AORTA-DERIVED MESOANGIOBLASTS FOR CELL THERAPY OF CARDIOMYOPATHY IN DUCHENNE MUSCULAR DYSTROPHY

BY

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DISSErTATION

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ABSTRACT

Aorta-derived mesoangioblasts (ADM) are multipotent adult stem cells that differentiate to adipocytes, smooth muscle cells, skeletal myotubes, oligodendrocyte precursors, and cardiomyocytes, indicating that they can be used for regeneration in severe disease in which multiple tissues are affected. Duchenne muscular dystrophy (DMD) is a fatal muscle-wasting disease, and patients with DMD do not survive beyond their early 30s. The most common causes of the death in DMD patients are cardiac and respiratory failure. Currently, there are advanced treatments and equipment for improving respiratory function so that it is possible to prolong the life of DMD patients. With increased lifespan dilated cardiomyopathy (DCM) has become prevalent in patients with DMD, and all patients develop DCM by 18 years of age about their mid-teen. However there have been not many studies that have focused on therapy for cardiomyopathy in DMD. This project is focused on determining whether ADM ameliorate cardiomyopathy in murine models for DMD, mdx and mdx/utrn^{−/−} mice, and whether ADM are good candidates for cell-based therapy of DCM in DMD.

In the first part of study, mdx/utrn^{−/−} mice were characterized to determine whether they develop DCM similar to patients with DMD as a good disease model of DMD. DCM was not observed in mdx/utrn^{−/−} mice at 5 weeks of age. However cardiac function and ventricular wall thickness decreased continuously at 10 and 15 weeks of age and an increase in fibrosis in the left ventricular posterior wall was observed, similar to patients with DMD. In the second part of the study we examined whether ADM differentiate into cardiomyocytes to determine whether they were a good source for stem cell therapy in
DMD. ADM differentiated to cardiomyocytes that expressed of cardiac specific mRNAs and proteins in vitro and in vivo, and restored dystrophin in the heart of young mdx mice after intramyocardial injection. In the last study ADM were transplanted into hearts of mdx and mdx/utrn<sup>−/−</sup> mice and the effect of ADM transplantation on DCM was examined. With ADM transplantation mdx/utrn<sup>−/−</sup> mice did not develop DCM in contrast to sham-injected mice which exhibited decreased heart function. Increased angiogenesis and proliferation were detected in mdx/utrn<sup>−/−</sup> heart after ADM transplantation which may contribute to the positive effects of ADM in mdx/utrn<sup>−/−</sup> cardiac muscle. In addition restored dystrophin was detected in mdx/utrn<sup>−/−</sup> heart after ADM injection. However, no cardiac functional improvement or dystrophin protein was detected in mdx mice with ADM transplantation.

To conclude development of DCM in mdx/utrn<sup>−/−</sup> mice was prevented or delayed by ADM transplantation correlating with angiogenesis and increased cell proliferation as well as restored dystrophin expression. ADM may therefore be a good stem cell source for treatment of DCM in DMD by preventing or delaying their development. However ADM are not a good stem cell source for treatment of pre-existing DCM at late disease stages.
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<td>Aorta-derived mesoangioblasts</td>
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<tr>
<td>CSC</td>
<td>Cardiac stem cell</td>
</tr>
<tr>
<td>DAP</td>
<td>Dystrophin associated protein</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
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<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>EBD</td>
<td>Evan’s blue dye</td>
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<tr>
<td>EDV</td>
<td>End diastolic volume</td>
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<tr>
<td>EF</td>
<td>Ejection fraction</td>
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<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ESV</td>
<td>End systolic volume</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>IVS</td>
<td>Interventricular septum</td>
</tr>
<tr>
<td>LVID</td>
<td>Left ventricular internal dimension</td>
</tr>
<tr>
<td>LVPW</td>
<td>Left ventricular posterior wall</td>
</tr>
<tr>
<td>MDSC</td>
<td>Muscle derived stem cells</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
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A. Duchenne muscular dystrophy

Duchenne Muscular Dystrophy (DMD) is a progressive, lethal muscle wasting disease caused by the absence of dystrophin. DMD is the most common X-linked (Xp21.2) recessive disorder and affects 1 in 3,500 boys born. In patients with DMD, dystrophin function is completely lost and patients need to use a wheelchair by early childhood. Patients with DMD rarely survive into their early 30s and the causes of death are frequently respiratory or cardiac failure. Unfortunately there is no cure for DMD.

Dystrophin is a component of the dystrophin associated protein (DAP) complex, linking the cytoskeleton to extracellular matrix components. Dystrophin is one of the largest genes in the genome and contains four structural domains including the NH2-terminal, rod, cysteine-rich, and COOH-terminal domains. The amino (N) terminus binds to γ-actin, which anchors dystrophin to cytoskeleton. The central rod domain of dystrophin gives flexibility and structural stiffness and the cysteine-rich domain in the carboxyl-terminus interacts with the intracellular portion of the β-dystroglycan transmembrane protein, anchoring dystrophin to the sarcolemma [Goodwin et al. 2005, Heydemann et al. 2007, Celec et al. 2008]. Dystrophin is important for the transmission of force from subsarcolemmal actin to the extracellular matrix and protects muscle cells from contraction-related muscle damage. Without dystrophin the cell membrane is vulnerable to damage and fails to stabilize itself during repeated contractions [Vatta et al.
2002] resulting in cell death and leading to necrosis, inflammation and replacement of muscle fibers by fat.

There are three common strategies proposed for treatment of DMD patients: pharmacologic agents, gene therapy, and cell therapy [Markert et al. 2009].

Pharmacological agents alleviate inflammation and slow progression of the disease, but are not enough to cure it because they cannot correct the genetic defect. In addition, they cause side effects from long-term administration similar to corticosteroids [Van Staa et al. 2000]. Gene therapy for DMD has yielded promising results in animal models, but is limited in scope because of the inability to deliver the full-length dystrophin gene due to its large size (total size of gene: 2.4mb, mRNA: 14kb, number of exons: 79). To circumvent this problem, mini- or micro- dystrophin genes with partial function were delivered to muscle in viral vectors. These studies yielded positive results in skeletal muscles of animal models for muscular dystrophy [Gregorevic et al. 2004]. However, these mini-or micro genes could not alleviate all aspects of cardiac dysfunction [Bostick et al. 2008, Townsend et al. 2009]. Another mechanism for gene therapy is to induce exon skipping mediated by antisense oligonucleotides to promote read-through of the dystrophin gene. By skipping a particular exon at the pre-RNA level functional dystrophin can be rescued [Goyenvalle et al. 2010, Cirak et al. 2012]. However the oligonucleotides have very low cellular uptake efficiency and personalized oligomers based on polymorphism of patients with DMD are needed. For treatment of DMD degenerated skeletal and cardiac muscle cells must be replaced to re-gain muscle function. Many stem cells can differentiate to cardiomyocytes in vitro, but do not maintain cardiac function. However, some stem cells improved cardiac function after

**B. Cardiomyopathy in DMD**

Cardiac involvement in DMD is common but occurs at a later age than skeletal muscle symptoms [Vatta et al. 2002, Mulder et al. 2009, Farini et al. 2009]. Many studies have focused on treatment of skeletal muscle, but increased activity after strengthening skeletal muscles provides significant cardiac dysfunction and advanced heart failure [Danialou et al. 2001, Goodwin et al. 2005, Townsend et al. 2008]. Although there have been many studies focused on regenerating or genetically correcting skeletal muscle in animal models for DMD, very few studies have been conducted to restore dystrophin in the heart and/or replace damaged cardiomyocytes.

Cardiac involvement in DMD is present in >90% of patients and causes the death of 20–40% of them [Bushby et al. 2003]. Pre-clinical cardiac involvement is observed in 25% of patients under 6 years old, and clinically apparent cardiomyopathy is diagnosed in 59% of them after 10 years of age. Nearly all patients develop cardiomyopathy by 18 years of age [Nigro et al. 1990]. Specifically, patients with DMD develop dilated cardiomyopathy (DCM) characterized by an enlarged left ventricular chamber, thinning ventricular wall, and decreased fractional shortening and ejection fraction [Nigro et al. 1990, Oldfors et al. 1994]. Absence of dystrophin elicits increasing intracellular calcium concentration because of the damaged plasma membranes and chronic intracellular calcium excess results in the degeneration of cardiomyocytes. The death of cardiomyocytes induces an inflammatory response in the dystrophic heart, as
macrophages are required to remove dead cells but also lead to fibrosis. Fibrosis is formed dominantly in the left ventricular wall, resulting in a loss of flexibility. Stretching of the inflexible area from blood filling the chamber induces thinning of the left ventricular wall, further decreasing its contractility, and eventually resulting in DCM [Kaspar et al. 2009]. Because it is asymptomatic, cardiac involvement is often severely advanced when diagnosed, rapidly progressing to heart failure. It is possible to delay the progress of cardiac degeneration if cardiac involvement can be regularly monitored from the time patients are first diagnosed with DMD. Clinically, it is now possible to improve the weakened skeletal muscle and support respiratory function to prolong the life of DMD patients [Eagle et al. 2007, Kamogawa et al. 2001]. With the extended lifespan, development of cardiomyopathy has become increasingly common in DMD patients, underscoring the importance of finding a method to treat cardiomyopathy in these patients [Townsend et al. 2009]. It is therefore necessary to develop treatment for both skeletal and cardiac muscle in DMD patients.

C. Animal models for DMD

Recognizing the need to treat cardiac involvement in DMD, it is essential to evaluate DMD animal models to determine whether they develop cardiomyopathy like DMD patients. Genetically manipulated mice have been used to study human diseases for several reasons: relatively short life cycle, fecundity, similar symptoms to human diseases, and effective manipulation of genes. Mdx, mdx/utrn-/- and mdx/MyoD mice have been used as murine model to study potential treatments for DMD. Mdx mice have a single mutation in the dystrophin gene which is genetically homologous to many DMD
patients, but have relatively mild skeletal phenotype and a normal life span, in contrast to DMD patients. Mdx mice develop cardiomyopathy with moderate myocardial necrosis and fibrosis by 6-8 months of age [Bridges et al. 1986]. Using echocardiography, DCM can be detected when mice are approximately 12-months-old, is associated with prominent heart pathology, and continues to worsen as mice age [Quinlan et al. 2004]. Abnormalities in mdx heart have also been detected by electrocardiography by 21 months of age [Bostick et al. 2008]. Although mdx mice develop DCM much later in life than patients with DMD they are a valuable model to use for studying therapies for DMD cardiac muscle because the mice develop pathology and heart dysfunction similar to patients. The mdx/utrν−/− mice lack expression of both dystrophin and utrophin and have severe clinical signs of DMD, similar to patients, including severe, progressive skeletal muscle pathology, kyphosis, and a severely shortened lifespan. Mdx/utrν−/− mice also show cardiac pathologic changes including fibrosis and cardiomyocyte membrane damage between 8 and 10 weeks of age [Grady et al. 1997]. However there has been no quantitative assessment of these parameters in mdx/utrν−/− mice or a functional study to determine whether the mice develop DCM like DMD patients. In Mdx/myoD mice cardiomyocyte membranes are extensively damaged and the mice have fibrosis and enlarged ventricles at 5 months of age. However functional data including echocardiography analysis and quantitation of ventricular dilation have not been reported in the mdx/myoD mice [Megeney et al. 1999, Duan 2006].

There are also other animal models of DMD. The golden retriever muscular dystrophy (GRMD) model and the beagle model of canine based x-linked muscular dystrophy (CXMD) are the most common canine models of DMD. They have severe
clinical symptoms similar to human patients with DMD, and GRMD develop cardiomyopathy similar to human DMD patients. However, CXMD have milder cardiac involvement than GRMD [Valentine et al. 1989, Yugeta et al. 2006]. Cats that lack dystrophin expression develop hypertrophic feline muscular dystrophy (HFMD). However these cats do not display muscle fibrosis or severe hypertrophy in diaphragm, in contrast to human patients with DMD. In addition it is rare to have severe cardiac failure in HFMD cats [Gaschen et al. 1999]. Recently a porcine DMD model has been generated by gene targeting with nuclear transfer. These animal have similar skeletal muscle defect to human patients with DMD, but development of DCM in this model has not been reported [Klymiuk et al. 2011].

D. Stem cell therapies in dystrophin–deficient cardiac muscle of DMD

Different types of stem cells have been tested for treatment of DMD, including embryonic stem cells (ESC) [Crisostomo et al. 2007, Schuldt et al. 2008, Stillwell et al. 2009, Vertesaljal et al. 2008], induced pluripotent stem cells (iPS) [Hansson et al. 2009], mesoangioblasts [Koh et al. 1995, Minasi et al. 2002, Cossu et al. 2003, Berry et al. 2007, Galvez et al. 2008], muscle derived stem cells (MDSCs) [Payne et al. 2005], satellite cells and mesenchymal stem cells (MSC) which were derived from bone marrow, adipose, and amniotic fluid and membrane [Duan et al. 2006, Gepstein et al. 2006, Crisostomo et al. 2007, Laflamme et al. 2007, Schuldt et al. 2008, Vertesaljal et al. 2008, Farini et al. 2009, Hansson et al. 2009, Otto et al. 2009]. However, only two of these studies have targeted cardiomyopathy in DMD. One of the studies, conducted by T. R. Payne and J Huard [Payne et al. 2005], reported restoration of dystrophin expression in
mdx/SCID hearts after intracardiac transplantation of MDSCs. However most of the dystrophin-positive myocytes expressed a skeletal muscle marker, indicating that the donor MDSC differentiate into skeletal muscle cells when transplanted into the heart. Most donor-derived myocytes did not form connexin 43 gap junctions, which are required for electrical coupling of donor and host cells. Moreover, it is unclear if transplanted MDSCs affect the function of dystrophic heart either positively or negatively, as heart function was not assessed. In a second study, Koh et al. injected fetal cardiomyocytes into the hearts of mdx mice and dystrophic dogs and found corresponding dystrophin-expressing cells in the heart and gap junctions between donor cell-derived cardiomyocytes and host myocardium. Arrhythmias were not detectable in electrocardiography analysis of dogs following cell injection, in agreement with histological data demonstrating that the donor cells were integrated into the host tissue and express connexin 43 [Koh et al. 1995]. These results are promising, and in agreement with engraftment of committed cardiomyocytes to normal heart. However, the use of fetal human stem cells is controversial, and they would likely be difficult to obtain for use in the clinic. Taken together, stem cells are attractive sources for treatment of DMD patients, but have not yet been demonstrated to be feasible for therapeutic use in the dystrophic heart of DMD patient.

