α). Their intracellular anti-oxidant levels in terms of glutathione and superoxide dismutase were also evaluated. The experimental schematic to study this in vitro stress resistance capacity is illustrated in Figure 13.
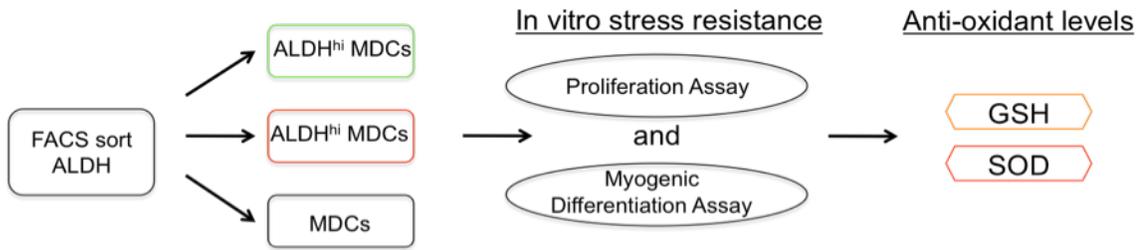
Figure 13. Experimental schematic to study stress resistance capacity of ALDH sorted murine preplate cells
ALDH^{lo} and ALDH^{hi} subpopulations of muscle derived cells (MDCs) were isolated by fluorescent activated cells sorting (FACS). Proliferative and myogenic differentiation capacity with were quantified in conditions of oxidative (H$_2$O$_2$) and inflammatory stress (TNF-α, not shown in schematic). Intracellular anti-oxidant levels, specifically reduced glutathione (GSH) and super-oxide dismutase (SOD) were assessed in each ALDH sorted subpopulation of MDCs.

The skeletal muscle regeneration capacity of the ALDH^{hi} and ALDH^{lo} subpopulations of murine preplate cells was evaluated in the gastrocnemius of an *mdx* mouse model. To conform to relevant studies of murine MDSCs, ALDH^{hi} and ALDH^{lo} subpopulations were transplanted into female *mdx* mice aged 6-8weeks for a period of 14d, as depicted in Figure 14 below[52].
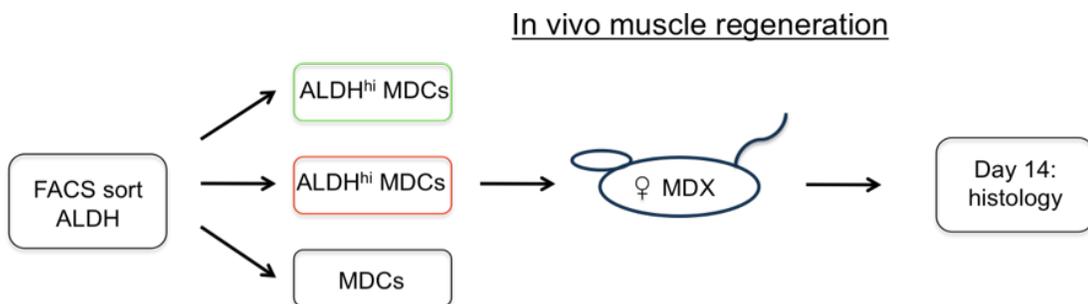
Figure 14. Experimental schematic to characterize the muscle regeneration capacity of ALDH sorted murine pre-plate cells
ALDH^{lo} and ALDH^{hi} cells were injected directly into the gastrocnemius muscles of female *mdx* mice aged 6-8 weeks. The regeneration index was quantified via histological evaluation of the rate of dystrophin expression of cryosectioned muscle 14 days post-injection.

3.4   METHODS

3.4.1   Murine myoblast and MDSC isolation via the preplate technique

Murine MDSCs and myoblasts are isolated from the skeletal muscle of 3-wk-old C57BL/6J mice as previously described using the modified preplate technique[42,79]. MDSCs were isolated from expansion of the sixth preplate (PP6) while early preplate myoblasts were isolated from preplates 2 through 4 (PP2-PP4). All cells were cultured and expanded in PM as described previously[79]. When applying the preplate technique to ALDH sorted cells (as shown in Figure 1h), the digestion process was identical to that described above; however, the digested muscle cells were sorted for ALDH activity using FACS prior to the performing the preplate protocol.

3.4.2   Flow cytometric analysis and sorting by ALDH activity

Cultured murine preplated cells were trypsinized, washed in cold PBS, and counted using a hemocytometer. Cells (10^6) of each population were resuspended in Aldefluor buffer, which contains an ABC transport inhibitor that prevents efflux of the aldefluor dye, and incubated at 37°C with BAAA according to the manufacturer's instructions (Aldagen Inc, Durham, NC). Cells were washed in Aldefluor buffer and maintained in 4°C throughout the cell sorting process. ALDH activity was assessed using the FL1 channel of a BD FACSAria Cell Sorting System and FACSDiva software (version 6.1.2) (Becton, Dickinson and Company, San Jose, CA). Collected cells were gated on their fluorescence intensity, which corresponds to their ALDH activity levels, as well as low side scatter (SCC^{lo}). Sorted cells were recaptured in cold (4°C)

proliferation media and immediately plated in collagen coated flasks and normal incubation conditions (5% CO_2 at 37°C).

3.4.3   In vitro proliferation capacity

Time-lapse live cell microscopy was employed to monitor the rate of proliferation under conditions of oxidative and inflammatory stress[246,247]. Sorted human muscle derived cells were plated in collagen-coated 24-well plates at an initial density of 1000 cells/cm^2 in PM and incubated under normal conditions for 24hrs. The media was exchanged with proliferation media treated to simulate oxidative (250µM, H_2O_2, Sigma-Aldrich) and inflammatory stress (2.5ng/ml, TNF-α, both mouse and human recombinant, R&D Systems) and immediately placed in live cell imaging incubator. Cells were imaged at 30min intervals in three 10x microscope fields per well over a 48 hr time period under normal incubation conditions (5% CO_2 at 37°C).

3.4.4   In vitro muscle differentiation capacity

The capacity of ALDH sorted cell populations to differentiate into MHC expressing cells under varying conditions of oxidative and inflammatory stress was quantified in vitro using a myogenic differentiation index (MDI) metric. Cells were plated at 1000 cells/cm^2 in 24-well plates for 2d in PM or until near confluence. PM was then exchanged for differentiation media (DM; 2% HS, 1% P/S in DMEM) treated to simulate oxidative stress (25µM, 100µM, 250µM, and 500µM, H_2O_2, Sigma-Aldrich) and inflammatory stress (1ng/ml, 2.5ng/ml, and 5ng/ml, TNF-α, R&D Systems). The media was exchanged daily to maintain constant stress levels[52]. Cells were allowed to differentiate and fuse for 4d then fixed with cold methanol (-20°C). The MDI metric

is calculated as a ratio of MHC expressing cells to the total number of cells detected following fixation. (Immunohistochemical staining methods for MDI measurement is described in the following "Immunohistochemistry" methods section.

3.4.5   Intracellular antioxidant capacity

The antioxidant capacity was measured in terms of the activity of the major intracellular anti-oxidant molecules: reduced glutathione (GSH) and superoxide dismutase (SOD). Levels of GSH were measured colorimetrically (Calbiochem, 354102) using a spectrophotometer (TECAN Infinite M200, Männedorf, Switzerland). GSH detection is mediated by capture of all thiol mercaptans (RSH) from mechanically homogenized cells into thioethers by 4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate followed by the formation of a chromophoric thione in those GSH specific thioethers using NaOH. GSH levels were quantified by chromophoric thione absorbance at 400nm. Total activity of SOD was measured using a colorimetric assay (Chemicon, Temecula, CA; APT290). SOD levels of chemically lysed cells (10 mM Tris, pH 7.5, 150mN NaCl, 0.1 mM EDTA, and 0.5% Triton X-100) were measured using a xanthine/xanthine oxidase system in which superoxide-chromagen absorbance (490nm) is lowered by the presence of SOD.

The intracellular antioxidant levels of ALDH^{lo} and ALDH^{hi} myoblasts were modified by a chemical antioxidant (XJB-5-131, generously donated by Prof. Peter Wipf at the University of Pittsburgh) and pro-oxidant (diethyl maleate (DEM), Sigma-Aldrich). XJB is a membrane permeable, mitochondrial targeted nitroxide anti-oxidant that has proven to be cytoprotective in disease states associated with oxidative stress, such as hemorrhagic shock, and mitigates apoptotic cell death in vitro[240,241]; whereas DEM, is a chemical that conjugates and inactivates

GSH thus decreasing the cell's capacity to resist oxidative stress[271]. To examine the role of antioxidant levels in ALDH sorted myoblasts, ALDH^{lo} myoblasts were pretreated with 500nM XJB in PM for 2hrs prior to exposure to oxidative stress (250µM H_2O_2 in PM) conditions. ALDH^{hi} myoblasts were pretreated with 50µM DEM for 2hrs prior to exposure to oxidative stress (250µM H_2O_2 in PM) conditions. In both cases the myoblasts were washed in PBS prior to stress exposure to insure all active antioxidants were intracellular. The rates of proliferation and survival of these pretreated myoblasts were quantified using a live cell imaging robot (Kairos Instruments LLC, Pittsburgh, PA) as described previously.

To examine the role of ALDH in the anti-oxidant capacity of ALDH sorted myoblasts a potent ALDH inhibitor, diethylaminobenzaldehyde, was used. Pretreatment of ALDH^{hi} myoblasts with 200µM of DEAB in PM was used 24hrs prior to oxidative stress exposure. A concentration of 200µM DEAB was maintained during the oxidative stress exposure to maintain ALDH inhibition throughout the experiment. The rates of proliferation and survival were quantified using the live cell imaging robot.

3.4.6   Skeletal muscle regeneration capacity

Skeletal muscle regeneration capacity of ALDH sorted myogenic cells was quantified using the regeneration index metric as previously described[42,52,81]. Briefly, the number of dystrophin positive myofibers per cryosection per 10^5 cells injected is recorded. ALDH sorted murine cells were injected into the gastrocnemius muscles of female *mdx* mice (C57BL/10ScSn-DMD^{mdx}/J, The Jackson Laboratory), which is a murine model of Duchenne muscular dystrophy using a protocol previously described[52,248]. The *mdx* mouse is a C57BL strain homozygous for a spontaneous X-linked mutation of the dystrophin gene and lacks dystrophin protein

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expression[248]. Animals were sacrificed 14d following injection. All gastrocnemius muscles were excised and frozen in 2-methyl-butane, cryosectioned (8μm), and mounted on glass slides. The animal research protocol was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and University of Pittsburgh (0902596).

3.4.7 Immunohistochemistry

Differentiation of myogenic cells into MHC expressing cells in vitro was quantified using the MDI metric described previously. Briefly, samples were blocked with 5% HS and incubated with monoclonal antibodies for MHC (Sigma; 1:300) followed by Cy3-conjugated anti-mouse antibodies. Nuclei were stained blue with 4, 6-diamidino-2-phenylindole (DAPI, Sigma; 100 ng/ml, 1:1000). The MDI is defined as the number of nuclei within MHC–expressing cells divided by the total number of nuclei[272]. At least 200 cell nuclei were counted for each MDI measurement.

All murine skeletal muscle tissue samples were frozen in 2-methylbutane cooled in liquid nitrogen, then stored at –80°C. 8μm cryosections were fixed in 5% formalin for 2 min and blocked in 5% donkey serum for 1h. Skeletal muscle sections were stained for dystrophin (DYS) using an anti-dystrophin primary antibody (1:300, Rabbit Anti-DYS, Abcam Ab15277) and a secondary anti-rabbit antibody (1:500, Donkey anti-Rabbit, Molecular Probes A21207) using a protocol previously described[249]. Nuclei were stained with DAPI. Fluorescence and brightfield microscopy was performed using a Nikon Eclipse E800 microscope (Melville, NY) equipped with a Retiga Exi digital camera (QImaging) and Olympus FV 500 confocal scanning microscope. All images were acquired and analyzed using Northern Eclipse software (version 6.0; Empix Imaging).

3.4.8   Osteogenic and chondrogenic differentiation capacity

ALDH^{hi}, ALDH^{lo} and unsorted cells were prepared in pellet form (2.5×10^5 cells) or as a monolayer (1.5×10^4 cells/well in 12 well plates). For osteogenesis, murine myoblasts were plated on collagen-coated 12 well plates and maintained for 4d in osteogenic media (Lonza, Walkersville, MD), which included dexamethasone, glutamine, ascorbate, penicillin/streptomycin (P/S), β-glycerophosphate and bone morphogenic protein-4 (50 ng/ml). The cells were stained for alkaline phosphatase following the manufacturer's instructions (Sigma-Aldrich, 86C-1KT). Chondrogenesis of murine myoblasts was studied by culturing the cells in a pellet culture by incubating the cell pellets for 21d in chondrogenic media (Lonza, PT3003), which contains dexamethasone, ascorbate, ITS+, P/S, sodium pyruvate, proline, glutamine and TGF-β3 (10ng/ml) (Lonza, PT4124). All pellets were fixed in 10% formalin for 24hrs followed by dehydration, paraffin embedding, sectioning, and Alcian blue staining.

3.4.9   Statistical Analysis

Data are expressed as a mean with its standard deviation. Direct comparisons between two cell populations were made using an unpaired, two-tailed Student's *t* test, where statistical significance was determined at a level $p < 0.05$. Comparisons of multiple groups were completed using one-way ANOVA followed by *Tukey* post-hoc comparisons.

3.5   RESULTS

3.5.1   Flow cytometric sorting of murine preplate cells

Cells with elevated ALDH levels become fluorescent when exposed to boron-dipyrromethene (BODIPY) labeled amino acetaldehyde (BAAA) (Aldefluor, StemCell Technologies) can be isolated using fluorescence activated cell sorting (FACS). The non-polar BAAA diffuses freely into the cytoplasm and is converted by ALDH1A1 to the negatively charged BIODIPY-aminoacetate, which trapped in the cytoplasm causes the cell to fluoresce with an emission peak at 513nm [189]. Cells, of low side scatter, that were significantly brighter than the DEAB inhibited population were deemed ALDH^{hi} and isolated from heterogeneous populations of murine and human muscle derived cells.

In order to isolate ALDH^{lo}SSC^{lo} and ALDH^{hi}SSC^{lo} sub-populations of cultured muscle derived cells, cells were treated with Aldefluor. ALDH^{hi} subpopulations were isolated from murine muscle cells segregated by the preplate technique, which included rapidly adhering myoblasts and slowly adhering MDSCs. The gating of ALDH^{hi} and ALDH^{lo} populations was determined by the low sidescatter extremes of the DEAB inhibited ALDH fluorescence (Figure 15a). ALDH^{lo} and ALDH^{hi} myoblasts represented roughly 1% and 3-5% of the total myoblast population respectively (Figure 15b) when dead cells were excluded using propidium iodide uptake (Figure 15c). An ALDH fluorescence frequency histogram illustrates the enrichment of ALDH^{hi} cells in murine MDSCs compared to myoblasts in Figure 15e. That is, MDSCs were shown to have a significantly elevated mean ALDH fluorescence when compared to murine myoblasts. In addition MDSCs appear to have a decreased ALDH distribution range indicating less heterogeneity in ALDH expression than murine myoblasts. It should also be noted that we

observed increased cell viability in ALDH^{hi} cells compared to ALDH^{lo} cells immediately following FACS isolation. That is, the mechanical stress of the cell segregation process of flow cytometry seemed to have more deleterious effect on the ALDH^{lo} cells compared to the ALDH^{hi} cells in terms of survival.
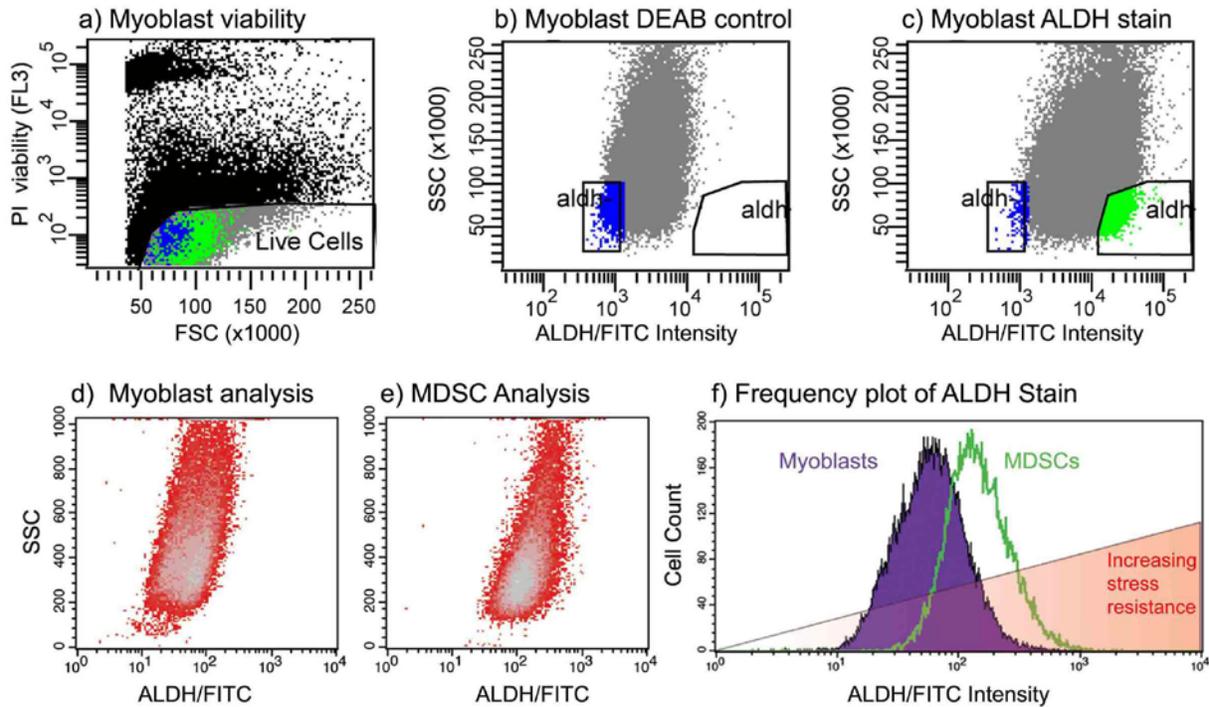
Figure 15. FACS analysis of murine myoblasts and MSDCs

Isolation of ALDH^{lo} and ALDH^{hi} subpopulations from cultured early preplate myoblasts was performed using FACS. (a) Dead cells were excluded from the isolation by detection of nuclear propidium iodide fluorescence. (b-c) ALDH^{hi} SCC^{lo} (high ALDH activity, low side scatter) cells were isolated from a heterogeneous population of myoblasts using DEAB, a potent inhibitor of ALDH, as a gating control. (d-e) Measurement of the FITC signal intensity of Aldefluor stained murine myoblasts and MDSCs demonstrated a shift in the distribution of signal intensity between the two populations, shown in (f), suggesting a increase in median ALDH activity in MDSCs compared to myoblasts. The shaded red overlay is meant to emphasize the trend of increasing stress resistance between myoblasts and MDSCs.

3.5.2   Enrichment of ALDH^{hi} cells in slowly adhering preplate cells

In order to determine whether the preplate process may select for ALDH^{hi} cells or enrich murine muscle derived cells with cells with elevated ALDH levels, Aldefluor intensities were measured in successive preplate cycle populations, illustrated in Figure 16a,b.   Despite an elevated median ALDH level and ALDH^{hi} proportion observed in PP1, a overall trend of increasing ALDH intensity and proportion of ALDH^{hi} cells was observed with each preplate cycle.   This result supports the previous observation of Figure 15 (d-f) that suggests an overall increase in ALDH levels in MDSCs (derived from PP6) and myoblasts (PP2-PP4).
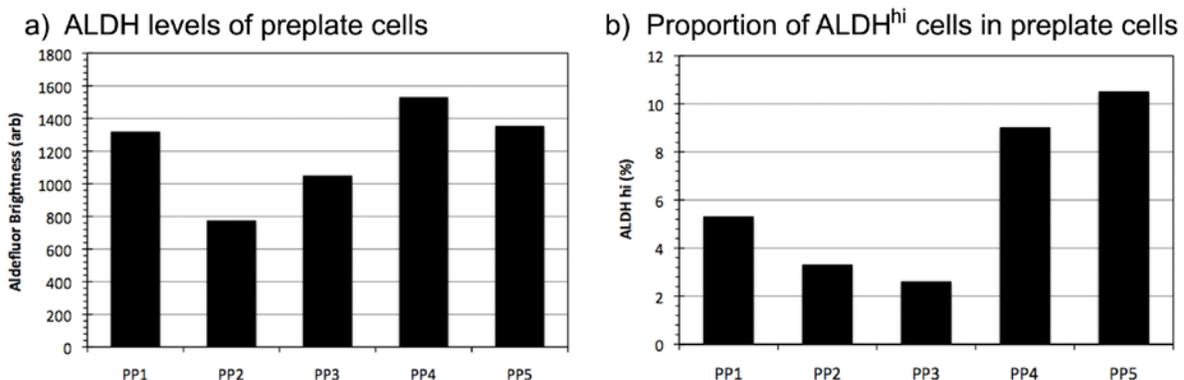
Figure 16. ALDH activity of murine muscle preplate cells via FACS

FACS analysis of preplated cells derived from murine skeletal muscle (PP1-PP5) indicates increasing ALDH activity with successive preplate cycles. (a)   Median FITC intensity measurements of Aldefluor stained preplate cells was measured by flow cytometry. A trend of increasing ALDH activity with preplate cycle was observed. (b) The percentage of ALDH^{hi} cells in each preplate was plotted as a function of preplate cycle. A trend of increasing proportion of ALDH^{hi} cells was observed as a function of preplate cycle.

To verify this observation of enrichment of ALDH^{hi} cells in later preplate cycles, freshly dissociated murine skeletal muscle cells were sorted into ALDH^{hi} and ALDH^{lo} sub-populations by FACS. These populations were then immediately subjected to the modified preplate process described previously. The ALDH^{lo} sub-population of cells not only suffered more cell death as a

result of the FACS process, observed in previous experiments as well, but of those cells that survived adhered rapidly to collagen-coated flasks. As depicted in Figure 17 below, ALDH^{lo} cells could only be preplated for two iterations over a 2hr period (PP1 to PP2). Although three cells were found in the fourth preplate this did not constitute a significant population, particularly given that no PP3 cells were observed. On the other hand the ALDH^{hi} subpopulation of muscle derived cells yielded a robust population of cells to preplate 4. While the ALDH^{hi} population did not yield any cells to preplate 6 (PP6) unlike the unsorted muscle derived cells, these cells were subjected to the high velocity mechanical stress of fluorescence activated cell sorting and likely suffered a loss in viability.
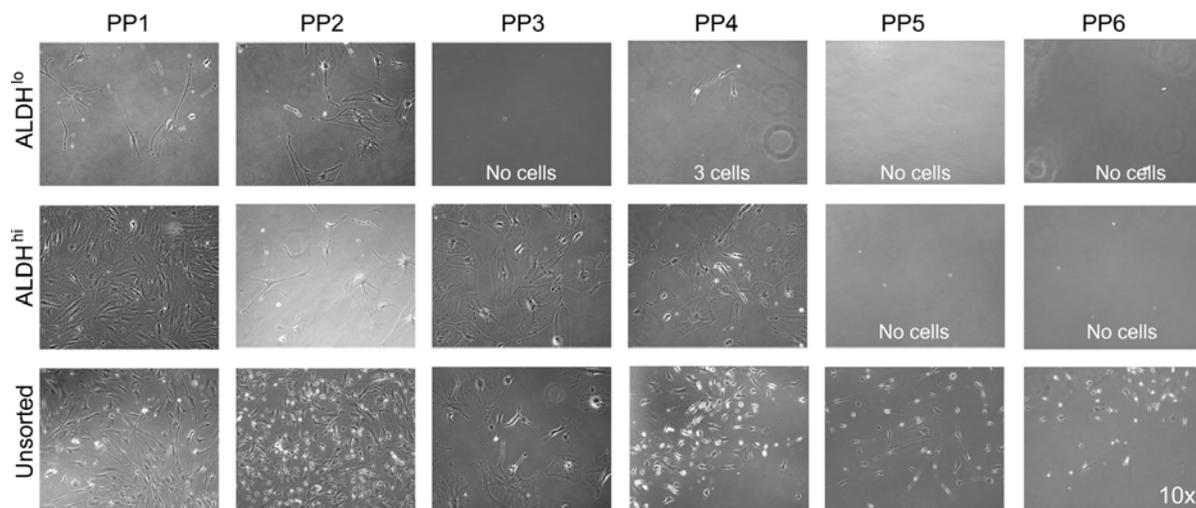
Figure 17. ALDH^{hi} muscle cells yield later preplate populations

Later preplate cycles were achieved using ALDH^{hi} cells (PP4) compared to ALDH^{lo} cells (PP2). MDSC equivalent preplate cells (PP5-PP6) were not observed following ALDH sorting likely due to loss of viability during the process of digestion and FACS isolation prior to initiating the preplate cycle.

3.5.3   Stress resistance of ALDH sorted myoblasts

Myoblasts isolated using the preplate technique have been characterized as a heterogeneous population in various states of quiescence, activation, and differentiation[27,42,81]. We therefore hypothesized that we may observe heterogeneity in their ALDH expression. Following FACS isolation of ALDH^{lo} and ALDH^{hi} subpopulations, we quantified the oxidative (hydrogen peroxide or H_2O_2) and inflammatory (TNF-α) stress resistance during proliferation and myogenic differentiation.   Hydrogen peroxide is a strong oxidant that is formed by the dismutation of superoxide and freely diffuses across the cell membrane.   TNF-α on the other hand is an acute phase cytokine that has been shown to inhibit myogenic differentiation via NF-κB induction, promote caspase mediated apoptosis, and induce reactive oxygen species accumulation in mitochondria[273,274].   Both H_2O_2 and TNF-α are active during inflammation[114].

Following FACS isolation of ALDH^{hi} and ALDH^{lo} subpopulations of murine myoblasts, oxidative (H_2O_2) and inflammatory (TNF-α) stress resistance during proliferation was quantified (Figure 18a and b). The proliferation rate of ALDH^{lo} and ALDH^{hi} subpopulations of murine myoblasts was quantified using live cell imaging microscopy in conditions of oxidative (250 μM, H_2O_2) and inflammatory (2.5 ng/ml, TNF-α) stress. A significant proliferation advantage of ALDH^{hi} cells is observed in the murine myoblasts when normalized cell numbers are compared to ALDH^{lo} cells as a function of time.
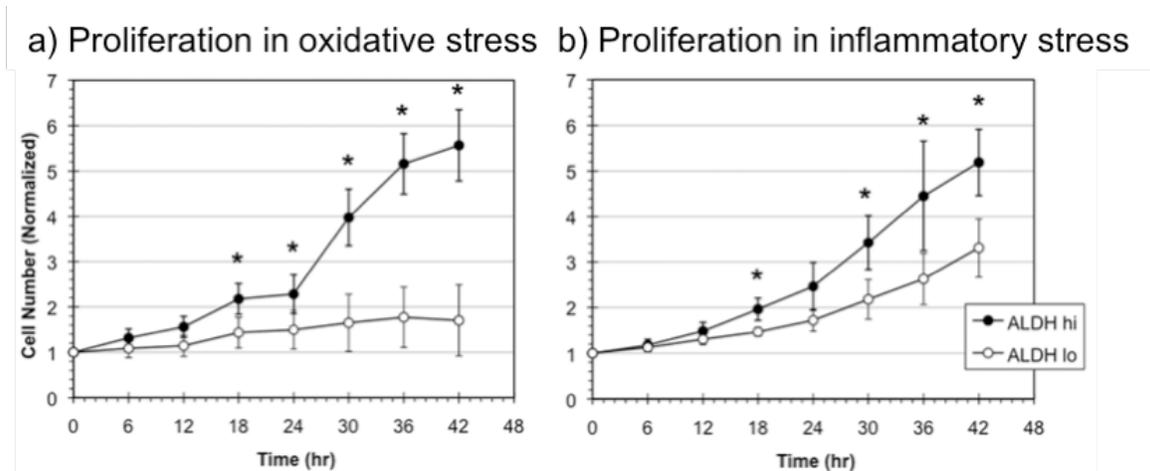
Figure 18. ALDH sorted myoblast proliferation in oxidative and inflammatory stress

(a) A significantly increased rate of proliferation of ALDH^{hi} early preplate myoblasts compared to ALDH^{lo} myoblasts was observed in conditions of oxidative stress (H_2O_2, 250μM) (* indicates $p < 0.05$). (b) Similarly ALDH^{hi} myoblasts demonstrated elevated proliferative capacity in inflammatory stress conditions (TNF-α, 2.5ng/ml).

The myogenic differentiation index (MDI), a measure of the proportion of cells expressing myosin heavy chain (MHC), of ALDH^{hi} and ALDH^{lo} murine and human muscle derived cells was quantified in conditions of oxidative (H_2O_2) and inflammatory (TNF-α) stress. Similarly, a significantly increased number of myosin heavy chain expressing cells was observed in ALDH^{hi} murine myoblasts compared to ALDH^{lo} myoblasts (Figure 19).

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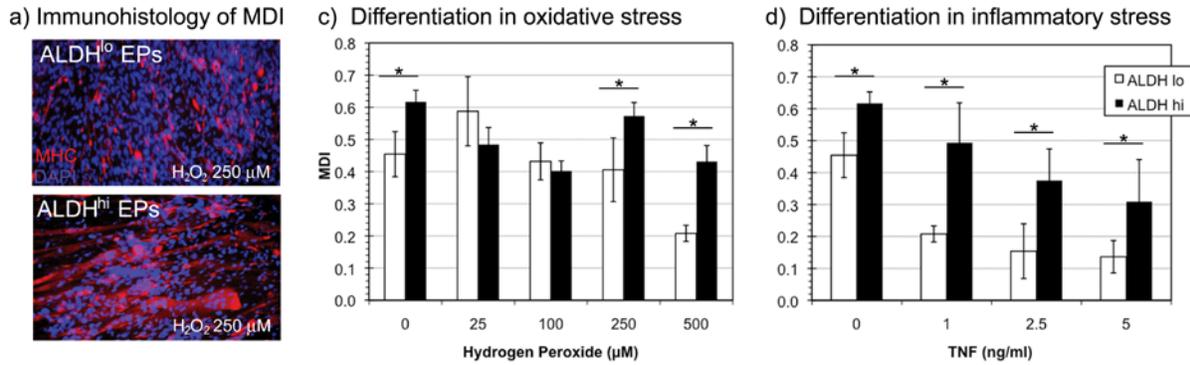
Figure 19. Myogenic differentiation of ALDH sorted myoblasts in conditions of oxidative and inflammatory stress

(a) ALDH^{lo} and ALDH^{hi} myoblasts underwent myogenic differentiation by fusing into MHC$^+$ myotubes (red) under oxidative stress conditions (250μM). Nuclei are stained with DAPI (blue). (b) Significantly increased myogenic differentiation indices (MDI) were observed ($p < 0.05$) in ALDH^{hi} myoblasts at several concentrations of oxidative stress (0, 250 and 500μM) when compared to ALDH^{lo} myoblasts.   (c) Significantly increased myogenic differentiation indices (MDI) of ALDH^{hi} cells were observed under all inflammatory stress conditions when compared to ALDH^{lo} myoblasts.