**E. Vessel-associated stem cells: Mesoangioblasts**

Originally mesoangioblasts (or meso-angioblasts) were identified by De Angelis et al. [De Angelis et al. 1999] in embryonic dorsal aorta while studying embryological origin of skeletal muscle. Later Minasi et al. designated the vessel-related progenitor cells
meso-angioblast, as multipotent stem cells, because they differentiate into most mesodermal tissues in vivo and maintain their multipotency in vitro and in vivo [Minasi et al., 2002]. Mesoangioblasts have been isolated from embryonic dorsal aorta [Minasi et al. 2002, Galvez et al. 2008], postnatal aorta [Berry et al. 2007], heart [Galvez et al. 2008], skeletal muscles [Sampaolesi et al. 2006, Morosetti et al. 2007]. Cardiac mesoangioblasts isolated from atria and ventricles have been transplanted to infarcted heart, increasing ventricular wall thickness and fractional shortening without ventricular chamber diameter. Injected cardiac mesoangioblasts also differentiated into cardiomyocytes and expressed sarcomeric myosin [Galvez et al. 2008]. These data demonstrated that cardiac muscle-derived mesoangioblasts regenerate cardiac muscle.

We have isolated multipotent mesoangioblasts (ADM) from aorta of wild-type newborn mice. These cells have a different cell surface marker profile than the mesoangioblasts isolated from skeletal or cardiac muscle muscles, or bone marrow, or embryonic dorsal aorta, but are most similar to those isolated from embryonic dorsal aorta, [Lei et al. 2011]. Our group has reported that ADM differentiate into smooth muscle cells, skeletal myotubes, oligodendrocyte precursors, and pre-adipocytes [Berry et al. 2007, Lei et al. 2011]. In addition, transplantation of ADM into the gastrocnemius resulted in engraftment into skeletal muscle, restoration of dystrophin expression and a decrease in the number of damaged muscle fibers in mdx/utn−/− mice [Berry et al. 2007]. This study suggested that ADM might be an excellent source for stem cell therapeutic purpose to treat DMD with damaged skeletal muscles. These results prompted us to perform this study to determine if ADM might improve damaged cardiac condition in DMD as well.
F. ADM as treatment of cardiomyopathy in murine models for DMD

The goal of this study was to examine the capacity of ADM to serve as a therapeutic approach in the heart of murine models for DMD to determine whether they had similar benefit in dystrophic heart as in the dystrophic skeletal muscles. Therefore I hypothesized that ADM will ameliorate DCM in murine model of DMD.

For addressing this goal I established three aims. 1) To determine whether the cardiomyopathy in the mdx/utrn<sup>−/−</sup> murine model for DMD is similar to DMD patients. This aim focused on characterizing the heart function and pathology in mdx/utrn<sup>−/−</sup> mice to determine whether it was similar to DMD patients, and whether mdx/utrn<sup>−/−</sup> mice are a feasible disease animal model to use in pre-clinical studies for therapy for cardiomyopathy in DMD. 2) To determine whether ADM differentiate into cardiomyocytes. This aim focused on inducing differentiation of ADM into cardiomyocytes in vitro and determining cell fate in vivo following transplantation into the dystrophic heart. 3) To determine whether ADM transplantation improved cardiac function and pathology in mdx and mdx/utrn<sup>−/−</sup> mice. This aim used echocardiography and histology to study whether ADM transplantation had a positive effect on dystrophic hearts.
Chapter II

Cardiac dysfunction and pathology in the dystrophin and utrophin-deficient mouse during development of dilated cardiomyopathy

A. Introduction

Cardiac involvement in Duchenne muscular dystrophy (DMD) is present in nearly all patients by adulthood [Nigro et al. 1990, Bushby et al. 2005, Wagner et al. 2007] and causes the death in approximately 20% of them [Bushby et al 2003, Baxter 2006, Spurney 2011]. Specifically, patients with DMD develop dilated cardiomyopathy characterized by an enlarged left ventricular chamber, thinning ventricular wall, and decreased fractional shortening and ejection fractions [Nigro et al. 1990]. Cardiac involvement in DMD has increased as a result of recent improvements in ambulation and respiratory support in patients [Kamogawa et al. 2001, Wagner et al. 2007, Kaspar et al. 2009, Spurney 2011]. These advances have made it possible to prolong the life of DMD patients [Eagle et al. 2002, Eagle et al. 2007]. However, with the extended lifespan, development of cardiomyopathy is increasingly common in the later stage of the disease.

There is evidence that ACE inhibitors, steroids, and beta blockers can delay decreases in heart function and development of ventricular dilation, and prolong the life of DMD patients [Bridges 1986, Simonds et al. 1998, Quinian et al. 2004, Duboc et al.

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1 This chapter appeared in its entirety in Neuromuscular Disorders journal and is referred to later in this dissertation. This article is reprinted with the permission of the publisher and is available from http://www.elsevier.com or http://dx.doi.org and using DOI:10.1016/j.nmd.2011.07.003
However, there is still no cure for cardiomyopathy in these patients [Townsend et al. 2009]. It is therefore valuable to have dystrophin-deficient animals with cardiac pathology and dysfunction similar to DMD patients on which to test experimental therapies. Genetically manipulated mice have been used to study human diseases and are good model organisms for several reasons: a relatively short life cycle, fecundity, similar symptoms to human diseases, and effective manipulation of genes.

There are three strains of dystrophin-deficient mice: the mdx, mdx/utrn−/− dystrophin/utrophin-deficient, and mdx/myoD−/− mice. Mdx mice have a single mutation in the dystrophin gene identical to many DMD patients, but have relatively mild skeletal muscle pathology that is not progressive, as well as a normal life span, in contrast to patients. However, similar to DMD patients, mdx mice develop moderate myocardial necrosis and fibrosis, detectable by 6–8 months of age [Bridges 1986], and prominent heart pathology appears when mice are approximately 12 months old and continues to worsen as mice age [Quinian et al. 2003], resulting in development of dilated cardiomyopathy at 12–21 months of age [Quinian et al. 2004, Bostick et al. 2008]. Because the mice develop cardiomyopathy, similar to patients, mdx is a valuable model to use for studying therapies for DMD cardiac muscle. Unfortunately, using mdx mice to mimic dilated cardiomyopathy in DMD is time-consuming and expensive because of the length of time necessary for development of pathology and ventricular dilation. Similarly, mdx/myoD mice also develop extensive damage to cardiomyocyte membranes, fibrosis, and enlarged ventricles at 5 months of age, although functional data by echocardiography and quantitation of ventricular dilation have not yet been performed [Duan 2006,
Megeney et al. 1999]. An animal that develops cardiomyopathy similar to DMD patients in a shorter time course would be cost-effective and would also facilitate the pace of the research. Mdx/utrn^−/− dystrophin/utrophin-deficient mice lack expression of both dystrophin and utrophin, and have severe and progressive skeletal muscle pathology, spinal curvature, limited mobility, and a severely shortened lifespan, similar to patients [Grady et al. 1997]. Mdx/utrn^−/− dystrophin/utrophin-deficient mice have also been reported to develop cardiac pathology including cardiomyocyte membrane damage and necrosis, between 8 and 10 weeks of age [Grady et al. 1997]. However there has not been a quantitative assessment of these parameters in mdx/utrn^−/− dystrophin/utrophin-deficient mice, and no functional analysis has been reported to determine whether the mice develop dilated cardiomyopathy similar to DMD patients.

In this study I characterized the cardiomyopathy and its development in mdx/utrn^−/− dystrophin/utrophin-deficient mice, including functional and histological changes as the mice age. Using echocardiography and analysis of post-mortem tissue, I demonstrated the evolution of cardiomyopathy in mdx/utrn^−/− dystrophin/utrophin-deficient mice, and determined that it is functionally and histologically similar to patients with DMD. In addition, because dilated cardiomyopathy occurs within weeks, at an earlier age than mdx or mdx/myoD mice, the mdx/utrn^−/− dystrophin/utrophin-deficient mice are an alternative, cost-effective model for testing potential therapeutic approaches for DMD-associated cardiac muscle disease.
B. Materials and Methods

B-1. Mice

All animal use was approved by the University of Illinois Institutional Animal Care and Use Committee. Mdx/utrn$^{-/-}$ dystrophin/utrophin-deficient mice were generated by mating mdx/utrn$^{+/-}$ male and female mice [Grady et al. 1997], and the progeny were genotyped as previously described [Burkin et al. 2001, Grange et al. 2002]. Age-matched wild-type controls were used for comparison in all experiments. Echocardiography was performed and mice were subsequently weighed and euthanized.

B-2. Echocardiography

Mdx/utrn$^{-/-}$ dystrophin/utrophin-deficient and wild-type hearts were examined by echocardiography at 5, 10, and 15 weeks of age. GE/Vingmed ultrasound Vivid7 was used for performing echocardiographs. Mice were anesthetized by halothane mixed in mineral oil. Before echocardiography the hair was clipped around the chest. Ultrasound gel was placed on the transducer (10S) and scanning was performed for approximately 10 min. Mdx/utrn$^{-/-}$ dystrophin/utrophin-deficient and wild-type mice were presented randomly to the echocardiographer (R.O.), who was unaware of the genotype.

Two-dimensional M-mode echocardiography was used to measure internal ventricular septum thickness, left ventricular internal dimension, and left ventricular posterior wall thickness, and these values were used to calculate left ventricular fractional shortening, end-diastolic volume, end-systolic volume, stroke volume, and ejection fraction. Left ventricular fractional shortening (FS) was calculated by the change between
filling (diastole; LVd) and emptying (systole; LVs) of the ventricle \((\text{LVd–LVs)/LVD} \times 100\). End-diastolic volume (EDV) was calculated by \(7\times(LVd)^{3}/(2.4 + LVd)\). End-systolic volume (ESV) was calculated as EDV–stroke volume (SV). Stroke volume (SV) was calculated as EDV–ESV. Ejection fraction (EF) is equal to \((\text{LVEDV–LVESV)/LVEDV} \times 100\). The equations for EF and EDV were used according to the Teicholz method.

**B-3. Statistics**

Echocardiography data were analyzed using the Proc Mixed procedure of SAS (SAS Inst. Inc., Cary, NC). The statistical model included effects of genotype, age, and their interaction, as fixed effects. There were six blocks, representing two genotypes by three time points. Block was a random effect. Differences between wild-type mice and mdx/utrn−/− dystrophin/utrophin-deficient type mice within each time point and among time points within each type of mice were tested by pair-wise comparisons when the main effects were significant.

**B-4. Tissue collection**

Hearts were dissected, weighed, and frozen in liquid nitrogen-cooled 2-methylbutane. Frozen hearts were sectioned to 10 µm thickness using a Thermo Fisher Scientific Shandon Cryotome FSE Cryostat (Pittsburgh, PA), at −17 °C.
**B-5. Masson’s trichrome staining**

Fibrosis was examined by analyzing collagen content in the heart tissue, visualized by Masson’s Trichrome stain. Briefly, frozen heart sections were warmed up to room temperature, and incubated in Bouin’s solution at 60°C for 1 h. After washing, the sections were stained with Weigert’s iron Hematoxylin, and then Biebrich scarlet-acid fuchsin solution followed by phosphomolybdic/phosphotungstic acid staining. After a quick water rinse, slides were placed in aniline blue solution, and were then placed into 1% acetic water solution followed by 95% and absolute alcohols and into xylene. Three slides per heart were used for quantitation of fibrosis. Images were captured with a Leica DFC320 Digital Camera (W. Nuhsbaum, Inc., McHenry, IL), mounted on a Leica Inverted DMI 4000B microscope (Nuhsbaum, McHenry, IL). Regions of fibrosis/Trichrome staining were quantitated using Image Pro Plus software (MediaCybernetics, Bethesda, MD).

**B-6. Immunofluorescent detection of collagen I**

Frozen heart sections were fixed with 3.7% formaldehyde for 10 min, and blocked with PBS containing 1% BSA and 5% horse serum for 1 h. The sections were incubated with anti-collagen type I rabbit polyclonal antibody (Abcam, Cambridge, MA) for 1 h. Sections were then washed and incubated with donkey anti-rabbit TRITC antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Slides were washed 3 times with 1xPBS, and mounted onto slides (Fisher Scientific, Pittsburgh, PA) using Vectashield mounting medium containing DAPI (Burlingame, CA). Images were
acquired with a Retiga 2000R digital camera (Q-imaging, Surrey, BC) mounted on a
Leica Inverted DMI 4000B microscope (Nuhsbaum, McHenry, IL), and colors were
assigned and images merged using Image Pro Plus software (MediaCybernetics
Bethesda, MD).