3.5.4   Skeletal muscle regeneration capacity of ALDH sorted myoblasts

ALDH^{hi} and ALDH^{lo} murine myoblasts were injected intramuscularly into the gastrocnemius of *mdx* mice to determine the degree of muscle regeneration. Muscles were excised and frozen for immunohistochemical characterization after a period of 14 days. The cell transplantations yielded robust engraftments and dystrophin (DYS) expressing myofibers (Figure 20a,b). The extent of myofiber formation was quantified using the regeneration index (RI) metric, a measure of DYS$^+$ myofibers in cryosectioned *mdx* muscle per 10^5 cells injected, as described previously[52]. Significantly greater RIs were observed in the muscles injected with ALDH^{hi} myoblasts compared to ALDH^{lo} myoblasts, suggesting that the survival, proliferation, and/or differentiation capacity of ALDH^{hi} myoblasts was greater in vivo than ALDH^{lo} myoblasts, which correlated with the results obtained in vitro.
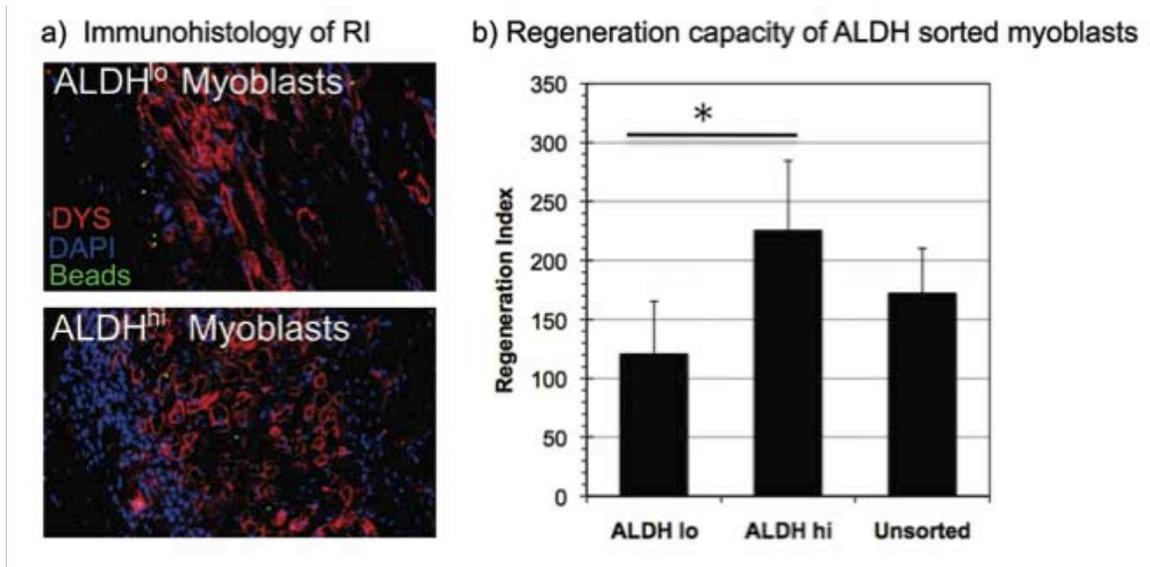
Figure 20. Skeletal muscle regeneration capacity of ALDH sorted myoblasts

(a) ALDH sorted myoblasts (2×10^5 cells) were injected intramuscularly into the gastrocnemius of *mdx* mice and analyzed after 14days. An increased number and density of dystrophin positive myofibers were observed in mice injected with ALDH^{hi} cells compared to those injected with ALDH[lo] myoblasts. Dystrophin (DYS) positive myofibers (stained in red) indicate transplanted ALDH[lo] cell myofiber generation or fusion with a host myofiber. Nuclei are DAPI (blue) stained. Fluorescent green Fluosphere beads localize the area of initial injection. (b) Significantly increased regeneration index was observed in ALDH[hi] transplanted mice compared to those injected with ALDH[lo] and unsorted cells. (* indicates $p < 0.05$).

To understand the increased stress resistance capacity of the ALDH[hi] myoblasts, we examined two major intracellular antioxidants, reduced GSH and SOD. Significantly increased concentrations of GSH and increased activity of SOD was observed in ALDH[hi] myoblasts compared ALDH[lo] myoblasts using spectrophotometric assays (Figure 21). The association of elevated ALDH activity with elevated intracellular anti-oxidant levels suggests a mechanism by which these cells are able to withstand oxidative and inflammatory stress conditions.
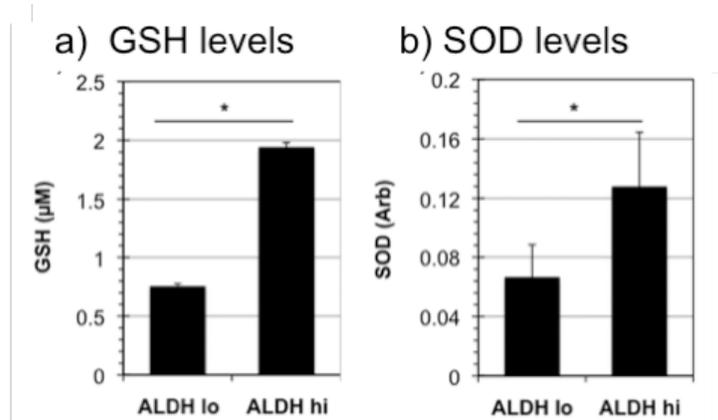
Figure 21. Intracellular anti-oxidant (GSH and SOD) levels of ALDH sorted myoblasts

(a-b) Measurements of intracellular antioxidant levels in terms of glutathione (GSH) and superoxide dismutase (SOD) demonstrated statistically significant elevation of both anti-oxidants in ALDH[hi] myoblasts compared to ALDH[lo] myoblasts. (* indicates $p < 0.05$).

3.5.5   Mechanism of increased stress resistance in ALDH[hi] murine myoblasts

To elucidate the role of intracellular anti-oxidant levels in the stress resistance capacity of ALDH sorted myoblasts, we altered the cells anti-oxidant levels prior to oxidative stress challenge. ALDH[lo] myoblasts were treated with a mitochondrial targeted anti-oxidant, XJB-5-131 (XJB) prior to exposure to hydrogen peroxide to determine whether the proliferation rate could be elevated to that of ALDH^{hi} cells[237]. On the other hand ALDH[hi] myoblasts were treated with a GSH sequestrator, diethyl maleate (DEM) prior to oxidative stress exposure in order to alter their intracellular anti-oxidant levels[275].

Alteration of the anti-oxidant levels of these ALDH sub-populations had a significant impact on their proliferation capacities in oxidative stress. Figure 22a depicts the proliferation advantage of ALDH[hi] myoblasts compared to ALDH[lo] myoblast as observed previously. By increasing the anti-oxidant levels of ALDH^{lo} cells with XJB prior to oxidative stress exposure

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the proliferation rate was significantly increased, to a level statistically equivalent to that observed in the ALDH^{hi} myoblasts (Figure 22b). On the other hand, when the anti-oxidant levels of ALDH^{hi} myoblasts were decreased by treatment with DEM, their proliferation rate was significantly decreased to that observed in the ALDH^{lo} myoblasts (Figure 22c). This result suggests that the differences in stress resistance between the ALDH^{hi} and ALDH^{lo} myoblasts is conferred by differences in their oxidative stress handling capacities, which can be easily modified using anti-oxidant and pro-oxidant treatment.
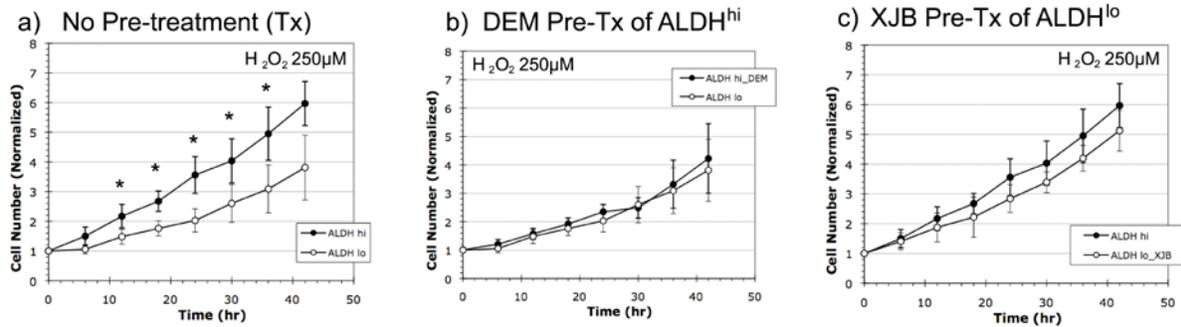
Figure 22. Anti-oxidant and pro-oxidant treatment of ALDH sorted myoblasts to elucidate the role of intracellular anti-oxidant levels on oxidative stress resistance

(a) As shown in Figure 18 ALDH^{hi} early preplate myoblasts have greater proliferative capacity in oxidative stress than ALDH^{lo} myoblasts. (* indicates $p < 0.05$). (b) However this proliferative advantage of ALDH^{hi} myoblasts was lost when these cells are pre-treated (Tx) with the GSH inhibitor, diethyl maleate (DEM). (c) When ALDH^{lo} myoblasts were treated with the anti-oxidant, XJB-5-131, their proliferative capacity was improved to a level observed in ALDH^{hi} myoblasts.

The next question that we addressed was whether ALDH was directly participating in the oxidative stress resistance of ALDH^{hi} myoblasts. Only in the ocular lens and cornea has ALDH been directly implicated in the mitigation of oxidative stress[276]; however it should be noted that in the course of its primary reaction of oxidation of aldehydes to carboxylic acids, it reduces the cofactor NADP$^+$ to NADPH. Given the role of NADPH in GSH recycling via glutathione reductase, we hypothesized that ALDH may in fact participate directly in the elevated anti-stress

resistance of ALDH[hi] myoblasts [199]. However, when we treated the ALDH^{hi} cells with DEAB (200μM), a potent inhibitor of ALDH, for 24hrs prior and during oxidative stress exposure we observed no difference in the proliferative capacity of ALDH^{hi} cells in oxidative stress conditions (Figure 23b,c). This result suggests that ALDH is not directly participating in the increased anti-oxidant capacity of ALDH[hi] myoblasts and that elevated ALDH activity may simply be a marker for myoblasts with elevated stress resistance capacity. Similarly no impact of DEAB on the proliferation capacity of ALDH[lo] myoblasts (Figure 23d,e).
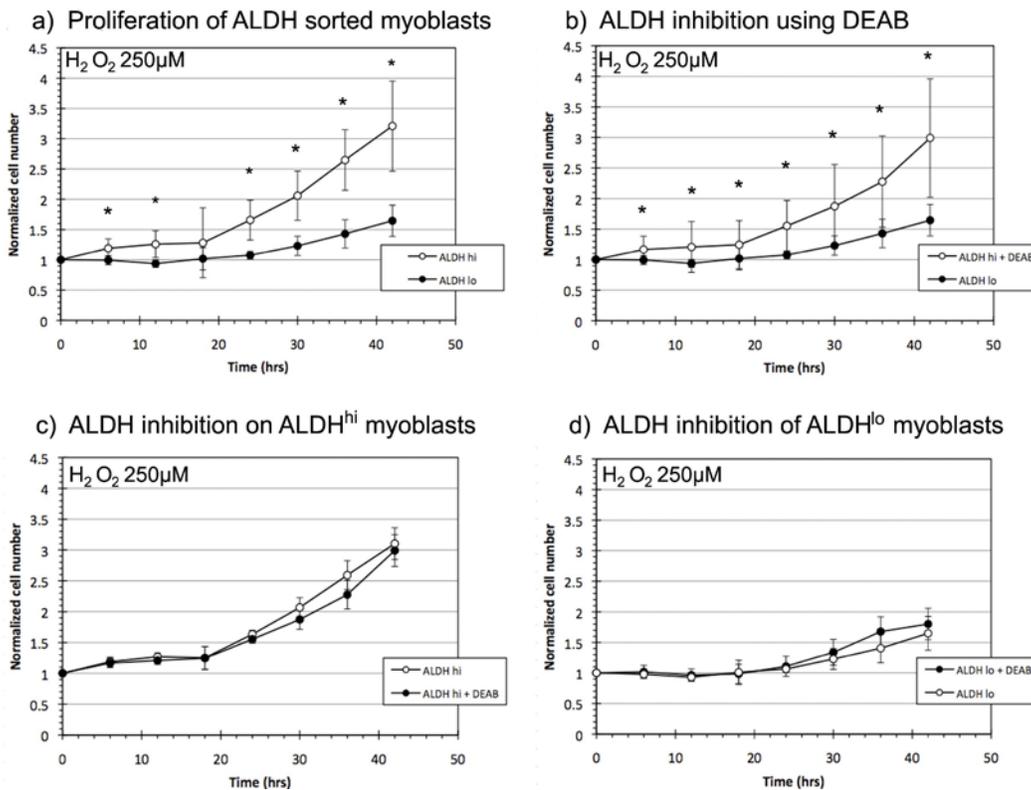
Figure 23. ALDH inhibition by DEAB does not alter stress resistance behavior of ALDH sorted myoblasts
DEAB is a strong inhibitor of ALDH activity, however it did not alter the proliferative behavior of ALDH sorted myoblasts in proliferation media treated with H_2O_2 (250 μM). (a) Similar to the result observed in Figure 18, an increased rate of ALDH[hi] myoblast proliferation in oxidative stress compared to ALDH[lo] myoblasts was observed. (b) DEAB inhibition of ALDH did not inhibit proliferation of ALDH[hi] myoblasts to that of ALDH[lo] myoblasts. (c-d) Nor did DEAB inhibition of ALDH alter the proliferative behavior of ALDH[hi] or ALDH[lo] myoblasts.

3.5.6 Chondrogenic and osteogenic differentiation of ALDH sorted myoblasts

Two clear features of myoblasts with elevated ALDH expression have emerged from the experiments discussed thus far: increased stress resistance capacity and myogenic differentiation capacity. In working with these cells, we questioned whether these two attributes were linked. That is, these cells may have increased myogenic differentiation capacities because of their increased stress resistance. One may consider the low serum conditions of myogenic differentiation medium as another kind of stress condition, which to has been shown to induce apoptosis in those cells that fail to differentiate into MHC$^+$ cells[253,254]. The cell's capacity to survive and function normally in adverse or altered media conditions is a necessary precondition to establish differentiation potential. We therefore hypothesized that elevated ALDH expression and stress resistance capacity could also favor osteogenic and chondrogenic differentiation in vitro, given the dramatic alteration in media conditions.

We studied the osteogenic and chondrogenic differentiation of ALDH sorted myoblasts and found that, indeed, ALDH^{hi} myoblasts demonstrate a greater capacity to undergo chondrogenic and osteogenic differentiation in vitro as illustrated in Figure 24. When chondrogenic differentiation was induced in ALDH sorted myoblasts, rapid pellet formation was observed in ALDH^{hi} myoblasts (24hrs) with robust production glycosaminoglycans (GAGs). This behavior mirrors that of the unsorted myoblasts (Figure 24a) suggesting that the chondrogenic potential of unsorted myoblasts may be conferred by the ALDH^{hi} myoblasts. However ALDH^{lo} myoblasts showed poor proliferation in the chondrogenic media and formed smaller, less dense pellets that required 2-3d to coalesce. Even after 21d of culture, increased cell density and glycosaminoglycans (GAG) formation was observed via Alcian blue staining in ALDH^{hi} myoblasts compared to ALDH^{lo} myoblasts (Figure 24a-c). Osteogenic differentiation of

murine myoblasts was induced via bone morphogenic protein-4 (BMP-4) stimulation. The unsorted myoblast population increased their alkaline phosphatase production in response to BMP stimulation (Figure 24d), which was also observed in ALDH^{hi} myoblasts (Figure 24e). However a poor alkaline phosphatase response was observed in ALDH^{lo} myoblasts. As was observed in chondrogenic media conditions, the osteogenic differentiation potential of unsorted myoblasts appears to be conferred by the ALDH^{hi} myoblasts (Figure 24d-f).

While the differences in osteogenic differentiation in ALDH sorted myoblasts may either be attributed to increased osteogenic capacity or increased media stress resistance, the case of chondrogenic differentiation capacities implicates another mechanism. It seems clear that ALDH^{lo} myoblasts experienced some form of growth retardation in chondrogenic media compared to ALDH^{hi} myoblasts to the extent that ALDH^{lo} myoblasts did not form a condensed pellet. Therefore the inability of ALDH^{lo} myoblasts to survive and proliferate in chondrogenic media impaired their capacity for chondrogenic differentiation, as measured by pellet size and amount of GAG production. Therefore, we believe that the increased survival and proliferation potential of ALDH^{hi} myoblasts may also improve the differentiation yield in not only myogenic differentiation but also chondrogenic and osteogenic differentiation.