B-7. Evan’s Blue Dye

One percent Evan’s Blue Dye was prepared in phosphate-buffered saline (PBS,
pH 7.5), passed through a 0.22 µm filter (Millpore, USA). Mice were injected
intraperitoneally with 1% volume of EBD relative to body weight. After injection mice
were housed in normal animal care conditions, and euthanized by CO₂ gas after 24 h.
Hearts were dissected, and frozen by 2-methylbutane cooled by liquid nitrogen. Frozen
sections were fixed with 3.7% formaldehyde for 10 min, and immunostained with FITC-
conjugated wheat germ agglutinin for 5 min. After washing with PBS the sections were
mounted using Vectashield mounting medium containing DAPI (Vector Laboratories,
Burlingame, CA). Images of Evans Blue Dye stain, FITC-conjugated wheat germ
agglutinin, and DAPI were acquired with a Retiga 2000R digital camera (Q-imaging,
Surrey, BC), mounted on a Leica Inverted DMI 4000B DMIL microscope (Nuhsbaum,
McHenry, IL) and colors were assigned and images merged using Image Pro Plus
software (MediaCybernetics, Bethesda, MD).
**B-8. Hematoxylin and Eosin staining**

Frozen sections were fixed in 95% ethanol, and then placed in 70% ethanol for 2 min. Sections were stained with Gills Hematoxylin (Fisher Scientific, Pittsburgh, PA) for 5 min, followed by a 2 min rinse in running H$_2$O, and then placed in Scott’s solution. Sections were immersed in eosin for 5 min, gradually dehydrated in ethanol, and cleared in xylene. Images for Trichrome staining of collagen or H&E were acquired using a Leica DMIL microscope, Leica Applications Suite Imaging Software (version 2.8.1), and a Leica DFC320 Digital Camera (W. Nuhsbaum, Inc., McHenry, IL).

**B-9. Transmission electron microscopy**

Heart tissues from mdx/utrn$^{-/-}$ and C57Bl6 mice at 15 weeks of age were frozen in liquid nitrogen-cooled 2-methylbutane. Samples were prepared and embedded for transmission electron microscopy (TEM) as described in Miller [Miller et al. 2001]. For TEM, 60–100 nm ultrathin sections were cut from embedded heart tissue samples with a Diatome Ultra 45° knife (DiATONE USA, PA). Sections were floated off onto water and picked up with 200 and 300 mesh copper grids and stained with uranyl acetate and lead citrate. Prepared samples were observed on an H600 Hitachi Electron Microscope (Hitachi High-Technologies Europe GmbH, Germany) at 75 kV. Images were taken onto Kodak Electron Microscopy Film 4489, developed and scanned in on a Microtek ScanMaker 9800XL with TMA (Microteck International, Inc., Taiwan) at 3200 dpi original scan.
C. Results

C-1. Mdx/utrn⁻/⁻ dystrophin/utrophin-deficient mice develop dilated cardiomyopathy, detected by echocardiography

Echocardiography of wild-type and mdx/utrn⁻/⁻ dystrophin/utrophin-deficient mice was performed at 5, 10, and 15 weeks of age, using motion (M)-mode scans to detect anatomical and functional parameters of the heart (Table 1-A and Fig. 1). At age 5 weeks salient differences were not detected between mdx/utrn⁻/⁻ dystrophin/utrophin-deficient and wild-type control mice with the exception of an accelerated heart rate. However, at 10 weeks of age there were multiple parameters that were significantly different. A decrease in the thickness of the left ventricular posterior wall in mdx/utrn⁻/⁻ dystrophin/utrophin-deficient mice at both diastole and systole was observed (Table 1-A). The interventricular septum was also significantly thinner in mdx/utrn⁻/⁻ dystrophin/utrophin-deficient mice than age-matched wild-type mice at diastole. Mdx/utrn⁻/⁻ dystrophin/utrophin-deficient hearts continued to deteriorate between 10 and 15 weeks, and a greater number of significant differences were prominent at 15 weeks of age. The left ventricular posterior wall was significantly thinner in a time-dependent manner in comparison to age-matched wild-type mice through 15 weeks of age (Table 1-A). In addition, the left ventricular internal dimension during diastolic phase gradually enlarged, reaching a statistically significant difference in size compared to wild-type heart (Table 1-A). An increase in left ventricular internal dimension at systole (Table 1-A) was also observed although not statistically significant.

There were also significant changes in systolic and diastolic function in mdx/utrn⁻/⁻ dystrophin/utrophin-deficient heart at 15 weeks. Fractional shortening, which
represents systolic function, was lower in mdx/utrn−/− dystrophin/utrophin-deficient hearts compared to age-matched wild-type mice hearts (Table 1-A and Fig. 1A). Ejection fraction also significantly decreased in mdx/utrn−/− dystrophin/utrophin-deficient versus wild-type mice (Table 1-A and Fig. 1B), and end-diastolic volume and end-systolic volume were significantly increased (Table 1-A and Fig. 1C). Differences in the overall heart and body weight ratio of the mdx/utrn−/− dystrophin/utrophin-deficient and wild-type mice could account for the observed differences in the echocardiograph parameters. As a result, the heart and body weight were recorded for each mouse following echocardiography, as well as their ratio, and were compared between genotypes (Table 1-B). Mdx/utrn−/− dystrophin/utrophin-deficient mice are significantly smaller than wild-type mice; however, there was not a significant difference in heart and body weight ratio between mdx/utrn−/− dystrophin/utrophin-deficient and wild-type mice (Table 1-B).

C-2. Cardiac fibrosis is present and increases over time in dystrophin/utrophin-deficient cardiac muscle

Fibrosis at the short axis of mdx/utrn−/− dystrophin/utrophin-deficient hearts was detected by Masson’s Trichrome staining for collagen, [Payne et al. 2005, Duan 2006, Bostick et al. 2008, Spurney et al. 2008, Au et al. 2011], as well as with an antibody to collagen I. Trichrome staining was used to compare collagen-rich regions in mdx/utrn−/− dystrophin/utrophin-deficient hearts with age-matched wild-type mice. There were small patches of collagen in both the left and right ventricles of mdx/utrn−/− dystrophin/utrophin-deficient, but not wild-type, mice at 5 weeks of age (Figs. 2B and 3). Fibrosis was detected at 10 weeks of age in similar regions (Figs. 2D and 3), and by the
age of 15 weeks, larger regions of fibrosis were visualized in both right and left
ventricles, and septum (Figs. 2F and 3). Fibrosis was detected throughout the heart at all
ages, with increasing size of lesions at late stages of disease, approaching the maximum
lifespan of the mdx/utn<sup>−/−</sup> dystrophin/utrophin-deficient mice (Fig. 3). Overall,
mdx/utn<sup>−/−</sup> dystrophin/utrophin-deficient mice at age of 15 weeks had approximately 17
times more cardiac fibrosis than those at age of 5 weeks, and 10 times more than those at
age of 10 weeks.

C-3. Membrane damage in cardiomyocytes and cellular necrosis are present in
ventricles of mdx/utn<sup>−/−</sup> dystrophin/utrophin-deficient mice

To examine other pathological features of mdx/utn<sup>−/−</sup> dystrophin/utrophin-
deficient mice Evan’s Blue Dye and hematoxylin and eosin staining were performed.
Evans Blue Dye binds the serum protein albumin, and therefore accumulates in muscle
cells or fibers that have sustained membrane damage, allowing serum proteins to leak
inside the cell [Hamer et al. 2002, Miller 2007]. Similar with fibrosis formation,
cardiomyocytes with Evan’s Blue Dye infiltration were present in both the right and left
ventricles (Fig. 4A and B, arrowheads, C and D, arrows), as well as the interventricular
septum of mdx/utn<sup>−/−</sup> dystrophin/utrophin-deficient mice at 15 weeks of age (Fig. 4A
and B, arrows). Evan’s Blue Dye infiltration was also detected in the heart of mdx mice
at 15 weeks of age (Fig. 4E and F), but in contrast to the in mdx/utn<sup>−/−</sup>
dystrophin/utrophin-deficient mice, was only detected in the septum of the mdx mice
(Fig. 4, white arrows) and not in the right or left ventricles. Preliminary results indicate
that there are higher numbers of Evan’s Blue Dye-positive cells in mdx/utn<sup>−/−</sup>
dystrophin/utrophin-deficient heart than in the mdx heart (0.51% in mdx/utrn−/− heart compared to .02% of total area in the mdx heart). Necrosis was also detected in both ventricles of the mdx/utrn−/− dystrophin/utrophin-deficient heart at 15 weeks of age (Fig. 4G, arrow), but not in mdx heart at this age (Fig. 4H).

C-4. Ultrastructural changes in mdx/utrn−/− dystrophin/utrophin-deficient cardiomyocytes

Transmission electron microscopy was performed on tissue from the ventricles of 15 week old wt and mdx/utrn−/− dystrophin/utrophin-deficient cardiac muscle (Fig. 5). In wt cardiomyocytes, mitochondria were regularly arranged between myofibrils, and were homogenous in size (Fig. 5A and B). In contrast, mitochondria in mdx/utrn−/− dystrophin/utrophin-deficient cardiomyocytes were disorganized, frequently disrupting myofibrils (Fig. 5C and D). Mitochondria were also irregularly shaped and the organization of cristae was altered (Fig. 5D). In addition, very few T-tubules were visible in mdx/utrn−/− dystrophin/utrophin-deficient cardiomyocytes in comparison to wt cells (Fig. 5B and D).

D. Discussion

Cardiac involvement in DMD patients has not been studied as extensively as skeletal muscle pathology and disease progression, in part because damaged skeletal muscle limits the movement of patients, and prevents overt symptoms of cardiac involvement. As a result, most of the studies in the last decade have focused on improving damaged skeletal muscles or supporting the respiratory system in Duchenne
patients. These studies have yielded promising results for management of these symptoms in DMD patients, prolonging life by several years, but resulting in significant clinical cardiac involvement in older patients with DMD [Eagle et al. 2002, Eagle et al. 2007, Kohler et al. 2009]. In contrast to the large number of studies focused on repairing or improving skeletal muscle, few studies have focused on treatment of cardiac muscle in DMD patients. However, recognizing the prevalence of cardiac involvement in this disease, the need to understand cardiomyopathy in DMD patients has resulted in an increase in the number of studies on this topic. It is therefore important to identify animals that develop cardiomyopathy similar to that of Duchenne patients, for use in preclinical studies to alleviate cardiac dysfunction resulting from dystrophin-deficiency.

Using echocardiography to monitor heart function and other parameters, we now report the development of progressive, dilated cardiomyopathy in the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mouse, similar to patients. At 5 weeks of age, the only significant difference between the wild-type and mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient heart is an elevated heart rate in the dystrophic mice. An elevated heart rate, or tachycardia, has been documented in patients with DMD [Heymsfield et al. 1978, Yotsukura et al. 1995, Bhattacharyya et al. 1997, Gulati et al. 2005, Kirchmann et al. 2005], at ages as young as 4 years [Heymsfield et al. 1978] and in patients as old as 24 years [Yotsukura et al. 1995]. Similarly, the difference in heart rate between the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient and wild-type mice is present at the earliest time point and persists until 15 weeks of age, with increasing statistical significance over time (Table 1). The presence of chronic tachycardia in DMD patients and mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice may exacerbate cardiomyopathy, as chronic
tachycardia can result in left ventricular dysfunction and dilation [Umana et al. 2003, Mohamed et al. 2007], including thinning of both the left and right ventricular walls.

At 10 and 15 weeks of age, changes indicative of dilated cardiomyopathy were apparent in the mdx/utrn−/− dystrophin/utrophin-deficient mice. These changes were consistent with the clinical criteria for diagnosis of dilated cardiomyopathy, which include reduced left ventricular function assessed by a decrease in fractional shortening and ejection fraction, and an increase in end-diastolic volume [Mestroni et al. 1999, Taylor et al. 2006]. Left ventricular fractional shortening is a common measurement of left ventricular function representing the ventricular size change between filling (diastole) and emptying (systole) of the ventricle. There is a significant decrease in fractional shortening in 15 week old mdx/utrn−/− dystrophin/utrophin-deficient mice (29.64%) in comparison to age-matched wild-type mice (38.62%). In fact, fractional shortening increases with age in wild-type mice, rather than decreasing, as in the mdx/utrn−/− dystrophin/utrophin-deficient mice. Ejection fraction, or the blood volume leaving the left ventricle with each contraction, is also significantly decreased in 15 week old mdx/utrn−/− dystrophin/utrophin-deficient mice (62.29%), in contrast to age-matched wild-type mice, which have an increase in ejection fraction as they mature (77.49%). In addition, there is also a difference in end-diastolic volume, which is the blood volume of the distended left ventricle at the end stage of diastole, often represented as the preload or force stretching the myocardium, between mdx/utrn−/− dystrophin/utrophin-deficient mice and wild-type mice. Mdx/utrn−/− dystrophin/utrophin-deficient mice have significantly increased end-diastolic volume (0.0841 ml) compared to age-matched wild-type (0.0533 ml), indicating
that more blood remains in the left ventricle following each contraction and therefore less blood is pumping out to the body. Together, the changes in these parameters support a gradual decrease in left ventricular function in the mdx/utrn−/− dystrophin/utrophin-deficient mouse heart. This also correlates with a gradual thinning of the left ventricular wall, and an increased overall diameter of the left ventricle, with a significantly larger chamber than that of wild-type mice by 15 weeks of age. In summary, the echocardiograph data indicate that mdx/utrn−/− dystrophin/utrophin-deficient mice develop dilated cardiomyopathy that is similar to Duchenne patients, with a sequence of events also similar to the human disease.