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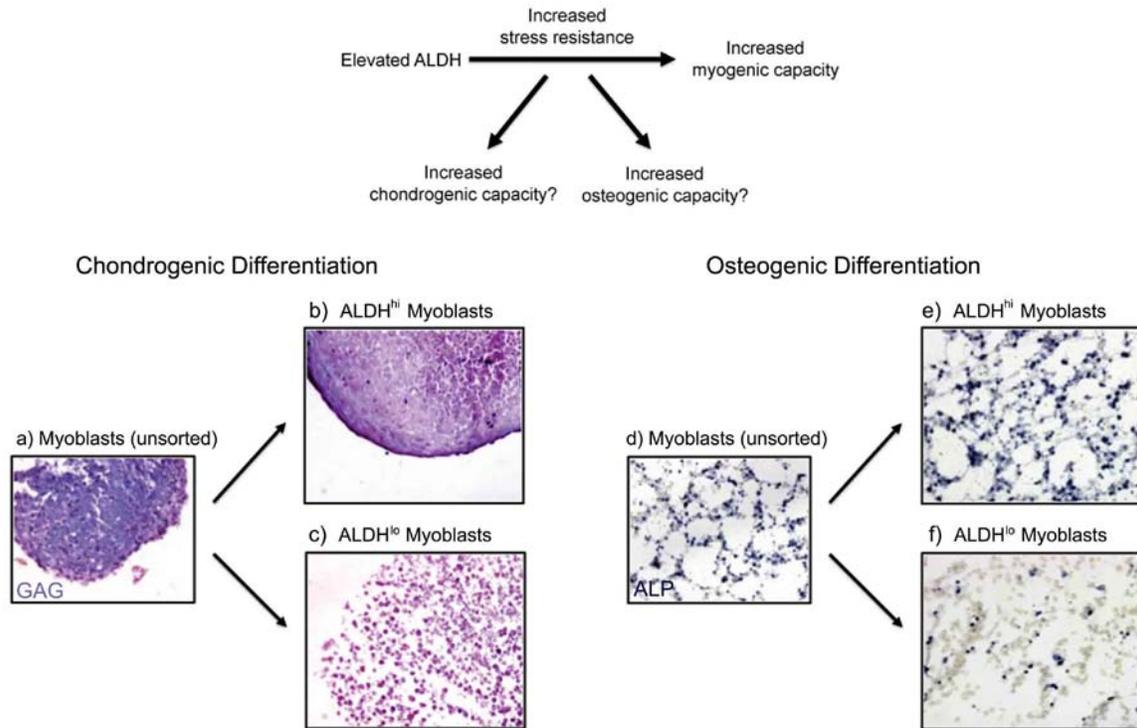
Figure 24. Chondrogenic and osteogenic differentiation capacity of ALDH sorted myoblasts in vitro

(a-c) Chondrogenic differentiation potential of ALDH^{hi} myoblasts was shown to be greater than ALDH^{lo} myoblasts in vitro in terms of pellet formation after 21d and glycosaminoglycan (GAG, stained in violet) production. (d-f) Osteogenic differentiation was induced in vitro by BMP-4 stimulation. Alkaline phosphatase (ALP, shown in blue) levels were significantly increased in ALDH^{hi} myoblasts compared to ALDH^{lo} myoblasts after a period of 4d.

3.5.7   Isolation and stress resistance of ALDH sorted MDSCs

Given the heterogeneity of ALDH activity and stress resistance in myoblasts, we hypothesized that perhaps similar heterogeneity may exist within the MDSC populations as well. We have previously observed increased stress resistance in MDSCs compared to myoblasts, however, we reasoned that there may exist an ALDH^{hi} subpopulation of MDSCs that is responsible for this enhanced stress resistance. ALDH^{hi} and ALDH^{lo} subpopulations of murine MDSCs were

isolated by FACS in the same fashion as murine myoblasts. Representative FACS isolation of ALDH[hi] and ALDH[lo] MDSCs is illustrated in Figure 25a.

In contrast to myoblasts, MDSCs did not display a high degree of heterogeneity in ALDH expression (Figure 25) or in stress resistance behavior when sorted into ALDH[hi] and ALDH[lo] subpopulations. This is not surprising given the already elevated stress resistance of MDSCs compared to myoblasts shown previously[52]. Isolation of ALDH[hi] and ALDH[lo] MDSCs yielded a subpopulation without a significant difference in proliferation capacity in stress conditions (Figure 25).
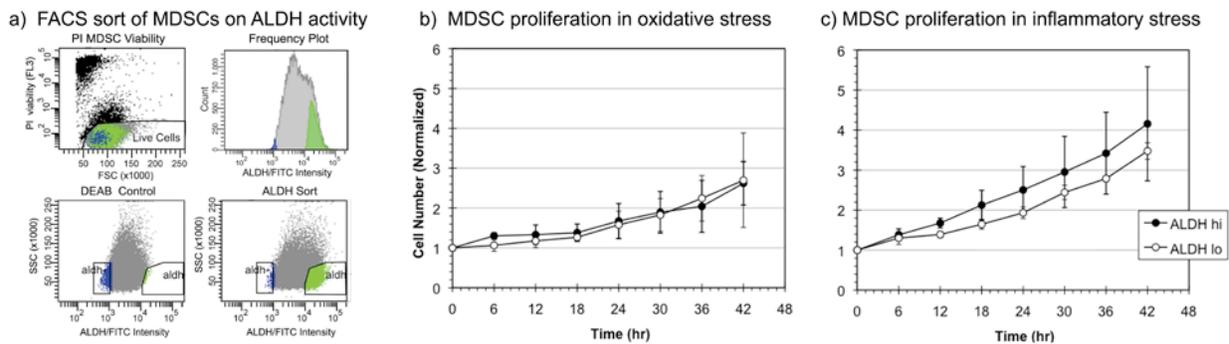
Figure 25. FACS isolation and proliferation capacity of ALDH sorted MDSCs in oxidative and inflammatory stress conditions

(a) In the same isolation protocol illustrated in Figure 15, ALDH[hi] SCC[lo] (high ALDH activity, low side scatter) cells were isolated from MDSCs using DEAB, an ALDH inhibitor, as a control. (b-c) No difference in proliferative capacity of ALDH sorted MDSCs were observed in either oxidative (H_2O_2, 250µM) or inflammatory stress (TNF-α, 2.5ng/ml)

Although a perceptible trend of increased myogenic differentiation index was observed in ALDH[hi] MDSCs when compared to ALDH[lo] MDSCs, these trends were not statistically significant except at one very high oxidative stress dose (H_2O_2, 500µM) and one intermediate inflammatory stress does (TNF-α, 1ng/ml).
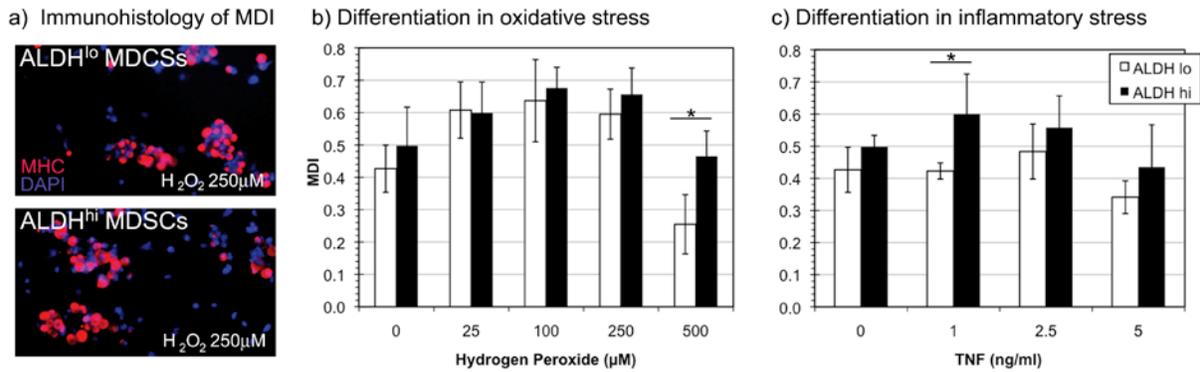
Figure 26. Myogenic differentiation of ALDH sorted MDSCs in oxidative and inflammatory stress

(a) ALDH sorted MDSCs underwent myogenic differentiation in oxidative stress (H_2O_2, 250μM) by expressing myosin heavy chain (MHC) and fusing into MHC^+ clusters rather than fusiform myotubes. (b) No significant differences in myogenic differentiation indices (MDI) was observed in $ALDH^{hi}$ MDSCs compared to $ALDH^{lo}$ MDSCs except at very high oxidative stress (H_2O_2, 500μM) and at one intermediate inflammatory stress level (TNF-α, 1ng/ml) ((indicated by *).

When injected intramuscularly into the *mdx* mouse gastrocnemius, both $ALDH^{lo}$ and $ALDH^{hi}$ MDSC subpopulations demonstrated robust engraftment; however, no significant difference in the RIs of $ALDH^{hi}$ and $ALDH^{lo}$ MDSCs was observed (Figure 27).

a) Immunohistology of RI
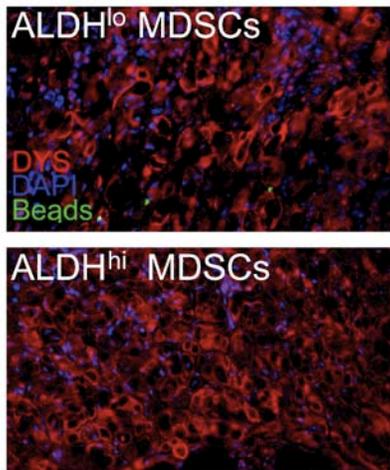
b) Regeneration capacity of ALDH sorted MDSCs
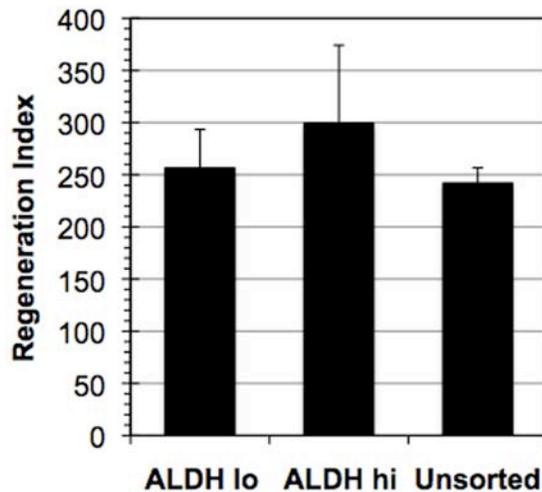
Figure 27. Skeletal muscle regeneration capacity of ALDH sorted MDSCs

(a) ALDH sorted MDSCs (2 x 10^5 cells) were injected intramuscularly into the gastrocnemius of *mdx* mice and analyzed after 14days. Robust engraftment of both MDSC sub-populations was observed. Dystrophin (DYS) positive myofibers (stained in red) indicate transplanted ALDH[lo] cell myofiber generation or fusion with a host myofiber. Nuclei are DAPI (blue) stained. Fluorescent green Fluosphere beads are observed to localize the area of initial injection. (b) No statistically significant differences in regeneration index were observed in ALDH[hi] transplanted mice compared to those injected with ALDH[lo] and unsorted cells.

Not surprisingly, there were no significant differences observed in the GSH or SOD levels between the two ALDH sorted sub-populations of MDSCs (Figure 28). This result suggested that no elevation in stress resistance or regenerative capacity could be achieved by isolating an ALDH[hi] subpopulation from MDSCs.
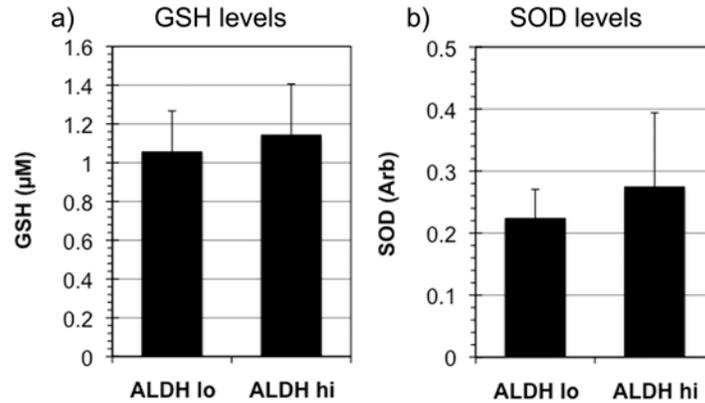
Figure 28. Intracellular antioxidant levels (GSH and SOD) of ALDH sorted MDSCs
(a-b) Measurements of intracellular antioxidant levels in terms of glutathione (GSH) or superoxide dismutase (SOD) levels indicated no statistically significant differences between ALDH[lo] and ALDH[hi] MDSCs.

Although no statistically significant difference in myogenic differentiation capacity was observed in ALDH[lo] and ALDH[hi] MDSCs, chondrogenic and osteogenic differentiation capacity was examined as well. Chondrogenic differentiation of ALDH sorted myoblasts was induced in chondrogenic media and in pellets. Mouse MDSC chondrogenesis was observed in monolayer form and stained for collagen type II after 4 days (Figure 29a-c). Although increased cell proliferation was observed in ALDH[hi] MDSCs and unsorted MDSCs compared to ALDH[lo] MDSCs, no differences in collagen II positivity per cell was observed. Osteogenic differentiation potential of MDSC was studied in plated cells under bone morphogenic protein-4 (BMP-4) stimulation. Increased alkaline phosphatase expression was observed in ALDH[hi] and unsorted MDSCs compared to ALDH[lo] MDSC (Figure 29d-f).
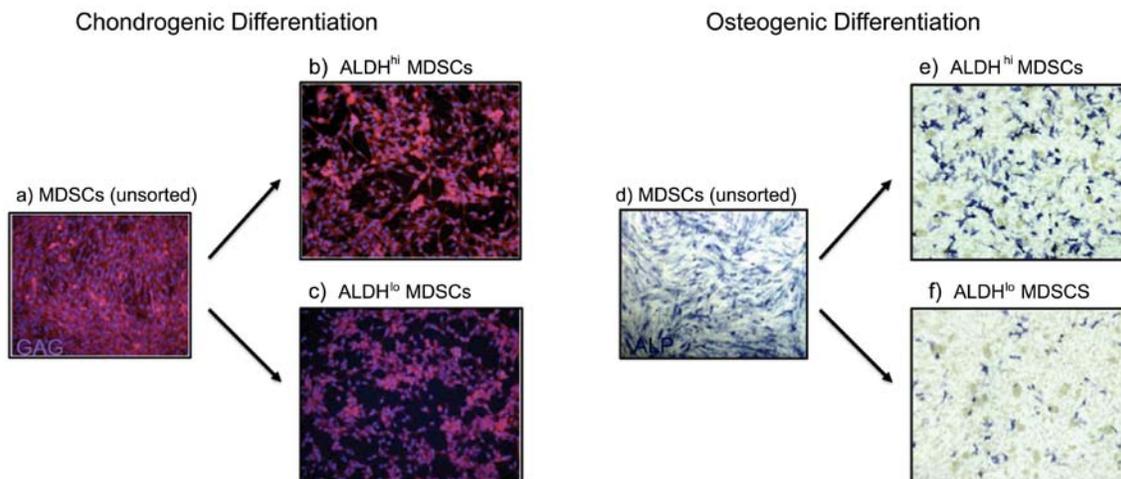
Figure 29. Osteogenic and chondrogenic capacity of ALDH sorted MDSCs

(a-c) Osteogenic differentiation of ALDH sorted MDSCs was induced in vitro by BMP-4 stimulation. Alkaline phosphatase (ALP, shown in blue) levels were increased in ALDH[hi] MDSCs compared to ALDH[lo] MDSCs. (d-f) However no observable differences in the chondrogenic differentiation potential of ALDH[hi] MDSCs could be shown by collagen II immunostaining (seen in red).

3.6   DISCUSSION

Our recent research on the muscle regenerative capacity of various myogenic progenitors suggests that stress resistance is predictive of muscle regeneration capacity[52,74,130]. Following transplantation, precipitous and acute cell loss is observed which has been attributed to inflammation, anoikis, and ischemia[200]. We have shown that by modulating the anti-oxidant levels of MDSCs we can alter its skeletal muscle regenerative capacity both in cardiac and skeletal muscle[52,130]. Similar studies using anti-oxidant and anti-inflammatory treatments such as SOD-mimetics[162], anti-inflammatories[45], such as anti-TNF-α and anti-neutrophil antibodies[277] further demonstrate the importance of stress resistance in the context of cell survival in cell therapy. While numerous immunosuppressive and anti-oxidant modalities have been identified to

increase cell survival following transplantation, cellular candidates for transplantation may also be screened for or isolated by their inherent survival capabilities[200,216]. It is this approach that has motivated the present study as well as other ongoing studies in our group.

The present study was motivated by an interest in isolating a myogenic progenitor population with elevated stress resistance capacity. We observed an enrichment of ALDH^{hi} cells in late preplate cells, a population with a known elevated oxidative stress resistance, which led us to hypothesize that ALDH may be a marker for elevated stress resistance. As discussed previously, this hypothesis was supported by evidence of enhanced stress resistance in ALDH^{hi} cells found in other cell lineages. Following transplantation, cell competence in terms of survival, proliferation, and myogenic differentiation largely determines efficacy in skeletal muscle regeneration. The conditions that donor cells encounter upon transplantation are deleterious to survival and tissue specific differentiation, as the host inflammatory process is initiated within minutes, exposing the cells to the oxidative stress from cytotoxic radicals and pro-inflammatory cytokines[44].

The improved proliferative capacity of ALDH[hi] myoblasts and hMDCs over that of ALDH[lo] myoblasts and hMDCs in the presence of H_2O_2 and TNF-α in vitro suggests that the ALDH^{hi} cells will have an increased proliferative capacity under conditions of inflammation in vivo. Similarly, an increased differentiation capacity in conditions of oxidative and inflammatory stress is likely to translate to an improvement in the differentiation capacity when the cells are exposed to post-transplantation inflammation. Indeed we observed improved muscle regeneration in muscles injected with ALDH[hi] myoblasts compared to ALDH[lo] myoblasts, which appears to be a function of improved survival, proliferation, and myogenic differentiation.

A possible mechanism for this improved regeneration capacity of ALDH^{hi} myogenic cells may be elevated intracellular levels of antioxidants such as GSH and SOD, an observation that was also made previously in MDSCs[52]. This hypothesis is supported by the finding that increasing the anti-oxidant levels of ALDH^{lo} myoblasts by treating them with XJB improved their proliferative capacity to a similar level observed in the ALDH^{hi} myoblasts. Furthermore, decreasing the GSH anti-oxidant levels of ALDH^{hi} cells by treating them with DEM, inhibited their proliferative capacity to a similar level observed in the ALDH^{lo} myoblasts.

While cytosolic ALDH (ALDH1A1) may be considered a detoxifying enzyme and an antioxidant in ocular tissues such the cornea and lens, it does not appear to actively participate in the antioxidant capacity of the cells studied here. ALDH eliminates aldehydes by an oxidation reaction that simultaneously reduces NADP$^+$ to NADPH, a cofactor for glutathione reductase recycling of GSH. However, ALDH does not appear to functionally mediate stress resistance in the cells isolated in this study, given that DEAB inhibition did not affect the stress resistance capacity of the ALDH^{hi} myoblasts.

That a similar improvement in stress resistance was not observed in ALDH^{hi} MDSCs suggests that MDSCs already have an elevated stress resistance capacity that is not further enhanced by ALDH sorting. Ongoing studies of ALDH sorting of freshly digested muscle may further elucidate the relationship between MDSCs and ALDH^{hi} muscle derived cells.

Osteogenic and chondrogenic lineage potential was shown to be substantially greater in ALDH^{hi} myoblasts than in ALDH^{lo} myoblasts; however, we posit that this result may simply be explained by an increased capacity of these cells to survive the chondrogenic and osteogenic media conditions, given the poor proliferation of ALDH^{lo} myoblasts in chondrogenic media and inability to form a dense pellet. ALDH^{hi} MDSCs underwent osteogenic differentiation at greater

rate than ALDH[lo] MDSCs however no significant difference was observed in their chondrogenic differentiation capacity. Further experiments may be required to understand this observation, particularly given that no obvious difference in stress resistance was observed between these two MDSC populations.

While numerous myogenic cells have been identified for muscle cell therapies, identification of definitive or even desirable markers for myogenic progenitor isolation remains elusive[45]. In our experience, a common feature of myogenic progenitors with high regeneration potential lies in their increased resistance to oxidative and inflammatory stress[52,74]. That is, these cells not only resist apoptosis, but they maintain increased rates of proliferation and myogenic differentiation capacity. In the current study, we isolated ALDH[hi] and ALDH[lo] subpopulations of cultured murine myoblasts and MDSCs in addition to human muscle derived cells. The utility of isolating myogenic cells with elevated ALDH expression is two-fold. Myogenic progenitors may be rapidly isolated from heterogeneous populations of muscle derived cells using a simple intracellular dye, Aldeflour. In addition, ALDH may be used as a marker to identify cells with an increased survival capacity when exposed to oxidative and inflammatory stress. Our studies suggest that increased survival and stress resistance is a primary but not often studied determinant of effective cell therapy. In our continued work, we seek a greater understanding of the mechanisms responsible for increased regeneration capacity and an understanding of what allows some progenitors greater survival abilities in the presence of oxidative and pro-inflammatory environments. The data presented in this study suggests that ALDH can be used as a marker to identify progenitors that are not only myogenic but have elevated oxidative and inflammatory stress resistance.

4.0   SPECIFIC AIM 3: ALDH ACTIVITY AS A MARKER FOR MYOGENIC CAPACITY AND STRESS RESISTANCE IN HUMAN MUSCLE DERIVED CELLS

4.1   BACKGROUND

4.1.1   Aldehyde dehydrogenase as a marker for early progenitor status in human cells

Enhanced ALDH activity has been observed in primitive progenitors of hematopeitic[180,181], mesenchymal[182], endothelial[183], and neural[184,185] lineages.   ALDH expression is associated with retinoic acid metabolism, a mediator of stem cell maintenance and differentiation, and confers oxazaphosphorine chemotherapy resistance in metastasis competent carcinoma stem cells[151,187,188]. Cells with elevated ALDH levels become fluorescent when exposed to boron-dipyrromethene (BODIPY) labeled amino acetaldehyde (BAAA) and can be isolated using fluorescence activated cell sorting (FACS). BAAA diffuses freely into the cytoplasm and is converted to BIODIPY-aminoacetate by ALDH, specifically ALDH1A1[189].

Vauchez et al. recently identified an ALDH$^+$ myogenic progenitor population in skeletal muscle, which occupies an endomysial niche similar to that of the satellite cell[186]. This group isolated ALDH$^+$ cells from human muscle biopsies and demonstrated their myogenic potential both in vitro and in vivo. Furthermore, they demonstrated that the ALDH$^+$CD34$^-$ (and CD56+) cells in this population were responsible for this myogenic potential. Another important result

from this paper was the robust engraftment of these ALDH$^+$CD34$^-$ cells in the skeletal muscle of SCID mice. They estimated that the engrafted cell population at 4 weeks post-transplantation was roughly 2-5 times the number of cells that were injected. These results are summarized in Figure 30 below. Although these results were very similar to our study on ALDH^{hi} muscle derived cells, our study focuses on the potential of these cells from myogenic therapy, particularly on the cells ability to withstand oxidative and inflammatory stress, and whether this sub-population of cells indeed demonstrate elevated regeneration capacity over the ALDH^{lo} subpopulation.

Vauchez, K., et al., *Mol Ther*, 2009. **17(11): p. 1948–1958.**
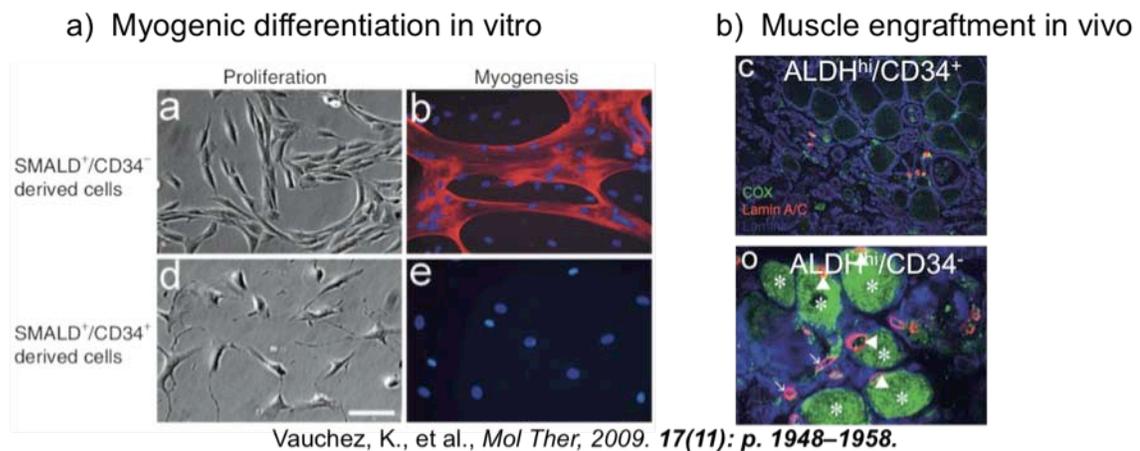
Figure 30. ALDH$^+$ myogenic progenitors isolated from human skeletal muscle
(a) Vauchez isolated ALDH$^+$ (SMALD$^+$ stands for Skeletal Muscle ALdehyde Dehydrogenase) cells from human skeletal muscle and demonstrated myogenic potential in vitro of ALDH$^+$CD34$^-$. (b) When these sub-populations were injected intramuscularly in to SCID mice, ALDH$^+$CD34$^-$ not only fused with existing myofibers but also took up residence in an endomysial position (indicated by arrows) similar to that occupied by the satellite cell. However, ALDH$^+$CD34$^+$ cells demonstrated poor engraftment and no myofiber fusion.

The co-authors identified the prevalence of several key surface markers of the ALDH and CD34 sorted skeletal muscle derive cells listed in Table 1[186]. Much of the ALDH sorting literature is devoted to isolating hematopoietic stem cells, which commonly uses the CD34

surface marker for identification of primitive hematopoietic cells. The co-authors sought to determine whether this is also an important marker for muscle progenitors as well. Therefore they isolated four subpopulations of dissociated muscle biopsy derived cells based on ALDH and CD34. A few important findings were described here. The majority of the muscle derived cells were SMALD⁻CD34⁻, which also coincided with the majority of the CD56 expressing cells. Each subpopulation was clearly heterogeneous in terms of lineage commitment because typically only a minority of cells from each sub-population were positive for the markers analyzed (CD31, CD44, CD45, CD56, CD90, CD105, and CD104b). The SMALD⁺CD34⁺ population was negative for CD45, CD56, CD105, and largely for CD31 as well. Whereas the SMALD⁺CD34⁻ population expressed a larger proportion of CD44⁺ cells than the SMALD⁺CD34⁺ cells. It should be noted that both SMALD⁺ sub-populations expressed no CD56⁺ unlike the double negative population, suggesting that the SMALD⁺ may not have made a myogenic lineage commitment at the time of isolation.

Table 1. Human ALDH sorted skeletal muscle cells surface marker analysis[186]

Populations[a]	Markers						
	CD31⁺ $n = 3$[b]	CD44⁺ $n = 4$	CD45⁺ $n = 3$	CD56⁺ $n = 12$	CD90⁺ $n = 5$	CD105⁺ $n = 4$	CD140b⁺ $n = 3$
SMALD⁺/CD34⁻	0%	19.6%[c] (0.0–51.0)	0%	0%	5.9% (1.2–8.5)	1.5% (0.0–2.6)	0%
SMALD⁺/CD34⁺	<1%	1.0% (0.0–1.5)	0%	0%	6.6% (0.7–39.0)	0%	1.6% (0.2–1.6)
SMALD⁻/CD34⁺	26.9% (5.1–55.5)	<1%	<1%	1.4% (0.0–21.5)	5.3% (3.5–16.9)	7.4% (2.0–27.7)	0%
SMALD⁻/CD34⁻	<1%	<1%	2.7% (0.7–4.2)	2.8% (0.4–43.4)	2.6% (1.8–21.8)	1.4% (0.1–3.1)	<1%

Abbreviation: SMALD, skeletal muscle aldehyde dehydrogenase.
[a]The four sub-populations were defined by ALDH activity and CD34 expression and analyzed for coexpression of extracellular markers. [b]Number of biopsies analyzed.
[c]Values correspond to median percentages. Higher and lower values are given within parentheses.

We have isolated similar populations of human ALDH^hi myogenic progenitors from unfractionated human muscle cells. As in our murine myogenic progenitor study, we hypothesized that *increased ALDH activity may identify myogenic progenitors with increased oxidative and inflammatory stress resistance.* As discussed previously, we believe this *increased stress resistance may confer an increased engraftment and muscle regeneration capacity over*

ALDH^{lo} and unsorted muscle derived populations. Like Vauchez et al, we observed survival of these ALDH^{hi} cells in myogenic culture conditions and therefore sought to isolate ALDH^{hi} cells from cultured myogenic progenitors.

4.2   RATIONALE

While Vauchez et al. observed increased myogenic capacity in an CD34^{+}ALDH^{hi} subpopulation of muscle derived cells, the authors did not address why this might be the case or whether ALDH alone is a marker for increased myogenic potential[186]. We seek to determine the whether anti-oxidant capacity or stress resistance plays a role in the myogenic capacity of human ALDH^{hi} cells. We have isolated ALDH^{hi} and ALDH^{lo} populations from human and murine muscle derived cells (hMDCs and mMDCs). We hypothesized that increased ALDH activity may identify myogenic progenitors with increased stress resistance thus conferring an increased engraftment and muscle regeneration capacity in vivo over the ALDH^{lo} and unsorted MDC populations. We sought to identify their intracellular anti-oxidant levels of ALDH^{hi} and ALDH^{lo} in terms of GSH and SOD. This tolerance will also be evaluated in terms of intracellular ROS accumulation.

4.3   EXPERIMENTAL DESIGN

ALDH^{hi} and ALDH^{lo} populations of hMDCs were isolated using fluorescence activated cell sorting (FACS). The proliferation and differentiation potential of these populations in the

presence of oxidative (H_2O_2) and inflammatory stress (TNF-α) were evaluated in vitro and compared to the unsorted hDMC population. The anti-oxidant levels in terms of glutathione and superoxide dismutase were evaluated and compared to the unsorted populated. Reactive oxygen species accumulation during oxidative challenge (H_2O_2) was measured as well, as depicted in the schematic in Figure 31 below:
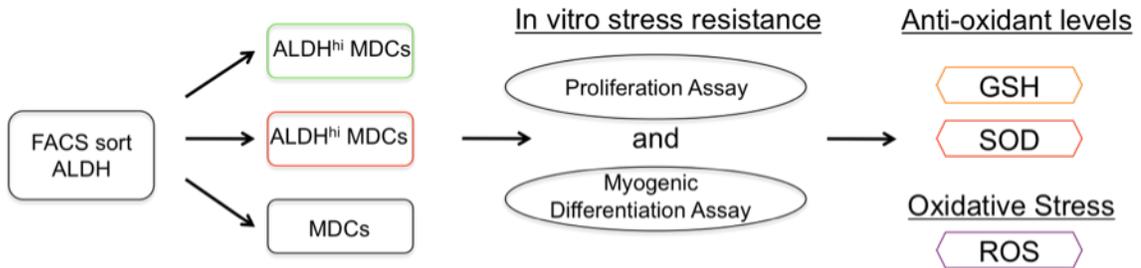
Figure 31. Experimental schematic to examine the stress resistance capacity of ALDH sorted hMDCs
ALDH^{lo} and ALDH^{hi} subpopulations of muscle derived cells (MDCs), human in this case, was isolated by fluorescent activated cells sorting (FACS). Proliferative and myogenic differentiation capacity was quantified in conditions of oxidative (H_2O_2) and inflammatory stress (TNF-α, not shown in schematic). Intracellular anti-oxidant levels, specifically reduced glutathione (GSH) and super-oxide dismutase (SOD) was assessed in each ALDH sorted subpopulation of hMDCs. The oxidative stress handling capacity of hMDCs was assessed during oxidative stress (H_2O_2) challenge by measuring changes in the intracellular reactive oxygen species (ROS) concentration.

The skeletal muscle regeneration capacity of the ALDH^{hi} and ALDH^{lo} subpopulations was evaluated in the gastrocnemius of an *mdx* mouse model. To conform to relevant in vivo studies of human myoendothelial cells[74], ALDH sorted hMDCs were transplanted into male *mdx*/SCID mice, as seen in the schematic in Figure 32 below:
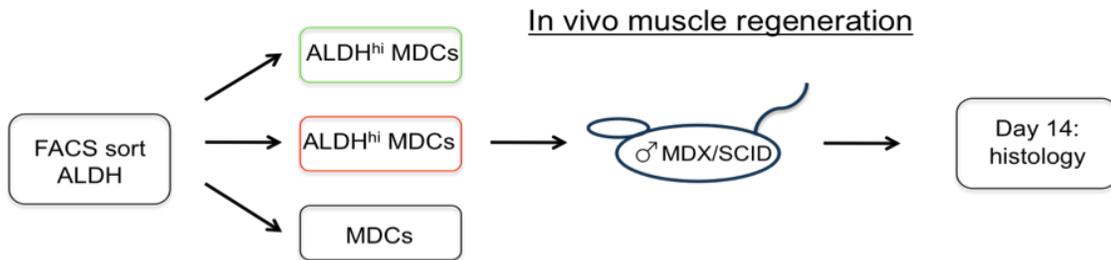
Figure 32. Experimental schematic to examine the muscle regenerative capacity of ALDH sorted hMDCs ALDH^{lo} and ALDH^{hi} hMDCs was injected directly into the gastrocnemius muscles of female *mdx* mice aged 6-8 weeks. The regeneration index was quantified via histological evaluation of the rate of dystrophin expression of cryosectioned muscle 14 days post-injection.