Mdx mice also exhibit dilated cardiomyopathy similar to DMD patients, but develop the condition more slowly than do the mdx/utrn−/− dystrophin/utrophin-deficient mice. Mdx mice develop a decrease in fractional shortening by 40–43 weeks of age [Quinlan et al. 2004, Spurney et al. 2008, Au et al. 2010], but do not exhibit differences in this parameter at earlier time points examined, including 8 and 29 weeks of age [Quinlan et al. 2004]. Ejection fraction is also decreased in mdx mice by 40 weeks of age [ Spurney et al. 2008] and ventricular dilation is observed by 36–42 weeks of age [Quinlan et al. 2004, Spurney et al. 2008] but not at 8 or 29 weeks of age [ Quinlan et al. 2004]. Mdx and mdx/utrn−/− dystrophin/utrophin-deficient mice therefore both develop symptoms indicating dilated cardiomyopathy but the mdx/utrn−/− dystrophin/utrophin-deficient mice exhibit changes much more rapidly than the mdx mice. Histopathology corresponding to the decline in heart function was detected in mdx/utrn−/− dystrophin/utrophin-deficient cardiac muscle. Regions of fibrosis were detected in right and left ventricles, as well as the interventricular septum, of mdx/utrn−/−
dystrophin/utrophin-deficient mice, with the most prominent involvement at the lateral left ventricular wall, where all mice examined had fibrosis at 5, 10, and 15 weeks of age. As the mice advanced in age, fibrosis was detected more frequently in the interventricular septum and later in the right ventricular wall. The size of the fibrotic areas directly correlated with increasing age, and was larger at the latest stage of the disease when ventricular dysfunction was also greatest. In addition, the large areas of fibrosis in 15 week old mice were in the outer half of the ventricular wall (Fig. 2F), which is consistent with, and distinctive for, Duchenne muscular dystrophy [reviewed in Kaspar and Allen 2009]. Damage to myocardial cell membranes was also detected in the mdx/utrn−/− dystrophin/utrophin-deficient mice, as previously reported by Grady et al. [Grady et al. 1997], by examining the presence of serum proteins within cardiomyocytes using Evan’s Blue Dye to bind albumin. The localization of damaged cells was not previously reported. In our study, Evan’s Blue Dye-positive cells were detected within the left and right ventricles as well as the interventricular septum (Fig. 4), correlating with regions of fibrosis. This differs from the mdx mice in our study, which had Evan’s Blue Dye-positive cardiomyocytes at 15 weeks of age, but only in the septum. This is in agreement with another study in which Evan’s Blue Dye-positive cells were reported in 3 month old mice [Erp et al. 2010]. A second study reported the absence of Evan’s Blue Dye-positive cells in the mdx heart at 10 weeks of age [Grady et al. 1997]. Collectively, these data suggest that membrane damage to cardiomyocytes occurs between 10 and 15 weeks of age in mdx mice as well as in the mdx/utrn−/− dystrophin/utrophin-deficient mice. Cellular necrosis had previously been detected in cardiac muscle of the mdx/utrn−/− dystrophin/utrophin-deficient mice [Grady et al. 1997] but the localization not reported.
We also detected necrosis, in both the left and right ventricles. The cell membrane damage and necrosis in the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice are consistent with reports of myocardial damage in Duchenne patients [Ramaciotti and Iannaccone 2003, Kirchmann et al. 2005].

Ultrastructure analysis of cardiomyocytes from 15 week old mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice revealed disruptions in myofibrils in the cardiomyocytes, which would likely negatively impact cell contraction and heart function. Myofibril disruptions were frequently due to mislocalized, disorganized mitochondria. We also observed abnormalities in mitochondria size and shape within the cell as well as cristae organization within the mitochondria (Fig. 5). A previous report of the ultrastructure of cardiomyocytes in 10 week old mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice did not indicate that abnormalities in mitochondria were present [Sanford et al. 2005]. However, similar results have been reported in cardiomyocytes of δ-sarcoglycan (δ-SG)-deficient hamsters that also develop dilated cardiomyopathy and do not express dystrophin in the heart [Kawada et al. 2003]. In that study, irregularly shaped mitochondria that varied in size and had loosely arranged cristae were detected at 25 weeks of age, although the overall organization of mitochondria was not reported to be abnormal. An increase in the numbers of mitochondria present in cardiomyocytes was also reported in 1–6 year old dogs with canine X-linked muscular dystrophy, although there was no evidence of abnormal organization of mitochondria within the cells, changes in shape of the mitochondria, or organization of cristae within the mitochondria [Valentine et al. 1989]. Although not specifically linked to alterations in members of the dystrophin–glycoprotein complex, mitochondrial abnormalities have also been reported
in patients with dilated cardiomyopathy in the clinic. Mitochondrial degeneration has been reported in children [NSimonds et al. 1999] and adults [Sekiguchi et al. 1978] reviewed in [NSimonds et al. 1999] with dilated cardiomyopathy, and mitochondriosis, or increased numbers of mitochondria, is present in cardiomyocytes of children with dilated cardiomyopathy [Schmaltz et al. 1990]. Oversized mitochondria and abnormalities in cristae were also observed in patients with dilated cardiomyopathy [Arbustini et al. 1998]. In addition, patients with mutations in mitochondrial DNA develop dilated cardiomyopathy [Arbustini et al. 1998], suggesting that mitochondrial defects and dysfunction may contribute to development of dilated cardiomyopathy. Accumulation of abnormal mitochondria in mdx/utrn−/− cardiac muscle may affect the metabolic rate in the heart and thereby exacerbate the heart dysfunction resulting from loss of cardiomyocytes.

The earlier onset of fibrosis and cardiac dysfunction in mdx/utrn−/− mice, in comparison to mdx mice, suggests that utrophin may functionally compensate for dystrophin. Utrophin-deficient mice do not exhibit a detectable cardiac phenotype [Deconinck et al. 1997], and Grady et al. reported that there was no detectable histological change in utrophin-deficient cardiac muscle [Grady et al. 1997]. However, Janssen et al. [Janssen et al. 2005] reported that mdx/utrn−/− dystrophin/utrophin-deficient mice exhibit contractile dysfunction to a greater degree than mdx mice, indicating that utrophin may be able to functionally compensate for dystrophin to some extent. This correlated with the up regulation and re-localization of utrophin in mdx hearts [Pons et al. 1994]. In mdx heart, utrophin localization is disrupted and the protein is expressed in regions of cardiomyocytes normally occupied by dystrophin, but not utrophin [Pons et al. 1994].
Based on the echocardiograph data and histological assays, mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice develop dilated cardiomyopathy similar to DMD patients, within 15 weeks (Fig. 6). The use of the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice for preclinical studies to treat the cardiomyopathy therefore may be advantageous over other mouse models for DMD, including the mdx and the mdx/myoD mouse. The latter two mouse models for DMD develop cardiomyopathic changes similar to patients as well, but in longer periods of time, 6–21 months [Bridges 1986, Quinlan et al. 2004, Bostick et al. 2008], and 5 months [Megeney et al. 1999, Duan 2006] respectively. Studies could be conducted at an accelerated pace in the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice, and would cost considerably less. However, mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice have severe and progressive skeletal muscle disease as well as cardiomyopathy, and as a result are fragile and may not survive 15 weeks, limiting their use for long-term studies. As a result, a combination of the three mouse models would allow both short- and long-term preclinical studies for potential therapies (Fig. 6).

In conclusion, the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mice have previously been established as an excellent phenotypic model for Duchenne muscular dystrophy because of the many similarities to patients in the development of skeletal muscle pathology [Grady et al. 1997]. Our study demonstrates that the mdx/utrn<sup>−/−</sup> dystrophin/utrophin-deficient mouse may also serve as a tool for simulating cardiac muscle dysfunction and pathology in Duchenne patients, developing dilated cardiomyopathy similar to patients with DMD in both functional and histological aspects (Fig. 6; [ Frankel and Rosser 1976, Miyoshi 1991, Moriuchi et al. 1993, Sasaki et al.].
1998, Nishimura et al. 2001, Connuck et al. 2008]). Additionally, the progression and time frame of cardiomyopathy in the mdx/utrn−/− dystrophin/utrophin-deficient mice is similar to that of Duchenne muscular dystrophy patients (Fig. 6). As a result, mdx/utrn−/− dystrophin/utrophin-deficient mice may be useful for advancing understanding of cardiomyopathy in Duchenne muscular dystrophy, as well as for testing potential experimental therapeutic approaches for cardiomyopathy in Duchenne patients.
### A. Echocardiography in comparison of mdx/utrn⁻ and wild type mice at different ages

<table>
<thead>
<tr>
<th>Item</th>
<th>Wild type</th>
<th>mdx/utrn⁻ type</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Week M-mode</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>5 (n=4)</td>
<td>10 (n=4)</td>
<td>15 (n=6)</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.081±3</td>
<td>0.100±7</td>
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</tr>
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<td>IVSS (cm)</td>
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<td>LVIDs (cm)</td>
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<td>LVPWs (cm)</td>
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<td>EDV (ml)</td>
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<td>ESV (ml)</td>
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<tr>
<td>EF (%)</td>
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<td>75.28±b</td>
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</tr>
<tr>
<td>FS (%)</td>
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<td>35.71±c</td>
<td>38.62±b</td>
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<td>SV (ml)</td>
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<td>HR (BPM)</td>
<td>506.13±a</td>
<td>452.25±b</td>
<td>482.18±b</td>
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</table>

IVSd: Interventricular septum at diastole, LVIDd: Left ventricular internal dimension at diastole, LVPWd: Left ventricular posterior wall at diastole, IVSS: Interventricular septum at systole, LVIDs: Left ventricular internal dimension at systole, LVPWs: Left ventricular posterior wall at systole, EDV: End-diastolic volume, ESV: End-systolic volume, EF: Ejection fraction, FS: Fractional shortening, SV: Stroke volume, HR: Heart rate, a, b, c Within a row, means without a common superscript differ in each type of mice (P < 0.05), 2PSEM= Pooled SEM, 3M*W= Interaction between mice type and week.

### B. Body and heart weight in comparison of mdx/utrn⁻ and wild type mice at different ages

<table>
<thead>
<tr>
<th>Item</th>
<th>Wild type</th>
<th>mdx/utrn⁻:</th>
<th>Main effects</th>
<th>P-value</th>
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</thead>
<tbody>
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<td></td>
<td>Week</td>
<td>5 (n=4)</td>
<td>10 (n=4)</td>
<td>15 (n=6)</td>
</tr>
<tr>
<td>Heart, g</td>
<td>0.1194±a</td>
<td>0.1596±b</td>
<td>0.1667±c</td>
<td>0.1248±b</td>
</tr>
<tr>
<td>Body, g</td>
<td>15.256±c</td>
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<td>21.138±b</td>
<td>14.462±b</td>
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a, b, c Within a row, means without a common superscript differ in each type of mice (P < 0.05), 2PSEM= Pooled SEM, 3M*W= Interaction between mice type and week.
Figure 1. Mdx/utrn⁻/⁻ left ventricular function at 15 weeks of age, in comparison to age-matched wild-type mice.

There is a significant decrease in Mdx/utrn⁻/⁻ left ventricular function at 15 weeks of age, in comparison to age-matched wild-type mice. Fractional shortening (A), ejection fraction (B), and end diastolic volume (C), of mdx/utrn⁻/⁻ and wild-type mice at ages of 5, 10, and 15 weeks. *: p<0.05.
Fibrosis in mdx/utrn−/− cardiac muscle. Trichrome staining of the left ventricle of mdx/utrn−/− (B, D, F) and age-matched wild-type (A, C, E) mice indicates that fibrosis is present in the mdx/utrn−/− heart as early as 5 weeks (B, arrow), and increases at 10 and 15 weeks of age (D, F, arrows). 4x
Figure 3. Increased fibrosis in the ventricles of mdx/utrn<sup>−/−</sup> mice over time

There is a significant increase in fibrosis in the ventricles of mdx/utrn<sup>−/−</sup> mice over time. A, short-axis view of the heart, with numbers designating specific regions where fibrosis was detected. RV, right ventricle; LV, left ventricle. B, localization of fibrosis in the mdx/utrn<sup>−/−</sup> cardiac muscle over time, including the number of mice with fibrosis in the regions indicated in ‘A’. C, Quantitation of fibrosis in mdx/utrn<sup>−/−</sup> or wild-type ventricles at 5, 10, and 15 weeks. D-G, Trichrome staining corresponds to the presence of collagen I in mdx/utrn<sup>−/−</sup> cardiac muscle. D, Trichrome staining, image taken at 4x magnification. E-F, Collagen I immunofluorescence (red, Collagen I primary antibody with TRITC-conjugated secondary antibody) in an adjacent serial section, G, TRITC-secondary antibody-only negative control for collagen staining. F, G, merged images with DAPI for visualizing nuclei. E-G, 10x
Figure 4. Damaged cardiomyocyte membranes and necrosis in cardiac muscle of 15 week old mdx/utrn\textsuperscript{-/-} and mdx mice.
Figure 4. Damaged cardiomyocyte membranes and necrosis in cardiac muscle of 15 week old mdx/utrn<sup>−/−</sup> and mdx mice. A-D, Evan’s Blue Dye-positive cells in the mdx/utrn<sup>−/−</sup> heart. A-B, Evan’s Blue Dye-positive cardiomyocytes in the right ventricle (arrowheads) and the interventricular septum (arrows). C-D, Evan’s Blue Dye-positive cardiomyocytes in the left ventricle (arrows). E-F, Evan’s Blue Dye-positive cells in the septum of the mdx heart. A-C, and E, Evan’s Blue Dye, red; wheat germ agglutinin, green; B, D, and F are merged images of A, C, and E with DAPI-labeled nuclei, blue, respectively. G, H, Necrosis was detected in mdx/utrn<sup>−/−</sup> heart (G) but not mdx heart (H) at 15 weeks of age with H&E staining. A-F, yellow arrows indicate chamber of the heart. Images for panels A-F acquired at 10x magnification; images for panels G and H acquired at 40x magnification.
Ultrastructure of mdx/utrn⁻/⁻ cardiac muscle. A and B, Mitochondria (M, arrowheads) have a regular arrangement in 15 week old wt cardiac muscle between fibrils. C and D, Mitochondria (M, arrowheads) are disarranged and of varying sizes in 15 week old mdx/utrn⁻/⁻ cardiac muscle and the Z-bands are frequently disrupted by the irregularly localized mitochondria (arrows). T, t-tubules. Magnification, 2000x, A, C; 10,000x, B, D.
Figure 6.