4.4   METHODS

4.4.1   Human muscle cell isolation

Skeletal muscle biopsies obtained during surgery, were placed on ice in Hanks' balanced salt solution (HBSS),finely minced, and digested for 60 min at 37 °C with type-I and type-IV collagenases (100 mg/ml) and dispase (1.2 mg/ml). Digested tissue was pelleted and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), triturated and then passed through a 40-mm filter to obtain a single cell suspension. Cells were either sorted by FACS or placed in collagen coated flasks in a proliferation media and cultured at 37°C and 5% CO_2.

4.4.2   Flow cytometric analysis and sorting by ALDH activity

Cultured human muscle derived cells were trypsinized, washed in cold PBS, and counted using a hemocytometer. To analyze and sort hDMCs on the basis of ALDH activity, cells (n=10^6) of each population were resuspended in ALDEFLUOR buffer and incubated at 37°C with BAAA according to manufacturer's instructions (Aldagen Inc, Durham, NC). Cells were washed in ALDEFLUOR buffer and maintained in 4°C. ALDH activity was assessed using the FL1 channel of a BD FACSAria Cell Sorting System and FACSDiva software. Collected cells were gated on their ALDH activity levels, as determined by a DEAB inhibited control sample, and low side scatter (SCC^{lo}).

4.4.3   In vitro proliferation capacity

Time-lapse live cell microscopy were employed to monitor the rate of proliferation under conditions of oxidative and inflammatory stress[246,247]. Sorted hMDCs were plated in collagen-coated 24-well plates at an initial density of 1000 cells/cm^2 in proliferation medium and incubated under normal conditions for 24hrs. Stress conditions of oxidative (250μM, H_2O_2) and inflammatory stress (2.5ng/ml, TNF-α) in PM were initiated 30min prior to placing the plate on live cell imaging incubator. Cell proliferation was quantified at 30min intervals in three 10x microscope fields over a period 48 hrs.

4.4.4   In vitro muscle differentiation capacity

Cells were plated at an initial density of 1000 cells/cm^2 in 24-well plates and allowed to proliferate for 2d or until near confluence in proliferation medium. Proliferation media were exchanged for differentiation media treated to simulate oxidative stress (0μM, 100μM, 250μM, and 500μM, H$_2$O$_2$) and inflammatory stress (1ng/ml, 2.5ng/ml, and 5ng/ml, TNF-α) and exchanged daily to maintain initial stress levels[52]. Cells were allowed to differentiate and fuse for 4d then fixed with cold methanol (-20°C). Differentiation of myogenic cells into myosin heavy chain (fast) expressing myotubes was quantified using the MDI metric described previously.

4.4.5   Intracellular antioxidant capacity

The antioxidant capacity was measured in terms of the major intracellular anti-oxidant molecules GSH and SOD activity as well as intracellular reactive oxygen species accumulation during H$_2$O$_2$ challenge. Levels of GSH were measured colorimetrically (Calbiochem, 354102) using a spectrophotometer (TECAN Infinite M200). Total activity of SOD was measured using a colorimetric assay (Chemicon, Temecula, CA; APT290) from cell lysates according to the manufacturer's directions. Intracellular ROS levels were measured fluorometrically using carboxy-H$_2$DCFDA. Briefly, cells were plated at an initial density of 1000 cells/cm^2 in 24 well plates and incubated for 24h. The cells were loaded with 5 mM carboxy-H$_2$DCFDA for 30 min in proliferation media, washed, and exposed to 250 μM H$_2$O$_2$ in proliferation medium. Cell fluorescence intensity was measured at 30 min intervals for 4 hrs using a fluorometric plate reader.

4.4.6   Skeletal muscle regeneration capacity

Sorted human muscle cells were injected into the gastrocnemius muscles (n=4) of male *mdx*/SCID mice (aged 6–8 weeks), using the protocol described previously. This experiment will require 16 mice per cell type (experimental groups include unsorted cells, ALDH^{hi}, ALDH^{lo}, and PBS control groups).

4.4.7   Statistical Analysis

 Data are expressed as a mean with its standard deviation. Direct comparisons between two cell populations were made using an unpaired, two-tailed Student's *t* test, where statistical significance was determined at a level $p < 0.05$.   Comparisons of multiple groups were completed using one-way ANOVA followed by *Tukey* post-hoc comparisons.

4.5   RESULTS

4.5.1   Stress resistance of ALDH sorted hMDCs

Following FACS isolation of ALDH^{hi} and ALDH^{lo} subpopulations of hDMCs, oxidative (H_2O_2) and inflammatory (TNF-α) stress resistance during proliferation was quantified (Figure 33a and b). The proliferation rate of ALDH^{lo} and ALDH^{hi} subpopulations of hMDCs was quantified

using live cell imaging microscopy in conditions of oxidative (250μM, H_2O_2) and inflammatory

(2.5ng/ml, TNF-α) stress. A significant proliferation advantage of ALDH[hi] hMDCs was

observed when normalized cell numbers are compared to ALDH[lo] hMDCs in both stress

conditions.
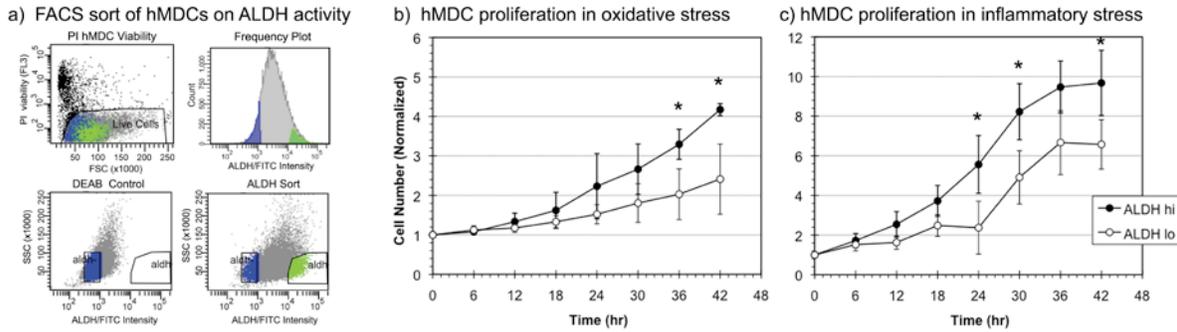
Figure 33. FACS sorting of hMDCs on ALDH activity and ALDH sorted hMDC proliferation in oxidative and inflammatory stress

(a) A significantly increased rate of proliferation of ALDH[hi] hMDCs compared to ALDH[lo] hMDCs was observed in conditions of oxidative stress (H_2O_2, 250μM) (* indicates $p < 0.05$). (b) Similarly ALDH[hi] hMDCs demonstrated elevated proliferative capacity in inflammatory stress conditions (TNF-α, 2.5ng/ml).

The myogenic differentiation index (MDI), a measure the proportion of cells expressing of

myosin heavy chain (MHC), of ALDH[hi] and ALDH[lo] hMDCs was quantified in conditions of

oxidative (H_2O_2) and inflammatory (TNF-α) stress. A significantly increased number of myosin

heavy chain expressing cells was observed in ALDH[hi] hDMCs compared to ALDH[lo] hMDCs

(Figure 34).
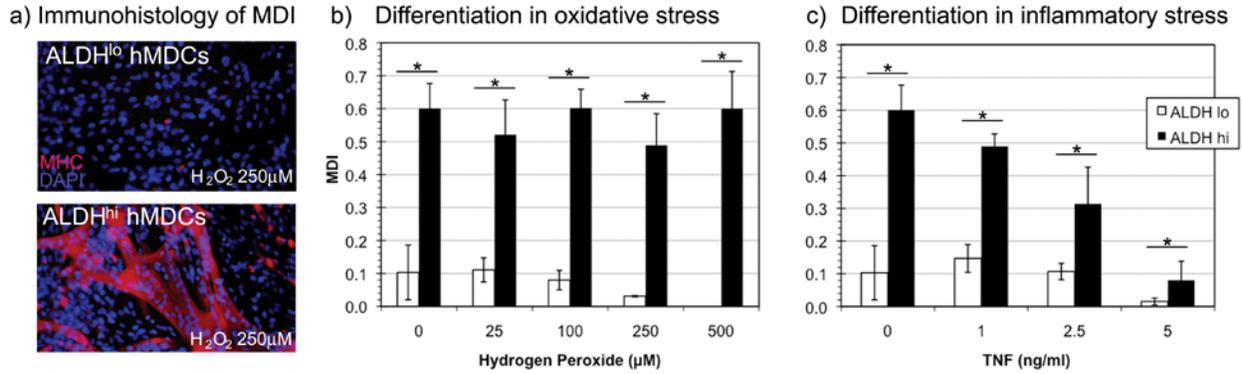
Figure 34. Myogenic differentiation of ALDH sorted hMDCs in oxidative and inflammatory stress
(a) ALDH[lo] and ALDH[hi] hDMCs underwent myogenic differentiation by fusing into MHC[+] myotubes (red) under oxidative stress conditions (H_2O_2, 250 μM). Nuclei are stained with DAPI (blue). (b) Significantly increased myogenic differentiation indices (MDI) were observed ($p < 0.05$) in ALDH[hi] hMDCs all concentrations of oxidative (H_2O_2) and inflammatory stress (TNF-α, 2.5/ml) when compared to ALDH[lo] hDMCs.

4.5.2 Skeletal muscle regeneration capacity of ALDH sorted hMDCs

ALDH[hi] and ALDH[lo] hMDCs injected intramuscularly in the gastrocnemius of *mdx/*SCID mice yielded robust engraftment and formation of dystrophin expressing myofibers (Figure 35). The extent of myofiber formation was quantified using the regeneration index metric, a measure of DYS[+] myofibers in a cryosectioned muscle per 10^5 cells injected, as described previously[52]. Significantly increased regeneration indices were observed in ALDH[hi] hMDCs compared to ALDH[lo] hMDCs.

a) Immunohistology of RI

b) Regeneration capacity of ALDH sorted hDMCs
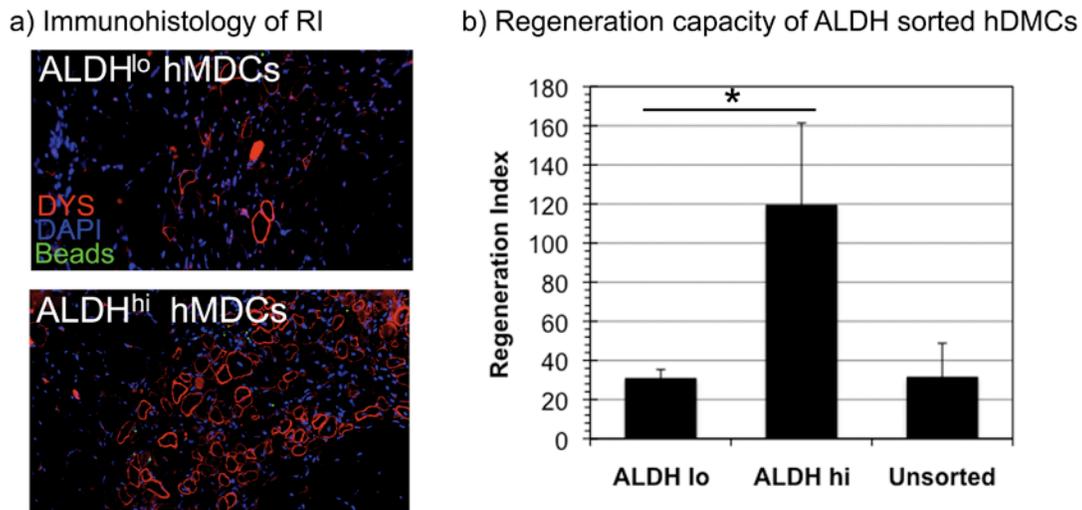
Figure 35. Skeletal muscle regeneration capacity of ALDH sorted hMDCs

(a) ALDH sorted hMDCs (2 x 10^5 cells) were injected intramuscularly into the gastrocnemius of *mdx* /SCID mice and analyzed after 14days. An increased number and density of dystrophin positive myofibers were observed in mice injected with ALDH^hi cells compared to those injected with ALDH^lo myoblasts. Dystrophin (DYS) positive myofibers (stained in red) indicate transplanted ALDH^lo cell myofiber generation or fusion with a host myofiber. Nuclei are DAPI (blue) stained. Fluorescent green beads can be observed to localize the area of initial injection. (c) Significantly increased regeneration index was observed in ALDH^hi hDMC transplanted mice compared to those injected with ALDH^lo and unsorted hMDCs. (* indicates $p < 0.05$).

4.5.3   Stress resistance capacity of the ALDH sorted hMDCs

To understand the increased stress resistance capacity of the ALDH^hi hMDCs, we examined two major intracellular antioxidants, reduced glutathione GSH and SOD. Although a trend of increased GSH was observed in ALDH^hi hMDCs compared to ALDH^lo, this trend was not statistically significant, however a significant difference in intracellular SOD was observed (Figure 36).

94
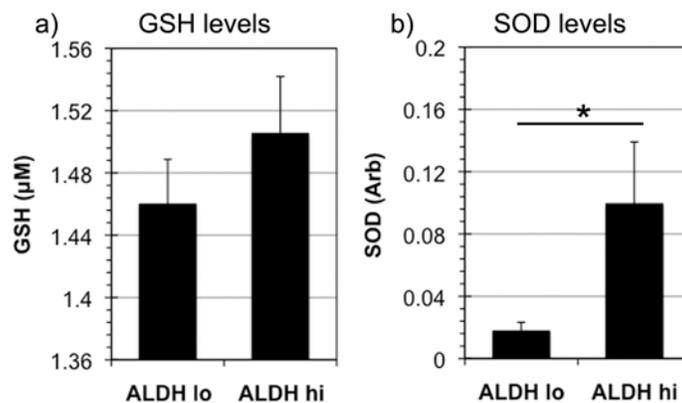
Figure 36. Intracellular anti-oxidant (GSH and SOD) levels of ALDH sorted hDMCs
(a-b) Measurements of intracellular antioxidant levels in terms of glutathione (GSH) and superoxide dismutase (SOD) levels demonstrated a trend in increased GSH levels (not statistically significant) and significant elevation of SOD in ALDH^{hi} hMDCs compared to ALDH^{lo} myoblasts. (* indicates $p < 0.05$).

The accumulation of intracellular ROS during oxidative stress challenge was used as a metric of oxidative stress handling. Intracellular ROS in ALDH^{hi} hMDCs was significantly less than ALDH^{lo} and unsorted hMDCs following hydrogen peroxide exposure (250μM, H_2O_2) at timepoints of 150min and beyond (Figure 37a). Similarly, decreased ROS accumulation in ALDH^{hi} hMDCs compared to ALDH^{lo} was observed at increasing oxidative stress levels (25-500μM H_2O_2) as illustrated in Figure 37b.
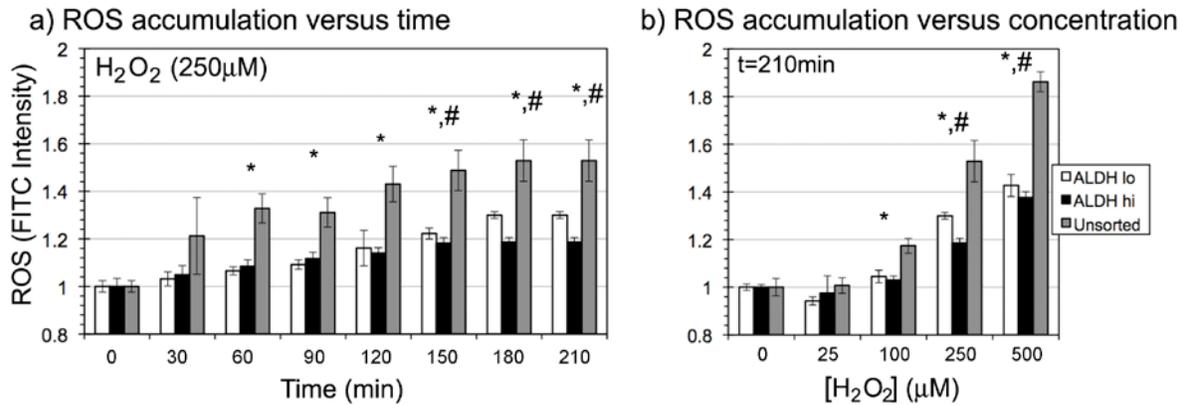
Figure 37. Reactive oxygen species accumulation in hMDCs during oxidative stress

Reactive oxygen species (ROS) accumulation in ALDH sorted hMDCs was measured during oxidative stress exposure (H_2O_2, 250μM). (a) Decreased ROS accumulation as a function of time was observed in ALDH[hi] hMDCs when compared to ALDH[lo] hMDCs (# indicates $p > 0.05$) and unsorted hDMCs (* indicates $p > 0.05$). (b) Similar trends in ROS accumulation was observed at different oxidative stress conditions, where significantly less intracellular ROS levels were observed in ALDH[hi] hMDCs compared to both ALDH[lo] hMDCs and unsorted hMDCs.

4.5.4   Osteogenic and chondrogenic differentiation potential of ALDH sorted hMDCs

In order to determine whether ALDH[hi] hMDCs elevated differentiation capacity is limited to myogenic differentiation, we studied the osteogenic and chondrogenic differentiation of ALDH sorted hMDCs. Osteogenic differentiation was induced in plated cells via BMP-4 stimulation. Because alkaline phosphatase was observed in all hMDCs even without prior BMP-4 stimulation, osteocalcin was used as a marker of osteogenic differentiation. However even in this case no observable difference in osteogenic differentiation capacity was observed in ALDH sorted hMDCs (Figure 38 a-c). Chondrogenic differentiation of plated, ALDH sorted hMDCs was induced in chondrogenic media and qualified via collagen II immunohistochemical staining. No increased collagen II positivity was observed in ALDHhi compared to ALDH[lo] or unsorted hMDCs (Figure 38 d-f). The high background observed in both of these stains has raised some

concern in terms of how to interpret this result. Therefore studies are underway to confirm this result.
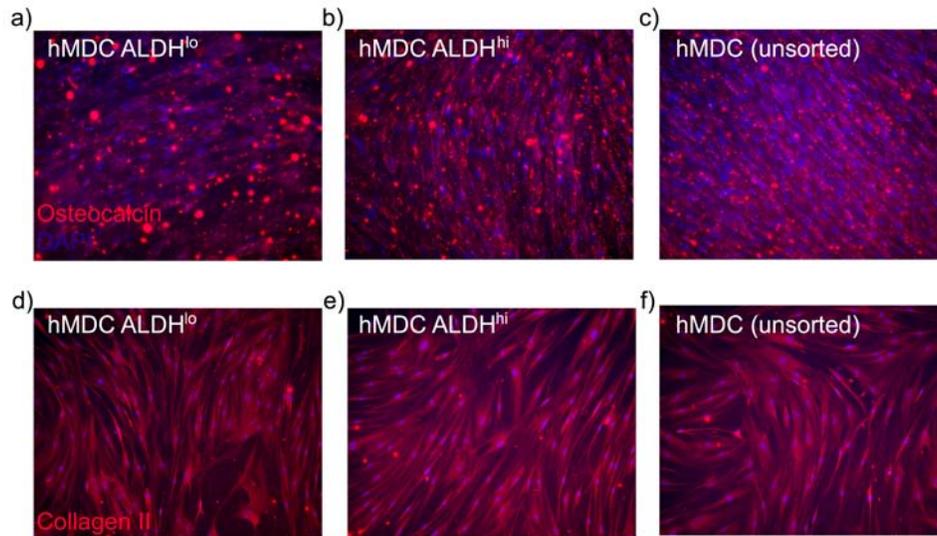
Figure 38. Osteogenic and chondrogenic differentiation capacity if ALDH sorted hMDCs in vitro
(a-c) Osteogenic differentiation of ALDH sorted MDSCs was induced in vitro by BMP-4 stimulation. Osteocalcin (shown in red with blue DAPI nuclear counterstain) immunostaining did not indicate significant changes in osteogenic differentiation capacity in ALDH sorted hMDCs. (d-f) Similarly no observable differences in the chondrogenic differentiation potential of ALDH[hi] MDSCs could be shown by collagen II immunostaining (shown in red).

4.6   DISCUSSION

In the current study, we have isolated ALDH[hi] and ALDH[lo] subpopulations of cultured human muscle derived cells. The isolation of ALDH[hi] progenitors yields a population of myogenic cells with enhanced oxidative and inflammatory stress resistance, which has been shown by our group to yield improved rates of engraftment and tissue regeneration. This works adds to the growing

view of aldehyde dehydrogenase as a marker of early progenitor status among multiple lineages of human cells.

The increased myogenic and stress resistance capacity of ALDH[hi] hMDCs also supports the earlier work of Vauchez et al; however, we posit that the increased stress resistance capacity of these cells plays a significant, and yet uncharacterized, role in the increased regenerative capacity of this sub-population of human muscle cells[186]. While Vauchez et al. isolated myogenic progenitors from freshly dissociated human muscle on the basis of elevated ALDH activity and CD34-negativity, we isolated ALDH^{hi} cells from un-fractionated human muscle derived cells previously expanded in culture or hMDCs. Primary human muscle cultures include a heterogeneous population of myoblasts, endothelial cells, fibroblasts and a multitude of other cell types; therefore, it is perhaps not surprising that we observed greater differences in oxidative stress resistance between ALDH[hi] and ALDH[lo] hMDCs given this heterogeneity. Elevated ALDH activity can effectively be used to select for myogenic progenitors that can more effectively withstand the effects of oxidative and inflammatory stressors from a heterogeneous population of cultured hMDCs. This result suggests that ALDH sorting of cultured human muscle derived cells may be as effective as sorting freshly digested muscle and selecting for ALDH positivity and CD34-negativity[186].

We observed that the behavior of ALDH sorted hMDCs was quite similar to that observed in murine myoblasts in terms of differences in stress resistance in vitro as well as regeneration indices in vivo. A significant proliferation advantage of ALDH^{hi} cells was observed in human hMDCs when exposed to either oxidative or inflammatory stress conditions. Similarly, a significantly increased MDI was observed for ALDH[hi] hMDCs at all oxidative and inflammatory stress doses, except for two lower doses of oxidative stress (25µM and 100µM,

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H$_2$O$_2$). In vivo, a significantly increased regeneration index was observed in ALDH^{hi} hMDCs transplanted into *mdx/*SCID mice compared to those injected with ALDH^{lo} and unsorted hMDCs. Although a trend of increased GSH was observed in ALDH^{hi} hMDCs compared to ALDH^{lo}, this trend was not significant, however a significant difference in intracellular SOD was observed.

Following transplantation, cell competence in terms of survival, proliferation, and myogenic differentiation will determine efficacy in skeletal muscle regeneration. The conditions the cell encounters upon injection may be hostile to these functions, as the host inflammatory process is initiated within minutes, which exposes the cells to hypoxia, numerous cytotoxic radicals, and pro-inflammatory cytokines. The improved proliferative capacity of ALDH^{hi} hMDCs over that of ALDH^{lo} hMDCs in the presence of H$_2$O$_2$ and TNF-a suggests that the ALDH^{hi} cells may have an increased proliferative capacity under conditions of neutrophil degranulation and other mediators of inflammation in the hours and days following transplantation.

Indeed, a strong correlation of stress resistance in vitro and regeneration index in vivo was observed. That is, ALDH sorting conferred a significant advantage in stress resistance in ALDH^{hi} hMDCs compared to ALDH^{lo} hMDCs which we believe is primarily responsible for the significant improvement in regeneration index when injected into the gastrocnemius muscle of the SCID mouse.

To determine if the enhanced proliferation and myogenic differentiation capacity of ALDH^{hi} cells in conditions of stress may be the result of increased intracellular anti-oxidants, we measured elevated levels of GSH and SOD in the ALDH^{hi} hMDC populations. This result should be viewed in the context of studies of the redox signaling in the differentiation of primitive progenitors in which numerous cell lineages exhibit a trend of earlier progenitors

maintaining a more reduced intracellular environment than their more differentiated counterparts[225]. Therefore this result not only suggests a mechanism for improved stress resistance but also lends evidence to elevated ALDH being a marker for more primitive cell status.

Osteogenic and chondrogenic lineage potential has yet to be adequately characterized and is the focus of ongoing studies. Our preliminary results to date do not suggest a difference in either lineage differentiation potential between ALDH^{lo} and ALDH^{hi} sub-populations of hMDCs. Ongoing work includes confirming the osteogenic and chondrogenic assay results shown in Figure 38. Given the substantial difference in stress resistance capacity between ALDH^{lo} and ALDH^{hi} hMDCs, we hypothesize that we will observe increased osteogenic and chondrogenic potential of ALDH^{hi} hMDCs as we observed in ALDH sorted murine myoblasts.

Of the numerous myogenic cells that have been identified as candidates for myogenic cell therapies, it is our view that the common feature of myogenic progenitors with high regeneration potential lies in their increased resistance to oxidative and inflammatory stress[45,52,74]. A vital criterion of myogenic cells is not only increased resistance to apoptosis, but increased rates of proliferation and myogenic differentiation capacity in conditions of stress. In the current study, we isolated ALDH^{hi} and ALDH^{lo} subpopulations of cultured human muscle derived cells to study these properties. Indeed, stress resistance appears to play an important role in the muscle regeneration capacity of ALDH^{hi} hMDCs.

Our studies suggest that increased survival and stress resistance is a major, but not often studied, determinant of effective cell therapy. Although it has been known for many years that stem and progenitor cells display unique behavior such as self-renewing and long term proliferation in addition to multipotent differentiation, our results suggest that survival and

resistance to stress should be added to that list[228]. These results suggest that stem cell behavior likely represents a better determinant for stem cell therapy than the use of marker profiles that identify a given cell population. In our continued work, we seek a greater understanding of the mechanisms responsible for increased regeneration capacity and an understanding of what allows some progenitors greater survival abilities in the presence of oxidative and pro-inflammatory environments. The data presented in this study suggests that ALDH can be used as a marker to identify progenitors that are not only myogenic but have elevated oxidative and inflammatory stress resistance.

5.0   DISSERTATION CONCLUSIONS

Myogenic cell therapies are based on the rationale that muscle regeneration may be induced or augmented by increasing the native myogenic progenitor or stem cell reserve. However, engraftment efficiency in myogenic cell transplantation is impaired by a precipitous loss of cell viability following transplantation that is associated with the oxidative and inflammatory stress environment of inflammation. This dissertation describes the isolation and characterization of murine and human populations of muscle derived cells that have an enhanced capacity to survive similar stress conditions induced by inflammation.

In the first specific aim of this dissertation (chapter 2), I examined the role stress resistance and survival during myogenic differentiation and muscle regeneration capacity of MDSCs and myoblasts derived from a modified preplate technique. As an extension of the recent publications of Urish et al. and Drowley et al., I sought to clarify the role of intracellular antioxidant capacity of myogenic cells using a mitochondrial targeted reactive oxygen species scavenger, XJB-5-131[52,262]. These studies suggest that survival during myogenic differentiation is an important is an important determinant of differentiation efficiency that should be considered when choosing a myogenic cell for cell therapeutic applications. Furthermore, it is clear that XJB anti-oxidant pre-treatment of cells can improve cell survival and myogenic differentiation in normal and oxidative stress conditions. While I did not observe statistically significant differences in the muscle regeneration capacity in treated and untreated myoblasts and

MDSCs, the trends of improved regeneration suggest that anti-oxidant pre-treatment can improve the engraftment and regeneration capacity of transplanted cells. This reinforces the hypothesis that oxidative stress resistance is a primary determinant of muscle regeneration capacity.

In the second specific aim (Chapter 3), I describe the isolation ALDH^{hi} and ALDH^{lo} subpopulations of cultured murine myoblasts and MDSCs that in the case of ALDH^{hi} myoblasts yielded improved regeneration capacity over their ALDH^{lo} and unsorted myoblast counterparts. The utility of isolating myogenic cells with elevated ALDH expression is two-fold. Myogenic progenitors may be rapidly isolated from heterogeneous populations of muscle derived cells using a simple intracellular dye, Aldeflour. In addition, ALDH may be used as a marker to identify cells with an increased survival capacity in oxidative and inflammatory stress. The improved proliferative capacity of ALDH^{hi} myoblasts over that of ALDH^{lo} myoblasts in the presence of H_2O_2 and TNF-α suggests that the ALDH^{hi} cells are likely to have an increased proliferative capacity under conditions of inflammation that is seen in the hours and days following transplantation. That a similar improvement in proliferative capacity was not observed in ALDH sorted MDSCs suggests one of two conclusions, either that the MDSCs already have an elevated stress resistance capacity that is not further enhanced by ALDH sorting or that MDSCs do not have a significant degree of heterogeneity in ALDH expression to effectively segregate ALDH^{lo} and ALDH^{hi} sub-populations. Osteogenic and chondrogenic lineage potential was shown to be substantially greater in ALDH^{hi} myoblasts than in ALDH^{lo} myoblasts; however, we posit that this result may simply be explained by an increased capacity of these cells to survive the chondrogenic and osteogenic media conditions, given the poor proliferation of ALDH^{lo} myoblasts in chondrogenic media and inability to form a dense pellet.

In the third specific aim (chapter 4), I describe the isolation of ALDH^{hi} and ALDH^{lo} subpopulations of cultured human muscle derived cells (hMDCs). Similar to murine myoblasts, ALDH hMDC sorting isolated a sub-population of ALDH^{hi} hMDCs with improved regeneration capacity over their ALDH^{lo} and unsorted myoblast counterparts. This improved muscle regeneration capacity following cell transplantation into the skeletal muscle of *mdx*/SCID mice appears to be largely conferred by the increased oxidative and inflammatory stress resistance of ALDH^{hi} hMDCs compared to ALDH^{lo} and unsorted hMDCs. Vauchez et al. describes a methodology of isolation ALDH^{hi} cells from freshly dissociated skeletal muscle, however the study described in this dissertation suggests that cells with elevated stress resistance can also be isolated from cultured hMDCs. This is significant because one may not need to subject these cells to the process of flow cytometry in addition to the exposure to the numerous digestive enzymes required for cell dissociation.

These studies were aimed at understanding the importance of survival and stress tolerance of myogenic cells in skeletal muscle cell therapy and how this tolerance modifies the efficacy of cell therapy. Each of the three studies described here all suggest that stress tolerance is central to the efficacy of cell therapy. Increased stress tolerance can be induced by treatment with an anti-oxidant such as XJB-5-131 or cells with an inherent increase in stress tolerance can be isolated from a myogenic cell population on the basis of elevated ALDH activity.

6.0   OTHER RELATED PROJECTS

The following chapter to devoted to projects that are either currently underway, constitute collaborative efforts in which I took part or projects that were initiated and discontinued.

6.1   THE ROLE OF ANOIKIS RESISTANCE IN ISOLATING MDSCS

6.1.1   Background

Myogenic cell therapies have been limited by rapid and precipitous cell death within days of transplantation[41,200,201]. Several mechanisms for this loss have been proposed including inflammatory and oxidative stress at the injection site[18,41,127,202], hypoxia/ischemia[203], and anoikis (apoptosis induced by loss of extracellular matrix (ECM) or adjacent cell contact)[204,205,278]. Anoikis is a poorly studied phenomenon in myoblast transfer therapy although has received increased attention relatively recently. Bouchentouf et al. in 2007 observed myoblast survival in vivo could be enhanced several fold by the co-injection of fibronectin, the silencing of Bit1 and FADD expression, or by over expression of Bcl-2, all mediators of anoikis[205]. Similarly, Silva-Barbosa et al. found that similar increases in engraftment occurred with co-injection of laminins[279]. However, anoikis specific signaling molecules have not be comprehensively elucidated given the overlap with other mechanisms of apoptosis[280]. This dramatic improvement

in post-transplantation survival suggests that anoikis as a mechanism of cell loss and its mitigation to improve cell therapy deserves closer study.

Work performed by our research group has shown that murine MDSCs, isolated by a modified preplate technique, display a high transplantation capacity in both skeletal and cardiac muscle[42,43]. The MDSCs' capacity for long-term proliferation and self-renewal, resistance to stress, ability to undergo multilineage differentiation, ability to induce neovascularization, and immune-privileged behavior—at least partially explains the high regenerative capacity of these cells in skeletal and cardiac muscles. It has been shown that MDSCs survive transplantation at significantly greater rates than more differentiated myoblasts. How much of this survival can be accounted by resistance to anoikis has not been evaluated.