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<th>↓ FS/EF and ↑ EDV/LVID</th>
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<tr>
<td>Fibrosis in left ventricle</td>
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Figure 6. Mdx/utrn−/− mice have a similar pattern of cardiac involvement to DMD patients. A comparison of the pathology, and the functional and structural data from the current study of the mdx/utrn−/− dystrophin/utrophin-deficient mouse, to reported data from Duchenne patients, reveals a similar sequence of events in the development of dilated cardiomyopathy. Mouse age was compared with human age based on Jugdutt et al., 2008 (51). CI; cardiac involvement, DCM; dilated cardiomyopathy, EDV; end diastolic volume, EF; ejection fraction, FS; fractional shortening, HR; heart rate, LVPW; left ventricular posterior wall thickness, LVID; left ventricular internal dimension.
Chapter III

Aorta-derived mesoangioblasts differentiate to cardiomyocytes

A. Introduction

In DMD contractile movement easily damages cardiomyocyte membrane integrity because of the absence of dystrophin. Damaged cell membranes trigger excessive extracellular calcium influx [Williams and Allen 2007], calcium related protease activity, and eventually induce cardiomyocyte cell death [Raymackers et al. 2003]. This process provokes an inflammatory response that leads to fibrosis, affecting the flexibility of cardiac muscles during contraction. This eventually results in development of dilated cardiomyopathy (DCM). The combination of cardiomyocyte loss and broad fibrous scar areas reduce heart function and recovery is rare.

As a therapeutic approach to DCM, it is important to replace dead/damaged cardiomyocytes and restore electromechanical function. Stem cells have unlimited self-renewal capacity and multilineage differentiation potency, which indicate that stem cell therapy may be a good mechanism for generating new cardiomyocytes in damaged heart. Stem cells have been isolated from different tissues including fertilized eggs: Embryonic stem cells from blastocysts, mesenchymal stem cells from bone marrow, adipose and umbilical cord, cardiac stem cells from hearts and spermatogonial stem cells from testis. These stem cells have differentiated to cardiomyocytes in vitro but rarely in vivo after transplantation [Guan et al. 2007, Smith et al. 2007, Van Dijk et al. 2008, Fan et al. 2011, Parson et al. 2011, Lin et al. 2012]. Unlike other stem cell types embryonic stem cells have been reported to differentiate into cardiomyocytes and are able to contract in vitro.
and in vivo [Kehat et al. 2001, Laflamme et al. 2007, Yamada et al. 2008]. However because of the possible immune rejection and teratoma/teratocarcinoma formation in addition to the ethical controversy surrounding destruction of embryos to attain them embryonic stem cells are not an attractive source of cells therapy.

Mesoangioblasts are multipotent stem cells, and were derived from embryonic dorsal aorta, or adult atria and ventricles of the heart [Galvez et al. 2008]. The cardiac mesoangioblasts differentiated to cardiomyocytes with sarcomeric myosin expression, and in vitro contractility was also observed following differentiation. The cardiac mesoangioblasts also formed new myocardium after intraventricular injection to the ischemic heart. Our lab has derived a similar population of cells, Aorta-derived mesoangioblasts (ADM), from postnatal aorta of juvenile mice that are myogenic and differentiate into smooth and skeletal muscle cells in vitro and in vitro. Moreover, after transplantation to gastronemius of mdx/utrn−/− mouse model of DMD, ADM were incorporated into the damaged skeletal muscles and decreased the number of damaged muscle fiber [Berry et al. 2007]. This finding, together with demonstrated cardiac differentiation of embryonic mesoangioblasts, supports further studies to determine whether myogenic ADM also differentiate to cardiomyocytes. ADM may therefore be used to treat two main defects of DMD: skeletal muscle degeneration and dilated cardiomyopathy.
B. Materials and methods

B-1. Cell culture of mesoangioblasts

ADM were cultured in Iscove’s Dulbecco Modified Eagle’s minimal essential medium (DMEM) containing 20% FBS, 0.1 U/ml penicillin, 0.1 µg/ml streptomycin, 2.0 mM L-glut, 0.1 mM nonessential amino acids, and minimal essential medium (MEM) vitamin solution from Gibco (Carlsbad, CA, USA) and 20 ng/ml purified leukemia inhibitory factor (LIF, Chemicon International, Temecula, CA, USA), hereafter referred to as ‘proliferation medium’. The cells were grown on 0.1% gelatin coated plates and maintained in a humidified incubator at 37°C in 5% CO₂.

B-2. In vitro cardiomyocyte differentiation assay

ADM were seeded on 0.1% gelatin coated plates with glass coverslips in proliferation medium. The 2% horse serum differentiation medium contains 2% horse serum in DMEM. The CMGS differentiation medium contains Cardiomyocyte Growth Supplement (100X) (ScienCell Research Laboratories, Cat. No. 6252) in 5% FBS proliferation medium. The N2B27 differentiation medium contained 10ul/ml NDiff Neuro-2 Supplement (200x) (Chemicon, Cat. No. SCM012), 20ul/ml B27 Serum free supplement (50x) (Gibco, Cat. No.17504-044) in 4.5g/L glucose DMEM without L-glutamine (Cellgro, Cat. No. 15-013-CM). Rat primary cardiomyocytes from neonate rat heart (ventricle) (ScienCell Research Laboratories, Cat. No. R6200) were used in 5% FBS DMEM as a positive control for cardiomyocytes differentiation. Cells were grown on 0.1% gelatin coated plates with glass coverslips. The media for each cell type was changed every other day. ADM were cultured in differentiation medium for 14 days, and
images were acquired every other day. Quantitation of cardiomyocyte differentiation was assessed by determining the number of cells in the total population with cardiomyocyte-like morphology.

**B-3. Immunocytochemistry assay**

Cells on glass coverslips were fixed in 3.7% formaldehyde for 10 minutes, and blocked in 2% horse serum and 5% BSA for 1 hour. Cardiac differentiation was determined by expression of cardiac specific proteins. Mouse monoclonal anti-Troponin I (1 to 300 dilution; Millipore Cat. No. MAB1691), sheep polyclonal anti-Tropomyosin (1 to 300 dilution; Abcam Cat. No. ab5441) and mouse monoclonal anti-actinin (1 to 100 dilution; Millipore Cat. No. MAB1682) primary antibodies were used. Cells were fixed with 3.7% formaldehyde for 10 minutes, and permeabilized with 0.25% Triton X-100 for 10 minutes at room temperature prior to incubation with primary and secondary antibody. Fixed cells were blocked with 2% BSA and 5% horse serum in PBS for 1 hour, incubated with primary antibodies for 1 hour at room temperature, washed with 1 X phosphate buffered saline, labeled with secondary antibodies (Jackson ImmunoResearch) for 1 hour, and mounted with Vectashield mounting medium including DAPI (Vector Laboratories, Burlingame, CA). Rabbit polyclonal anti-Connexin 43 (1 to 300 dilution; Sigma Cat. No. C6219) was used for detecting gap junctions. Fluorescent images were acquired with a Retiga 200R digital camera (Q-imaging, Surrey, BC) (Nuhsbaum, McHenry, IL). Image Pro Plus software (MediaCybernetics, Bethesda, MD) was used to assign and merge colors.
B-4. RT-PCR analysis

Expression of cardiac specific transcription factors in ADM after 14 days in differentiation medium was detected by RT-PCR. The total RNA from cells grown as a monolayer was isolated by Trizol Reagent (Invitrogen, Cat. No. 15596-018), and cDNA was synthesized by QIAGEN LongRange 2Step RT-PCR kit (Qiagen, Cat. No. 205920) as per the manufacture’s instruction. The primers used in RT-PCR are listed in Table 2.

B-5. Animals

Mice were cared for according to the Illinois Institutional Animal Care and Use Committee guidelines. Mdx/utrn+/− mice were used for generating mdx/utrn−/− mice as described in previous studies [Burkin D. J. 2001, Grange R. W. 2002]. After weaning, all mdx/utrn−/− mice were placed on the heating pad with moisturized food and extra bedding on the cage bottom until they were euthanized.

B-6. GFP transfection of ADM

For the preparation of GFP labeled ADM, the cells were maintained on gelatin coated plates in proliferation medium. The cells were transfected with pIRES2-AcGFP1 (Clontech, CA, USA) using the LipofectaminTM2000 reagent (Invitrogen, CA, USA), according to the product instruction manual. After transfection the cells were cultured in proliferation medium with 400mg/ml of G418. The transfected cells were sorted by Fluorescence Activated Cell Sorting (FACS) using an iCyte® Automated Imaging Cytometer) based on positive GFP signal. Non-transfected control cells were used to set the background fluorescence. GFP positive cells were detected by using 520 λ keep the
FITC channel, and sorted for purity at 2,000 cells/sec. After sorting, GFP positive cells were maintained in proliferation medium with 20 ng/ml LIF and 400mg/ml of G418.

**B-7. DiI labeling of ADM**

ADM were incubated with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) at a concentration of $1 \times 10^6$ cells/ml with 5µl with 5µl DiI/ml in proliferation medium for 30 minutes in a humidified incubator at 37°C in 5% CO₂ prior to the transplantation. After washing with Hank’s Balanced Salt Solution (HBSS) three times, DiI labeled ADM were suspended in HBSS with the concentration of $5 \times 10^6$cells/50µl. For double labeling GFP transfected ADM were labeled with DiI prior to the transplantation.

**B-8. Cardiac injection of ADM**

Mice were anesthetized by halothane (Halocarbon, USA) mixed in mineral oil (7.5 ml halothane/40ml of mineral oil). Bactoshield CHG 4% solution (STERIS, MO) was used to scrub the anterior chest area of mice. Labeled ADM were transplanted to the wall of left ventricle as previously described [Odintsov et al. 2011]. Briefly, insulin syringes (3/10 cc with a 29 G needle) with $5 \times 10^6$cells in 50µl HBSS were injected into the fifth intercostal space to the left side of the sternum where left ventricular wall is located. The plunger was slowly pushed, and the needle was retracted gently. Following injection of cells mice were placed on heating pad and their condition was observed until they were fully recovered.
C. Results

C-1. Aorta-derived mesoangioblasts differentiate to cardiomyocytes in vitro

The differentiation of ADM to cardiomyocytes was induced with three different supplements 1) 2% horse serum 2) cardiomyocyte growth supplement, and 3) N2B27 serum free supplement. Among them, N2B27 serum free supplement in DMEM was the most effective for induction of ADM differentiation to cardiomyocyte based on morphological change (data not shown). ADM in proliferation medium has spindle shape (Fig7-A), and has changed its morphology similar to rat cardiomyocyte with a large and paper-thin flat cytoplasm in differentiation medium (Fig7-B and C). In addition, ADM in differentiation medium made tight contact with adjacent cells, unlike ADM in proliferation medium (Fig7-C).

At day 9 in N2B27 differentiation medium less than 40% of cells had cardiomyocyte-like morphology, but nearly all, 97.82%, of cells had cardiomyocytes-like morphology at day 14 (Fig10-A). After induction of differentiation to cardiomyocytes, we examined ADM for expression of cardiac specific mRNA and proteins. By RT-PCR cardiac specific homeobox transcription factor Nkx2.5, cardiac troponin I (cTnI) and tropomyosin (cTm) were detected in differentiated ADM (Fig. 8). In addition cTnI, cTm and α-actinin proteins were also detected by immunohistochemistry (Fig. 9). At day 14 in N2B27 differentiation medium 69% of ADM expressed cTm protein compared to 7% of ADM in proliferation medium (Fig10-B). In addition, connexin 43, a gap junction protein was also expressed in the tight contact regions with adjacent cells (Fig9-G).
C-2. Aorta-derived mesoangioblasts differentiate into cardiomyocytes in vivo

To investigate in vivo differentiation, ADM were transplanted into mdx cardiac muscle. For tracking transplanted cells, ADM were double labeled with DiI and GFP to ensure that cells could still be detected if the DiI label was diluted by cell proliferation. First ADM were transfected with GFP. 96.33% of cells (Fig. 11) were GFP positive which were labeled with DiI prior to the transplantation. Cells co-labeled with both GFP and DiI were localized in the ventricles of the heart (Fig. 12). After verifying that DiI was not lost from ADM after injection due to co-localization of both labels in cells, ADM were singly labeled with DiI for subsequent experiments. ADM silenced GFP expression over time, and that GFP prevent ADM differentiation into some mature cell types after transfection with GFP (data not shown).

To determine whether the transplanted ADM differentiated into cardiomyocytes, immunohistochemistry was performed using an antibody to cardiac troponin I (cTnI), marker of cardiomyocytes (Fig. 13-A). DiI positive cells co-localized with cTnI positive labeling, indicating that the donor ADM expressed cTnI 6 weeks after injection into the heart (Fig 13-B-C).

Next, immunostaining was used to examine whether dystrophin was restored in cardiac muscle of mdx mice after ADM transplantation. Dystrophin was detected in the specific pattern around the cytoplasm in longitudinal and cross sections of muscle tissue from the ventricles (Fig. 14). DiI particles were detected in the cytoplasm of cardiac myocytes positive for dystrophin staining. These data confirm that ADM express dystrophin in the heart after injection, indicating that the stem cells have differentiated into muscle cells in vivo. However the intensity of detected dystrophin was weaker than
wild type positive control and dystrophin was detected in only limited regions of the heart rather than throughout the entire ventricle.

D. Discussion

Because of the absence of dystrophin, cardiomyocytes in DMD are exposed to the continuous damage by mechanical stress, which will eventually induce the death of cardiomyocytes. Unfortunately hearts rapidly lose their regenerative ability after birth, and leave little option for treatment following damage except heart transplantation [Bergmann et al. 2009]. Cell therapy with transplantation of exogenous stem cells is currently being investigated for the treatment of damaged cardiac muscles. Although multiple types of stem cell have been tested for regeneration of skeletal muscle in DMD animal models and DMD patients, few have been tested for treatment of dilated cardiomyopathy in DMD.

First, ADM were induced to differentiate to cardiomyocytes in three different medium conditions; 2% horse serum, cardiomyocytes growth supplement, and N2B27, Cardiomyocyte-like cells with a flat and thin cytoplasm were detected in all three medium conditions, although the serum free condition showed the most dramatic result. In N2B27 differentiation medium most of ADM changed their morphology to cardiomyocyte-like in 14 days and expressed cardiac specific cDNAs (Nkx2.5, cTnI and cTm) and proteins (cTnI, cTm and α-actinin). We did not expect ADM to differentiate into cardiac-like cells in this medium condition because N2 and B27 supplements were formulated for the growth of rat neuroblastoma cells and survival of hippocampal neurons in a serum free culture system [Bottenstein et al.1979, Brewer et al. 1993].
However, cardiomyogenic differentiation may share some common molecular players and pathways with neuronal differentiation during embryogenesis. Murashov et al. induced cardiomyocyte differentiation by neuronal differentiation medium [Murashov et al. 2004], and also reported that embryonic stem cells differentiated to functional cardiomyocytes with neuronal cells in aggregation [Murashove et al. 2005]. In addition a transcription factor, serum response factor (SRF) is involved in cardiomyocyte differentiation [Zhao et al. 2005] and also in the central nervous system [Stringer et al. 2002]. These data offer an explanation and precedent for our observations of efficient ADM differentiation to cardiomyocytes in the medium with N2B27 neuronal supplements.

Connexin 43 is a predominant gap junction molecule in the heart, which serves to electrically couple cardiomyocytes in the heart, allowing the heart to maintain a rhythm during contraction. In the differentiation medium, ADM formed tight contact with adjacent cells and expressed connexin 43 at the junctions. Connexin 43 expression by ADM strongly supports the possibility that these cells would be a good stem cell source that would functionally integrate with cardiomyocytes in vivo to provide functional benefit.

GFP and DiI positive cells were found to co-localize in the heart 3 weeks after transplantation. DiI co-localized with cTnI in cells in the ventricle of the heart. These data indicate that ADM express cardiac-specific markers up to 6 weeks following transplantation, indicating that the cells survive and differentiate into cardiomyocytes. ADM may therefore be used to replace damaged/dead cardiomyocytes. Modest dystrophin expression was detected in the heart after injection of ADM, and co-localized
to DiI positive cells in the left ventricle. This indicates that injected ADM may differentiate to cardiomyocytes and restore dystrophin expression in dystrophic hearts.

For regeneration of cardiac muscles in patients with DMD there are two main aspects to be considered. First damaged cardiomyocytes must be replaced/regenerated to recover heart function. Second, cardiac muscle needs to be protected from further damage by contractile stress, for example, through restoring dystrophin. In this regard stem cells carrying a full-length dystrophin gene for therapeutic application in DMD are strongly suggested to study their capability of cardiomyocytes differentiation and dystrophin restoration in vitro and in vivo. With this, in this study ADM has been tested its differentiation ability to cardiomyocytes and restore dystrophin in dystrophic hearts.
Figure 7. Induction of ADM differentiation into cardiomyocytes in vitro

Induction of ADM differentiation into cardiomyocytes in vitro. ADM in proliferation medium (A), primary rat cardiomyocytes (B) and ADM in differentiation medium with N2B27 (C).
ADM express cardiac specific mRNA. Heart: tissue from cardiac muscle was used as a positive control from heart tissues, Adipose was used as a negative control for cardiac markers, HS: horse serum, CMGS: cardiomyocyte growth supplement, GAPDH: an internal loading control. cTnI: cardiac troponin I, cTm: cardiac tropomyosin
Cardiac specific protein expression in ADM. Primary rat cardiomyocytes were used as a positive control for cardiac proteins (A-C), ADM in differentiation medium also express cardiac markers (D-F), cardiac tropomyosin (A and D), cardiac troponin I (B and E), alpha-actinin (C and F), connexin 43 (G), Blue: DAPI.
Figure 10. Quantitative analysis of ADM cardiac differentiation in vitro.

Quantitative analysis of ADM cardiac differentiation in vitro. Cardiac morphological change (A) and cardiac tropomyosin expression (B) of ADM in differentiation medium with N2B27 supplements.
pIRES2-AcGFP1 transfection and characterized by Fluorescent Activated Cell Sorting (FACS) of ADM. Untransfected cells were used as a negative control (A), ADM expressing GFP were sorted with FACS (B), and observed using fluorescence microscopy (C).
Figure 12. ADM are detected in vivo using GFP and DiI after transplantation to mdx heart

Using GFP and DiI labeling were used to track ADM after transplantation into mdx heart. DiI (A), GFP (B), DiI with DAPI (C) and GFP with DAPI (D).
Figure 13. DiI labeled ADM express cardiac markers after transplantation to the heart of mdx mice.

DiI-labeled ADM express cardiac marker after transplantation to the heart of mdx mice. Cardiomyocytes co-localized with DiI in heart section were after DiI labeled ADM transplantation to the heart of mdx mice. Cardiac troponin I (green) with DAPI (A), DiI (red) with DAPI (B), cardiac troponin I positive cells with DiI and DAPI (C), and secondary (FITC) antibody negative control (D).
Figure 14. DiI labeled ADM exhibit dystrophin expression in the heart of mdx mice.

DiI-labeled ADM exhibit dystrophin expression in the heart of mdx mice.
Dystrophin (green) with DiI (red) (A) and dystrophin co-localized with DiI and DAPI in longitudinal heart section (B), and dystrophin with DiI (C) and dystrophin co-localized with DiI with DAPI D) in heart cross section.
Table 2. Primer sequences for RT-PCR.

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<td>Troponin I</td>
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<td>Nkx 2.5</td>
<td>100 bp</td>
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<td>GAPDH</td>
<td>150 bp</td>
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Chapter IV

Injection of vessel derived stem cells prevents onset of dilated cardiomyopathy in \(mdx/utrn^-\) dystrophin/utrophin-deficient mice but fails to improve pre-existing dilated cardiomyopathy in aged \(mdx\) mice

A. Introduction

Cardiac involvement in Duchenne muscular dystrophy (DMD) is covert, unlike severe skeletal muscle defects observed in this disease, and patients often exhibit an advanced stage of dilated cardiomyopathy when it is diagnosed. Approximately 90% of patients are diagnosed with cardiomyopathy and 25% of them exhibit pre-clinical cardiac involvement by 6 years of age. 20–40% of patients die because of cardiomyopathy by their third decade of life. [Nigro et al. 1990, Bushby et al. 2003, Baxter et al. 2006].

In the past decade, medical advances have been developed that extend the lifespan of DMD patients from their early teens into their early 30s. As disease progression continues over this time, an increase in the incidence of cardiac dysfunction has been observed [Kamogawa et al. 2001, Eagle et al. 2002, Eagle et al. 2007]. This underscores the importance of finding a method to treat cardiomyopathy in these patients [Townsand et al. 2009]. As a result, follow-up monitoring for the development of cardiomyopathy has been recommended for patients after a diagnosis of DMD, to allow management of progressing cardiomyopathy [Ahuja et al. 2000, Bouhouch et al. 2008]. In this regard it is important to investigate the effect of candidate treatments of DCM in DMD during different stages of disease in the heart.
For treatment of dilated cardiomyopathy in DMD there are two aspects of concern: 1) replacing damaged/dead cardiomyocytes and 2) restoring dystrophin expression. The absence of dystrophin leads to loss of cell membrane integrity and eventually to cell death. For recovering cardiac function it is important to replace damaged/dead cardiomyocytes. Hearts have limited regeneration capacity from endogenous cardiomyocytes. Transplantation of exogenous stem cells with a normal copy of the dystrophin gene might contribute to replace damaged/dead cardiomyocytes and restoration of dystrophin in hearts with non-functional dystrophin. However, simply replacing cardiomyocytes is not sufficient. Because cardiac myocytes that do not express dystrophin are continuously exposed to damage from mechanical stress during contraction, restoration of dystrophin is also essential for protecting cardiac muscles from further damage.

We isolated ADM from aortas of 1~3 day old wild type C57BL10 mice and confirmed that they differentiate into cardiomyocytes with cardiac specific gene and protein expression in vitro and in vivo. ADM transplantation also restored dystrophin expression in the heart of mdx mice (Fig. 14). Moreover ADM might functionally cooperate with resident cardiac myocytes because in vitro studies indicate that ADM express a gap junction molecule, connexin 43, which allows electrical coupling of cardiomyocytes for contraction in cardiac muscle. All of these characteristics indicate that ADM may be useful for treating cardiomyopathy in DMD. Here I hypothesized that ADM transplantation would restore dystrophin expression, and prevent dilated cardiomyopathy in dystrophic heart of murine models of DMD.
In this study two different DMD animal models were used: mdx and mdx/utrn−/− mice. Mdx develop dilated cardiomyopathy at 12-21 months of age, and mdx/utrn−/− mice develop dilated cardiomyopathy at 10 to 15 weeks of age [Chun et al. 2012]. Using these two murine models, I have determined that ADM transplantation does not ameliorate dilated cardiomyopathy (mdx mice) but that it does prevent or delay the development of cardiomyopathy (mdx/utrn−/− mice) in dystrophic heart.

B. Materials and methods

B-1. Animal care.

All animal use was approved by the University of Illinois Institutional Animal Care and Use Committee. Mdx/utrn−/− mice were generated by mating mdx/utrn+/− male and female mice [Grady et al. 1997], and the progeny were genotyped as previously described [Burkin et al. 2001, Grange et al 2002]. As a control group age-matched wild-type mice were used for comparison in all experiments. After intramyocardiac injection all mice were placed on a heating pad until they were fully recovered. Mdx/utrn−/− mice in cages were placed on a heating pad with moisturized food for the duration of the experiment until euthanization.

B-2. Echocardiography

Two-dimensional M-mode echocardiography was used to measure heart function. A baseline echocardiograph was performed on 5 week old mdx/utrn−/− mice and 14~16 month old mdx mice prior to cell transplantation. Echocardiography was performed once again 5 weeks after cell transplantation in mdx/utrn−/− mice and 10 weeks after cell
transplantation in mdx mice. GE/Vingmed ultrasound Vivid7 (GE healthcare) was used for performing echocardiograph with mdx mice, and Ultrasound-Visual Sonics Vevo 2100 (Visualsonics, Toronro, Canada) was used for performing echocardiograph with mdx/utrn⁻/⁻ mice. Mice were anesthetized by halothane in mineral oil as described above. The chest hairs were clipped, ultrasound gel was placed on the transducer, the transducer was placed on top of the chest, and images were acquired. Mdx and mdx/utrn⁻/⁻ mice were presented with age matched wild type mice randomly to the echocardiographer, who was blinded to the genotype of mice.

B-3. Statistics

The Proc Mixed procedure of SAS (SAS Inst. Inc., Cary, NC) was used to analyze the echocardiograph data. The statistical model included effects of genotype, ages, time point of performing echocardiographs, and their interaction as fixed effects. Differences between before and after cell transplantation within each genotype and among genotypes within each time point of performing echocardiography were tested by pair-wise comparisons when the main effects were significant.

B-4. Cell Culture

ADM were grown on 0.1% gelatin coated plates in Iscove’s Dulbecco Modified Eagle’s minimal essential medium (DMEM) containing 20% FBS, 0.1 U/ml penicillin, 0.1 µg/ml streptomycin, 2.0 mM L-glut, 0.1 mM nonessential amino acids, and minimal essential medium (MEM) vitamin solution from Gibco (Carlsbad, CA, USA) and 20
ng/ml purified leukemia inhibitory factor (LIF, Chemicon International, Temecula, CA, USA). The cells were maintained in a humidified incubator at 37°C in 5% CO₂.

B-5. Labeling and injection of stem cells

Prior to the transplantation ADM were incubated with 5 µl of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) at density of 1 million cells per 1 ml of proliferation medium for 30 minutes in a humidified incubator at 37°C in 5% CO₂. DiI labeled ADM were washed with Hank’s Balanced Salt Solution (HBSS) three times and suspended in HBSS at a concentration of 5x10⁶ cells/50µl.

B-6. Cardiac injection of ADM

Mice were anesthetized by halothane (Halocarbon, USA) mixed in mineral oil (7.5 ml halothane/40ml of mineral oil). Bactoshield CHG 4% solution (STERIS, MO) was used to scrub the anterior chest area of mice. Intramyocardiac injection was performed in the wall of the left ventricle as previously described [Odintsov et al. 2011]. Briefly, insulin syringes (3/10 cc with a 29 G needle) with 5x10⁶ cells in 50µl HBSS or 50µl HBSS without ADM were injected into the fifth intercostal space to the left side of the sternum where the left ventricular wall is located, the plunger was slowly pushed, and needle was retracted gently. After cell injection mice were placed on a heating pad and their condition was observed until fully recovered.
B-7. Western blot

Protein samples were prepared from frozen heart with 15~20 sections with 10 µm thickness per sample. Briefly frozen heart sections were collected in an eppendorf tube and lysed in 2X SDS buffer (10% SDS, 100 mM Tris-HCl (pH8.0), 10mM EDTA, 10% glycerol, 1µg/ml Aprotinin and 100 µg/ml PMSF), boiled at 100 °C for 10 minutes, centrifuged 10,000 rpm for 10 minutes, and supernatants were collected and stored at -80 °C for further Western blotting assays. 40 µg of protein sample was used for Western analysis. Whole protein lysate was separated by a SDS-polyacrylamide gel composed with 5 and 10 % polyacrylamide for 1.5 hours at 150V. Proteins were transferred to a PVDF membrane at 0.05A overnight at 4°C. After blocking in 5% non-fat dry milk in 1X TBST the membrane was incubated with rabbit polyclonal anti dystrophin antibody (1 to 1,000 dilution; Abcam, Cat. No. ab15277) overnight at 4 °C, and with anti-rabbit IgG HRP-linked antibody (1 to 500 dilution; Cell Signaling, Cat. No. 7074) for 1 hour at room temperature. The target protein was visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Cat. No. 4077) on X-ray film (Denville Scientific Inc., Cat. No. E3018). GAPDH was used as a loading control. GAPDH antibody (Sigma, Cat. No. G9545) was used at a concentration of 1:1,000.

B-8. Immunohistochemistry assay

After a second echocardiograph, mice were euthanized by CO2, and hearts were isolated and weighed. Hearts were frozen in liquid nitrogen-cooled 2-methylbutane, and stored -80 °C. Frozen hearts were sectioned at 10 µm, and tissue sections were fixed prior
to incubation with 2% horse serum and 1% BSA blocking solution in PBS. (Primary antibodies used are listed in Table 3)

B-9. Data Analysis

For digital images a Retiga 2000R digital camera (Q-imaging, Surrey, BC) was used with a Leica Inverted DMI 4000B microscope (Nuhsbaum, McHenry, IL). Images were assigned color and merged using Image Pro Plus software (MediaCybernetics, Bethesda, MD), which was also used for counting and measuring primary antibody stained positive areas. The average of each protein expression was counted based on the positive area or cell number on sections, and divided by whole area of same section or whole cell number in the same section.