The preplate technique by which MDSCs are isolated suggest that these cells may have an enhanced ability to resist anoikis. The preplate segregates cells that adhere rapidly to collagen coated flask surfaces from cells that are slow to adhere. Cells that fail to adhere over a course of 5 successive preplate passages are collected in a 6th preplate flask and are allowed to adhere over the course of several days. It is these cells that are termed the muscle derived stem cells[79]. It seems logical that these cells may perform better following transplantation in vivo partially because of their enhanced ability to resist anoikis however this ability has yet to be studied directly.

We have isolated myogenic cells from skeletal muscle with elevated aldehyde dehydrogenase (ALDH). ALDH expression is associated with retinoic acid metabolism, a mediator of stem cell maintenance and differentiation, and confers oxazaphosphorine chemotherapy resistance in metastasis competent carcinoma stem cells[151,187,188]. Elevated expression of aldehyde dehydrogenase (ALDH) has been shown to be a marker of early

progenitor status in hematopeitic[180,181], mesenchymal[182], endothelial[183], neural[184,185], and recently skeletal muscle[186] populations. We have found that these ALDH[hi] sub-populations not only resist oxidative and inflammatory stress in vitro but have enhanced survival and engraftment in murine skeletal muscle[281]. Anoikis resistance may play an important role in the improved transplantation characteristics of these cells, particularly in light of the increase in the use of ALDH to isolate and characterize tumorigenic and cancer stem cells capable of metastasis[267,268,282,283].

When cancer cells metastasize to distant sites in the body, they may do so by hematogenous or lymphatic spread which requires that they degrade and detach from the extracellular matrix of the primary tumor in a process similar to an epithelial to mesenchymal transition (EMT). The cells must be able to survive this loss of cell contacts that would normally initiate anoikis, travel for some period of time before finding some hospitable tissue to re-initiate tumor growth[284]. Frish and Screaton argue that anoikis resistance confers a selective advantage to metastasizing cells because it effectively increases the survival time after detachment[284]. Numerous authors have observed that ALDH is marker of poor prognosis given these cells propensity to survive radiation and chemotherapy (e.g. cyclophosphamide) and more importantly to metastasize, and recapitulate the tumor at a distant site. These are properties that essentially define a cancer stem cell. Is ALDH a marker for cells capable of shedding their extra-cellular matrix contacts? Is it possible that ALDH has some essential function in blocking integrin mediated cell death? There are questions that have yet to be addressed in the literature.

As discussed previously, the use of ALDH to isolate stem cells is not limited to cancer. In fact, ALDH used to isolate hematopoietic stem cells from peripheral blood. Clearly these

cells are capable of surviving being suspended in blood[285-287]. These cells (particularly those that are CD45-negative) have been used to home to various tissues[285].

We are still quite a ways away from finding an effective cell therapy for skeletal muscle diseases such as DMD and traumatic injuries. However, one of the most significant impediments, poor cell survival immediately following transplantation, may be mitigated by selecting for cells that have improved survival in the muscle transplantation environment.

6.1.2 Experimental design

We chose to explore the relationship between ALDH expression and anoikis resistance with two experiments. The first entails measuring capacity ALDH^{hi} and ALDH^{lo} cells isolated from a murine skeletal muscle biopsy to survive under conditions of the preplate technique. That is, we will examine the number of preplate cycles that ALDH^{hi} and ALDH^{lo} cells survive and proliferate adhered to a collagen-coated flask as illustrated in the experimental schematic below (Figure 39).
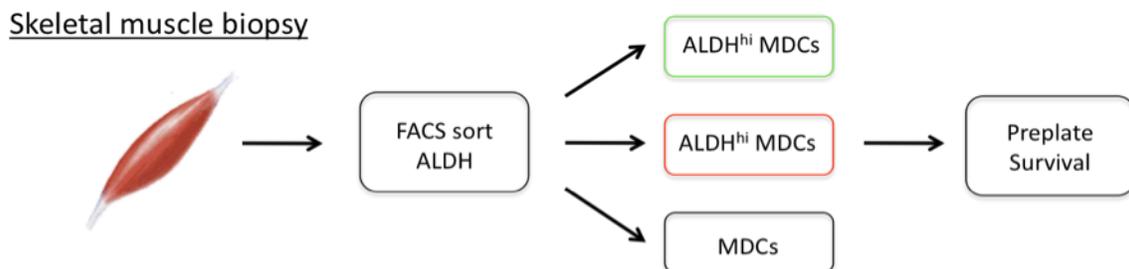
Figure 39. Experimental schematic to examine preplate survival of ALDH sorted murine muscle derived cells

The second experiment is designed to study the anoikis resistance of ALDH sorted muscle derived cells. Cells dissociated from murine (female, WT 6J) skeletal muscle and placed immediately in media in a spinner bottle to prevent cell adhesion through settling or cell clustering (myosphere formation)[288].. Spinner bottles of uncoated glass were used to maintain cells in culture for progressive durations (2d, 4d, and 6d) chosen to approximate the duration of the preplate technique.
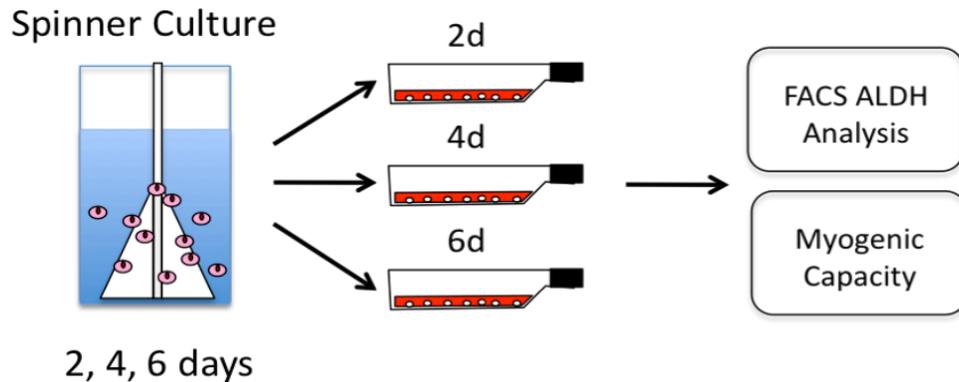
Figure 40. Experimental schematic of to examine anoikis resistance cells for ALDH activity

6.1.3 Results

Freshly dissociated murine skeletal muscle cells were sorted into ALDH^{hi} and ALDH^{lo} sub-populations by FACS. These populations were then immediately subjected to the modified preplate process described previously. The unsorted murine muscle derived cells did not undergo the same FACS process however. The ALDH^{lo} sub-population of cells not only suffered more cell death as a result of the FACS process, an observation made in many prior FACS isolations, but of those cells that survived adhered rapidly to collagen-coated flasks. As depicted in Figure 41 (also depicted in Figure 17 in section 3.5.2) below, ALDH^{lo} cells could

only be preplated for two iterations over a 2hr period (PP1 to PP2). It should be noted that three cells were found in the fourth preplate following a thorough search of the entire T-75 flask, however this would hardly constitute a significant population especially given that no PP3 cells were observed. On the other hand the ALDH^{hi} subpopulation of muscle derived cells yielded a robust population of cells to preplate 4. While the ALDH^{hi} cell did not yield any cells to preplate 6 (PP6) unlike the unsorted muscle derived cells, it should be born in mind that these cells were subjected to the high velocity mechanical stress of fluorescence activated cell sorting.
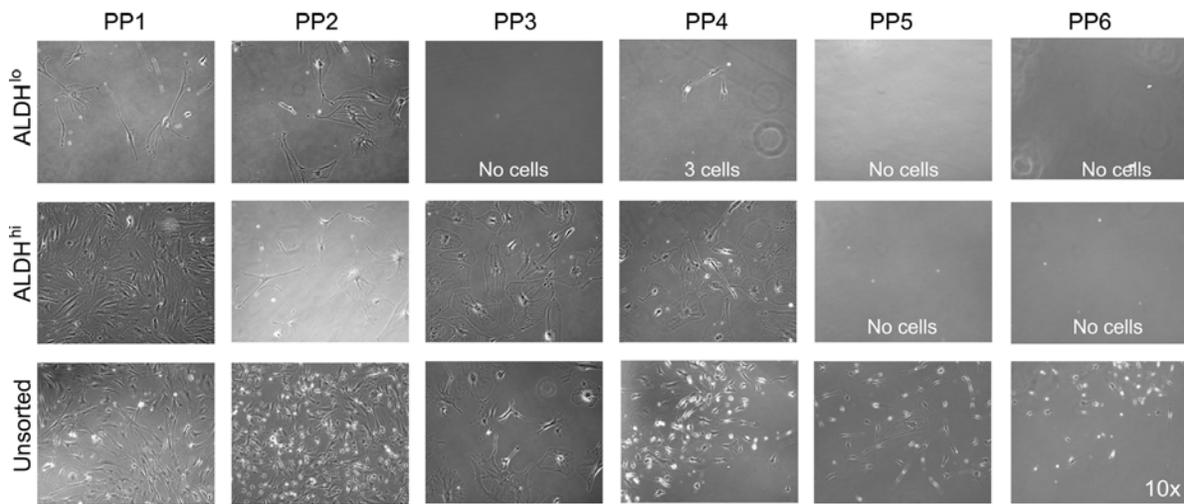
Figure 41. Preplate survival of ALDH sorted murine muscle derived cells

Freshly dissociated murine skeletal muscle cells sorted into ALDH^{hi} and ALDH^{lo} sub-populations underwent the modified preplate process. ALDH^{lo} cells could only be preplated for two iterations over a 2hr period (PP1 to PP2). The ALDH^{hi} subpopulation of muscle derived cells yielded a robust population of cells to preplate 4.

We have initiated experiments on the spinner flask culture of murine muscle derived cells. We have determined the spinner conditions to prevent myosphere, which we observed initially when the spinner culture was spun at very revolutions per minute (20 RPM). To date we have observed good viability of cultured murine muscle derived cells for a period of 4 days in spinner culture. Due to mechanical issues of the spinner plate, experiments had to be discontinued to date. We will need proceed with experiments at longer spinner culture times to

determine if good viability can be achieved for longer time period and whether these culture conditions may enrich the cell population with MDSCs in a manner similar to the preplate technique.

6.1.4 Discussion and future directions

Two important conclusions can be drawn from the preplate experiment of ALDH sorted cells. The first is that ALDH^{hi} cells have a greater propensity to survive the FACS process which is not surprising given their increased stress resistance in vitro and in vivo, observed in chapters 3 and 4. The second is that the ALDH^{hi} population is enriched with cells that are slow to adhere a behavior that is similar to MDSCs. This should not be surprising either given that MDSCs appear to be enriched with ALDH^{hi} cells as seen in Figure 15.

The spinner function is important to examine whether cells cultured in a spinner bottle that prevents adhesion will select for cells that are either ALDH^{hi}, have stem cell like properties, have improved stress resistance, or improved muscular engraftment properties. Of course, digested muscle will have numerous peripheral blood cells that may have a propensity to survive in the absence of an extracellular matrix, which is why I will need to verify the myogenic capacity of these cells. Similarly, I have planned experiments to examine the survival rate of muscle derived cells sorted on the basis of ALDH activity in the spinner bottle environment.

6.2 MEASURING MUSCULAR VASCULARITY USING MICRO X-RAY TOMOGRAPHY

6.2.1 Experimental design

To qualify and quantify vascular changes in Ercc1 knock out mouse musculature, a murine model of progeria or accelerated aging[289], I developed a method of muscle vasculature visualization in the mouse using polymer corrosion casting[290,291]. Polymer casts can be scanned and digitally reconstructed using confocal as well as micro-computed tomography (microCT). In this way we were able to quantify microvascular density in three dimensions (i.e. across an entire muscle), to quantify gross morphological changes as a function of age and Ercc1 activity.

Polymer perfusion methodology is described elsewhere[290]. Briefly, the left ventricle was cannullated with a 24 gauge IV catheter and the right atrium is incised following thoracotomy. Blood was flushed from the vasculature using 4ml Heparin/PBS (100 units/mL), 4mL 10% Formalin, 4mL Heparin/PBS, followed by 3mL of polymer (Batson's No. 17 methylmethacrylate, Polysciences for confocal and SE microscopy or Microfil or FlowTech for microCT). Casting solution was allowed to polymerize overnight at 4°C. For confocal microscopy of the tissue of interest, the gastrocnemius muscle in this case, was cryosectioned at 40μm prior to imaging. However for μCT imaging, the intact muscle can be imaged. An intact cast of the vasculature was also obtained by allowing the tissue to be digested for 1 week in 10% NaOH, exchanged daily.

6.2.2 Results

Perfusions were performed in wt mice as well as in Ercc1 -/Δ (20wks) were performed as seen in Figure 42 below. A fluorescent Microfil polymer was used for confocal microscopy. The confocal image was used to characterize capillary density in the gastrocnemius muscle. A radio-opaque Flotech polymer was used for μCT (micron scale computed tomography), which was used to reconstruct the vasculature of the intact gastrocnemius muscle.
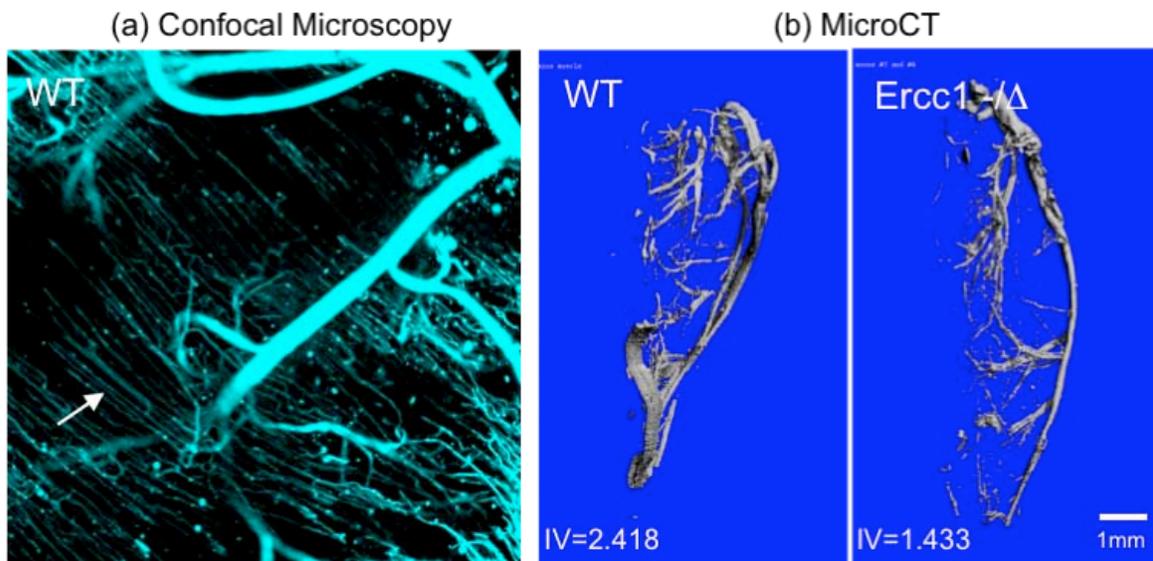
Figure 42. Radio-opaque polymer casting of skeletal muscle vasculature
The intravascular volume or capillary density can quantified by perfusing the mouse with a fluorescent and radio-opaque polymer. (a) Confocal microscopy was used to image capillaries in the gastrocnemius muscle. (b) μCT (micron scale computed tomography) was used to reconstruct the vasculature of the intact gastrocnemius muscle following perfusion.

6.2.3 Discussion

The methodology described here has been used extensively to perfuse murine central organs such as the lung and liver, however to my knowledge this has not been used in peripheral tissues

such as the gastrocnemius. I worked closely with Donna Stolz and Atsu Nagao to quantify the

capillary density and intravascular volume of the ERCC KO mouse, which was suspected to

have decreased vascular density in its skeletal muscle.   Two limitations of this technique arose

during the course of these experiments. The first is that due to the viscosity of the polymer,

relatively high pressures are required to drive the polymer into the systemic vasculature of the

mouse. These high pressures have a propensity to rupture vessels in an unpredictable pattern.

Therefore the technique does not always yield well perfused peripheral tissues suitable for

imaging, however when the tissue is perfused properly, high quality confocal microscopy images

can be obtained. Gross intravascular pattern and volume characterization of intact tissues can be

quickly obtained by µCT however the µCT machine the we use has a 10 µm resolution limit.

Therefore capillaries are highly pixilated if they are detected at all. Given the quality of the

capillary images obtained by confocal microscopy, it appears that the lack of detection is not a

matter of poor capillary perfusion rather it appears to be a µCT resolution issue. There are

however (non-synchrotron) µCT machines with practical resolution limits of 1µm, such as the

Xradia MicroXCT.

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