C. Results

C-1. Echocardiography of mdx/utrn−/− mice with ADM transplantation

Echocardiography was performed before and after ADM transplantation and resulting data compared to study the changes related to stem cell transplantation. Age-matched wild type mice and mdx/utrn−/− mice were used as control groups (Table 4). Thinning of the septum was observed in both mdx/utrn−/− mice injected with saline and mdx/utrn−/− mice injected with ADM (Table 16 and Fig. 6). However, the left ventricle was enlarged, characteristic of dilated cardiomyopathy, only in mdx/utrn−/− mice injected with saline solution. LVIDd and LVID significantly increased in mdx/utrn−/− mice injected with saline solution, but not in wild type mice with saline injection and mdx/utrn−/− mice injected with saline solution, in which the LVIDs decreased following stem cell
injection (Fig. 17). The wall of the left ventricle also became significantly thinner in mdx/utrn−/− mice injected with saline solution. LVPW was significantly decreased in mdx/utrn−/− mice injected with saline solution, but mdx/utrn−/− mice injected with ADM had significantly increased LVPWs (Fig. 18). However EDV was significantly increased, also characteristic of dilated cardiomyopathy, in mdx/utrn−/− mice injected with saline solution (Fig. 19). Overall, wild type mice with saline injection showed no change except decreased LVID at systole.

C-2. Effect of ADM on extracellular remodeling and cardiac fibroblasts in mdx/utrn−/− mice

Collagen I has important roles regarding the structural and functional integrity of heart. Excessive accumulation of collagen I is related to fibrosis formation and development of dilated cardiomyopathy. For detecting fibrotic tissues anti-collagen I antibody was used. There was no significant difference in collagen content between mdx/utrn−/− mice with saline injection or with stem cell injection. The collagen receptor Discoidin Domain Receptor 2 (DDR2) is a specific marker for cardiac fibroblasts [Goldsmith et al., 2004], which produce collagens [Eghmali et al. 1988, Chapman et al. 1990]. Anti-DDR2 antibody was used to detect cardiac fibroblast which is related to the expression of collagen I. There was no significant difference in the number of DDR2 cells in mdx/utrn−/− mice injected with saline or ADM (Fig. 25), but there is higher number of DDR2 positive cells in wild type control mice compared to mdx/utrn−/− mice (Fig. 25).
C-3. Effect of ADM on expression of a vascular adhesion molecule in mdx/utrn⁻/⁻ mice

CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is involved in angiogenesis and expressed by endothelial cells [Albelda et al. 1991, Newman et al., 1997]. CD31 expression was used to assess whether there was a change in vasculature of the heart after ADM transplantation (Fig. 28). The expression of CD31 was significantly higher in mdx/utrn⁻/⁻ mice with ADM injection compared to the mdx/utrn⁻/⁻ mice with saline injection (Fig. 29). Interestingly the CD31 expression in mdx/utrn⁻/⁻ mice with ADM injection was even higher than in age-matched wild type control mice (Fig. 29-A). No significant difference in CD31 expression was observed in wild type control mice or mdx/utrn⁻/⁻ mice injected with saline injection (Fig. 29-A).

C-4. Effect of ADM on proliferating cells in mdx/utrn⁻/⁻ mice

Ki-67 is a nuclear protein which is associated with cellular proliferation and is present during interphase of cell cycle (G₁, S, G₂, and mitosis). For detection of proliferating cells in the heart, anti-Ki-67 antibody was used. The number of Ki-67 positive cells was not statistically different between mdx/utrn⁻/- mice with saline injection and ADM injection (Fig. 27-A). However there was a statistical tendency ($p<0.06$) toward an increase in the number of Ki-67 positive cells in mdx/utrn⁻/⁻ mice injected with ADM, but not following injection of saline (Fig. 27-A).
C-5. Effect of ADM on expression of connexin 43 in mdx/utrn−/− mice

In heart, electrical cell-cell communication between cardiomyocytes is achieved through gap junctions. Connexin 43 is the most abundant type of gap junction molecule in cardiac muscle [Beyer et al. 1989]. Connexin 43 was measured but was not different between mdx/utrn−/− mice injected with saline or ADM (Fig. 30). However more connexin 43 was expressed in wild type control mice (Fig. 31).

C-6. Effect of ADM on dilated cardiomyopathy in mdx mice

Echocardiography was performed before ADM transplantation and at 10 weeks after ADM transplantation. Echocardiograph data were compared to study the changes of cardiac morphology and function related to ADM transplantation. Aged–matched wild type mice and mdx mice with saline injection were used as control groups (Table 4). Interventricular Septum (IVS) at systole was significantly decreased in wild type with saline injection and mdx with saline injection, but not in mdx with ADM injection. IVS at diastole decreased in all groups (wt/HBSS, mdx/HBSS and mdx/ADM) (Table 4). Left LVID was significantly enlarged in mdx with ADM injection but not in wild type with saline injection and mdx with saline injection (Fig. 33). LVPW was significantly decreased in mdx with saline injection but it was notably increased in mdx with ADM injection (Fig. 34). As functional parameters EF and FS were significantly decreased in both mdx with saline injection and mdx with ADM injection (Fig. 35 and Fig. 36). However, only mdx with ADM injection displayed a significant increase with EDV and ESV (Fig. 37 and Table 4).
C-7. Effect of ADM on extracellular remodeling and cardiac fibroblasts in mdx mice

Higher collagen I expression was detected in mdx mice injected with either ADM or saline injection in comparison to age matched wild type controls (Fig. 38 and Fig. 39). However there was no significant difference in collagen content with stem cell injection between mdx with ADM injection and mdx with saline injection (Fig. 39). The collagen receptor Discoidin Domain Receptor 2 (DDR2) is a specific marker for cardiac fibroblasts [Goldsmith et al. 2004], which produce collagen [Eghmali et al. 1988, Chapman et al. 1990]. There was no significant difference in numbers of DDR2 cells between mdx mice injected with saline or ADM (Fig. 40 and 41). Interestingly higher levels of collagen I were detected in both groups of mdx mice than in wild type with saline injection although there was no difference in numbers of DDR2 positive cells among wild type with saline injection, mdx with saline injection and mdx with ADM injection(Fig 41).

C-8. Effect of ADM on expression of a vascular adhesion molecule in mdx mice

Anti-CD31 antibody was used to detect the vasculature in the heart to determine whether ADM transplantation altered this parameter. CD31 positive areas were measured, and the average was calculated (Fig. 44 and 45). There was no significant difference in CD31 expression among mdx with saline injection and mdx with ADM injection including wild type mice with saline injection (Fig. 45).
C-9. Effect of ADM on proliferating cells in the mdx heart

Compared to wild type mice with saline injection there were significantly fewer Ki-67 positive cells in mdx mice injected with saline and mdx mice injected with ADM (Fig. 43). However, there was no statistical difference in the number of Ki67 positive cells between mdx injected with saline and mdx injected with ADM.

C-10. Effect of ADM on expression of connexin 43 in mdx mice

To determine whether gap junctions were altered during disease progression or following ADM injection, expression of connexin 43, a gap junction protein, was examined (Fig. 46). There was no significant difference in expression of connexin 43 among wild type mice injected with saline injection, mdx with injected with saline or mdx mice injected with ADM (Fig. 47). However there was slight tendency (p<0.07) towards an increase in mdx mice injected with ADM in comparison to mdx mice injected with saline (Fig. 47-A).

C-11. Restoration of dystrophin after ADM transplantation in mdx/utrn\(^{-/-}\) and mdx mice

Dystrophin was detected in the heart of mdx and mdx/utrn\(^{-/-}\) mice after ADM injection. In mdx/utrn\(^{-/-}\) mice with ADM injection, restored dystrophin was detected (Fig. 47) by immunohistochemistry, but not detected by western blot (Fig. 48). There were two different expression patterns of restored dystrophin (Fig. 47-A-F) in immunohistochemistry. The pattern in relatively larger areas consisted of weak dystrophin positive signal throughout(Fig. 47-C-D and E-F), although a different pattern,
including strong dystrophin positive staining, was present around individual cells in small regions (Fig. 47). However restored dystrophin was found in limited areas rather than in all heart sections. In mdx mice with ADM transplantation we did not detect dystrophin expression.

D. Discussion

As we previously reported, at the age of 5 weeks, when ADM were injected, mdx/utrn<sup>−/−</sup> mice showed no sign of cardiomyopathy [Chun et al. 2012]. However at 10 weeks of age, thinning of the IVS and LVPW, as well as enlarged LVID, was observed in mdx/utrn<sup>−/−</sup> mice injected with saline. This is also similar to our previous report, as dilated cardiomyopathy is beginning to develop at this time [Chun et al. 2012]. In contrast, mdx/utrn<sup>−/−</sup> mice injected with ADM did not exhibit thinning of the ventricle wall or an enlarged chamber (Fig. 16 and 18). There were no significant changes in EF and FS in all groups (Fig. 20 and 21). However a significant increase in EDV was detected in mdx/utrn<sup>−/−</sup> mice injected with ADM (Fig. 19). That is, mdx/utrn<sup>−/−</sup> mice injected with ADM experienced improvement in systolic function based on IVSs, LVPWs and LVIDs and remained healthy without developing left ventricular dilation. In summary ADM transplantation to hearts prevent development of dilated cardiomyopathy in mdx/utrn<sup>−/−</sup> mice with systolic functional improvement.

To determine why ADM may prevent delay cardiomyopathy in mdx/utrn<sup>−/−</sup> mice, we examined the heart of mice to determine whether pathology was decreased after stem cell injection. Fibrosis formation in mdx/utrn<sup>−/−</sup> heart was measured by assessing collagen
I. There was significantly more collagen I in the heart of wild type control mice than in either mdx/utrn−/− mice injected with saline or injected with ADM (Fig. 23-A). However, there was no difference in collagen content between mdx/utrn−/− mice injected with saline or ADM (Fig. 23). Similarly, there was no difference in the number of DDR2 positive fibroblasts between mdx/utrn−/− mice injected with saline or ADM, and both groups have fewer DDR2 positive cells compared to wild type control mice (Fig. 25). These data demonstrate that the collagen content and cardiac fibroblasts vary with genotype, but are not altered by stem cell injection. The fibrillar collagen network is an important component of the extracellular matrix and disruption of this network induces structural cardiac remodeling which is related to declining cardiac function [Spinale 2007, Fedak et al. 2008]. The reduced collagen I expression in mdx/utrn−/− mice in comparison to wild type mice may reflect an abnormal collagen network caused by absence of dystrophin.

It is possible that the injected stem cells differentiate into cardiomyocytes therefore inducing functional benefit in mdx/utrn−/− mice. High levels of red auto fluorescence, particularly in aged mdx heart, hampered detection of DiI-labeled donor cells. As a result, no DiI positive cells were detected in mdx heart injected with ADM, and donor cells were positively identified in only one mdx/utrn−/− mice heart injected with donor cells. We therefore were not able to examine the fate of donor cells in the heart. However, the presence of dystrophin in the heart of mdx/utrn−/− mice injected with ADM is indicative of donor cell differentiation into cardiomyocytes (Fig. 47).

The heart of mdx/utrn−/− mice was also examined for indirect effects from ADM injection. There was also no statistically significant difference in the number of Ki-67 positive cells between mdx/utrn−/− mice injected with saline and mdx/utrn−/− mice injected
with ADM, and there was also significantly less proliferating cells in both groups than in wild type control mice (Fig. 27). However there was a slight statistical trend (p<0.07) of greater numbers of Ki67 positive proliferating cells in mdx/utrn\textsuperscript{−/−} mice injected with ADM in comparison to mdx/utrn\textsuperscript{−/−} mice injected with saline. It is likely that this trend was not statistically significant because of the large variation among individual mice in the different treatment groups. To determine whether this trend is significant, increased number of mdx/utrn\textsuperscript{−/−} mice should be examined. The number of proliferating cells in mdx/utrn\textsuperscript{−/−} heart with ADM injection is similar to wild type mice with saline injection (Fig. 27-A). An increase in Ki67 positive cells with ADM injection may indicate that the increased proliferating cells are contributing to regenerate damaged cardiac muscles.

Angiogenesis is a well-known indirect effect of stem cell therapy known to improve heart function. It has been reported that angiogenesis facilitates regeneration of damaged tissues with stem cell transplantation [Zhang et al. 2010, Katare et al. 2011]. To determine whether ADM may prevent heart dysfunction by enhancing blood flow to the heart, we examined the vasculature in the heart of mdx/utrn\textsuperscript{−/−} mice. Mdx/utrn\textsuperscript{−/−} hearts injected with ADM had more CD31 positive staining compared to the hearts of age-matched wild type control mice and mdx/utrn\textsuperscript{−/−} heart injected with saline, indicating that ADM may stimulate angiogenesis in the heart after transplantation (Fig. 28 and 29). Therefore angiogenesis could be the reason why there was cardiac functional benefit by ADM transplantation together with increased number of proliferating cells in mdx/utrn\textsuperscript{−/−} mice.

Interestingly in mdx/utrn\textsuperscript{−/−} heart injected with ADM restored dystrophin was detected with immunohistochemistry (Fig. 48) but not with western blotting (Fig. 49).
This may indicate that the amount of restored dystrophin is too small to be detected by Western analysis, or that protein degradation might occur during frozen section preparation for western blotting. In agreement with this, dystrophin staining detected with immunohistochemistry was faint (Fig. 47) in many regions, although in some areas a small number of intensely stained cells were also detected (Fig. 47-C and D). This low level of restored dystrophin may contribute to the functional benefit on mdx/utrn−/− mice, however, as previous studies have shown functional benefit from restoration dystrophin to 30% of wild-type levels [Wu et al. 2010, Van Putten et al. 2012].

In contrast to mdx/utrn−/− mice, injection of stem cells into the heart of mdx mice with existing cardiomyopathy does not result in functional benefit. Mdx mice have dilated cardiomyopathy by 10~12 months of age that is similar to patients with DMD, including excessive fibrosis formation and decreased cardiac function similar to the patients with DMD [Quinlan et al. 2003, Spurney et al. 2008, Bostick et al. 2008, Van Erp et al. 2010]. Based on these reports, we injected ADM into 14-16 month old mdx mice. IVS and LVPW became thinner in mdx mice after saline injection and the EF and FS were also significantly decreased 10 weeks after HBSS cardiac injection, indicating that mdx mice were developing progressive dilated cardiomyopathy with aging. A significantly enlarged LVID, increased EDV and ESV, and decreased EF and FS with thickened LVPW were detected in mdx mice injected with ADM. From this finding, there was no beneficial effect by ADM transplantation in cardiac function of mdx mice which have dilated cardiomyopathy, and in fact the cells exacerbated the condition.

Fibrosis is one of the well-known characteristics of dilated cardiomyopathy in patients with DMD. There is more collagen I detected in mdx heart injected with saline
than age-matched wild type mice, similar to reports in previous studies [Quinlan et al. 2003, Bostick et al. 2008, Spurney et al. 2008, Erp et al. 2010]. Cardiac fibroblasts are involved in production of collagen [Eghmali et al. 1988, Chapman et al. 1990], and it was assumed that collagen I expression is in proportion as DDR2 expression. However there was a decrease in the number of DDR2 positive cells in mdx mice in comparison to age-matched wild type mice, although the collagen I content of the mdx heart was higher in comparison to age-matched wild type controls. It has been reported that collagen extracts from aged mice have a decreased diameter and density of collagen fibrils with loosened collagen network [Damodarasamy et al. 2010]. Collagen I in aged mdx mice may therefore be expressed in relatively larger areas because of loosened collagen network with less density.

Hearts of mdx mice were studied for indirect effects from ADM transplantation including proliferation, angiogenesis and gap junction formation. There were significantly lower numbers of Ki-67 positive cells in both mdx mice injected with saline or with ADM compared to age-matched wild type controls, but there was no difference between mdx mice injected with saline and mdx mice injected with ADM, indicating that there was no effect of ADM transplantation on overall cell proliferation. There was no difference of CD31 expression among mdx with saline injection and mdx with ADM injection including wild type with saline injection. There was also no significant effect of donor cell injection on the gap junction protein, connexin 43 compared to wild type control mice, and also no significant difference between mdx mice with saline injection and mdx with ADM injection. However there was a tendency towards higher connexin 43 expression in the mdx with ADM injection group in comparison to the mdx with saline
injection group ($p<0.08$; Fig. 47-A). Together, ADM transplantation into mdx heart after onset of dilated cardiomyopathy and fibrosis resulted in no detectable indirect effect on endogenous regeneration. There was also likely no direct differentiation of ADM into cardiomyocytes, as no dystrophin protein was detected by either immunohistochemistry or western blotting assay (Fig. 48 and 49). These results are unexpected because we found cardiac specific protein was co-localized with DiI labeled ADM and restored dystrophin expression after transplantation into mdx heart in Chapter III. Unlike the young mdx mice which were used in Chapter III, aged mdx mice have severe dilated cardiomyopathic heart. It could be an influence of the different microenvironment present in the disease condition, related to ages of mdx mice, that influences the direct differentiation of ADM into cardiomyocytes.

In stem cell therapy there are two main ways that stem cells may improve function of damaged cardiac muscles: 1) by direct differentiation to cardiomyocyte, and 2) from indirect mechanisms such as release of paracrine factors like vascular endothelial growth factor (VEGF) and fibroblasts growth factor (FGF). Multiple types of stem cells which promote functional benefit in damaged heart differentiate to cardiomyocytes, including skeletal muscle derived stem cells (SMDS), cardiac stem cells (CSC), and embryonic stem cells (ESC) [Payne et al. 2005, Smith et al. 2007, Schuldt et al. 2008]. However the rate of differentiation in vivo was very low. Instead, the major benefits were likely due to cardiac regeneration by endogenous cells that were activated by a paracrine mechanism through the donor cells. This was initially surprising because previously cardiomyocytes were believed to be terminally differentiated cells and hearts were thought to harbor few or no stem cells for regeneration. However there have been recent
studies demonstrating that hearts have an endogenous cardiac stem cell population (CSC) that contributes to regeneration of damaged cardiac muscle. This can occur by stimulating endogenous CSC in vivo or by ex-vivo expansion and transplantation of CSC. Thus, one mechanism by which exogenous cells may promote repair of damage to the heart is through secretion of various factors that induce proliferation of endogenous cells in the heart including cardiac stem/progenitor cells and cardiomyocytes, or differentiation of stem/progenitor cells that contribute to repair or regeneration of the cardiac structure.

In addition to the effect of ADM on dystrophin-deficient cardiac muscle, this study has yielded interesting facts regarding disease-related changes in the heart of mdx and mdx/utrn−/− mouse models for DMD. For example mdx and mdx/utrn−/− mice have fewer DDR2 positive cardiac fibroblasts than hearts of age-matched wild type mice, which could affect stable extracellular matrix structure. However, mdx mice had fewer DDR2 cells with increased collagen I expression, in contrast to mdx/utrn−/− mice which had also lower numbers of DDR2-positive cells but decreased collagen I expression. It may be DDR2 positive cells produce different amount of collagen I in mdx and mdx/utrn−/− mice. Mdx and mdx/utrn−/− mice also have fewer proliferating cells in the heart than age matched wild type mice, which may indicate that mdx and mdx/utrn−/− mice have less capacity to maintain endogenous regeneration than wild type mice. This could be useful characteristics of these DMD mice models for future studies.

In summary ADM transplantation has a different effect on mdx and mdx/utrn−/− mice. ADM transplantation into the heart of mdx mice did not stimulate functional improvement result in direct differentiation of donor cells to cardiomyocytes, or cause indirect effects. However in mdx/utrn−/− heart, ADM transplantation prevented
development of dilated cardiomyopathy. Our data suggest that this is due to both direct and indirect effects, including restored dystrophin. Therefore ADM delay development of dilated cardiomyopathy by transplantation prior to the onset of disease. However ADM do not improve function of dystrophic hearts when injected during advanced stages of dilated cardiomyopathy.
Figure 15. Scheme of ADM transplantation into mdx/utrn^-/- mice prior to the onset of dilated cardiomyopathy.

Scheme of ADM transplantation to mdx/utrn^-/- mice prior to the onset of dilated cardiomyopathy. Mdx/utrn^-/- mice develop dilated cardiomyopathy with aging. At 5 weeks of age when the mice are still relatively healthy, echocardiography was performed with mdx/utrn^-/- mice. ADM were then transplanted, and second echocardiography was performed with the same mice 5 weeks after ADM transplantation, and then mice were euthanized.
Table 3. Primary antibodies for immunohistochemistry

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<th>Catalog</th>
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<tr>
<td>Dystrophin</td>
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Table 4. Echocardiography in comparison of pre- and post ADM transplantation (Mdx/utrn⁻⁻)

<table>
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<tr>
<th>Item</th>
<th>Treatment</th>
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<th>Post-injection</th>
<th>P-value</th>
<th>Main effects</th>
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<td>dKO-ADM</td>
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<td>IVSD (mm)</td>
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1. a, b, c Within a row, means without a common superscript differ in each type of mice (P < 0.05).
2. PSEM= Pooled SEM.
3. T*M= Interaction between time and mice.
Table 4. Echocardiography comparison of before and after ADM transplantation in mdx/utrn−/− mice. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn−/− mice with saline injection and dKO/ADM: mdx/utrn−/− mice with ADM injection. IVSd: Interventricular septum at diastole, LVIDd: Left ventricular internal dimension at diastole, LVPWd: Left ventricular posterior wall at diastole, IVSs: Interventricular septum at systole, LVIDs: Left ventricular internal dimension at systole, LVPWs: Left ventricular posterior wall at systole, EDV: End-diastolic volume, ESV: End-systolic volume, EF: Ejection fraction, FS: Fractional shortening, SV: Stroke volume, HR: Heart rate.
Figure 16. Internal ventricular septum thickness (IVS) in mdx/utrn<sup>−/−</sup> mice

Internal ventricular septum thickness (IVS) in mdx/utrn<sup>−/−</sup> mice. ADM injection prevented thinning of the septum. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM: mdx/utrn<sup>−/−</sup> mice with ADM injection. *: p<0.05
Figure 17. Left ventricular internal dimension at diastole (LVIDd) in mdx/utrn<sup>−/−</sup> mice

Left ventricular internal dimension at diastole (LVIDd) in mdx/utrn<sup>−/−</sup> mice. ADM injection prevented ventricular dilation. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM: mdx/utrn<sup>−/−</sup> mice with ADM injection. *: p<0.05.
Figure 18. Left ventricular posterior wall thickness at diastole (LVPWd) in mdx/utrn\(^{-/-}\) mice.

Left ventricular posterior wall thickness at diastole (LVPWd) in mdx/utrn\(^{-/-}\) mice.

ADM injection prevented the thinning of the left ventricular wall. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn\(^{-/-}\) mice with saline injection and dKO/ADM: mdx/utrn\(^{-/-}\) mice with ADM injection. *: \(p<0.05\).
Figure 19. End diastolic volume (EDV) in mdx/utrn<sup>−/−</sup> mice

End diastolic volume (EDV) in mdx/utrn<sup>−/−</sup> mice. ADM injection prevented the increase in EDV. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM:mdx/utrn<sup>−/−</sup> mice with ADM injection. *: p<0.05.
Figure 20. Ejection fraction (EF) in mdx/utrn⁻/⁻ mice

Ejection fraction (EF) in mdx/utrn⁻/⁻ mice. WT/HBSS: wild type mice with saline injection. ADM injection did not affect to EF. dKO/HBSS: mdx/utrn⁻/⁻ mice with saline injection and dKO/ADM:mdx/utrn⁻/⁻ mice with ADM injection.
Figure 21. Fractional shortening (FS) in mdx/utrn^−/− mice

Fractional shortening (FS) in mdx/utrn^−/− mice. WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn^−/− mice with saline injection and dKO/ADM:mdx/utrn^−/− mice with ADM injection.
Collagen I expression in hearts. Wild type mice with saline injection (WT/HBSS) (A), mdx/utrn<sup>−/−</sup> with saline injection (dKO/HBSS) (B) and mdx/utrn<sup>−/−</sup> mice with ADM injection (dKO/ADM) (C). Collagen I: green, 20x magnification.
Quantitative data of collagen I expression in hearts of mdx/utrn<sup>−/−</sup> mice. Average collagen I positive areas of graph (A). WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM: mdx/utrn<sup>−/−</sup> mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of collagen I positive area (arrow) (B, 20x magnification).
Figure 24. DDR2 expression in hearts of mdx/utrn\(^{-/-}\) mice. Wild type mice with saline injection (WT/HBSS) (A and B), mdx/utrn\(^{-/-}\) mice with saline injection (dKO/HBSS) (C and D) and mdx/utrn\(^{-/-}\) mice with ADM injection (dKO/ADM) (E and F). A, C and E: DDR2 (green), B, D and F: DDR2 (green) and DAPI (blue), 20x magnification.
Figure 25. Quantitative data of DDR2 expressing cardiac fibroblasts in hearts of mdx/utrn\(^{-/-}\) mice. Average DDR2 positive cell number of graph (A), WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn\(^{-/-}\) mice with saline injection and dKO/ADM: mdx/utrn\(^{-/-}\) mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of DDR2 positive cells (arrow) (B, 20x magnification).
Figure 26. Ki-67 expression in hearts

Ki-67 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx/utrn<sup>−/−</sup> mice with saline injection (dKO/HBSS) (C and D) and mdx/utrn<sup>−/−</sup> mice with ADM injection (dKO/ADM) (E and F). A, C and E: Ki-67 (green), B, D and F: Ki-67 (green) and DAPI (blue), 20x magnification.
Figure 27. Quantitative analysis of Ki-67 expression in hearts

Quantitative data of Ki-67 expression in hearts. Average Ki-67 positive cells of graph (A). WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM: mdx/utrn<sup>−/−</sup> mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of Ki-67 positive cells (arrow) (B, 20x magnification).
Figure 28. CD31 expression in hearts.

CD31 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx/utrtn⁻/⁻ mice with saline injection (dKO/HBSS) (C and D) and mdx/utrtn⁻/⁻ mice with ADM injection (dKO/ADM) (E and F). A, C and E: CD31 (green), B, D and F: CD31 (green) and DAPI (blue), 20x magnification.
Figure 29. Quantitative data of CD31 expression in hearts

Quantitative data of CD31 expression in hearts. Average CD31 positive cells of graph (A). WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn⁻/⁻ mice with saline injection and dKO/ADM: mdx/utrn⁻/⁻ mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of CD31 positive cells (arrow) (B, 20x magnification).
Figure 30. Connexin 43 expression in hearts

Connexin 43 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx/utrnc−/− mice with saline injection (dKO/HBSS) (C and D) and mdx/utrnc−/− mice with ADM injection (dKO/ADM) (E and F). A, C and E: Connexin 43 (green), B, D and F: Connexin 43 (green) and DAPI (blue), 20x magnification.
Quantitative data of connexin 43 expression in hearts. Average connexin 43 positive cells of graph (A). WT/HBSS: wild type mice with saline injection, dKO/HBSS: mdx/utrn<sup>−/−</sup> mice with saline injection and dKO/ADM: mdx/utrn<sup>−/−</sup> mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of connexin positive cells (arrow) (B, 20x magnification).
Scheme of ADM transplantation to mdx mice with dilated cardiomyopathy. Mdx mice develop dilated cardiomyopathy at later their life about 12 months old. At 14-16 months old when the mice have developed dilated cardiomyopathy echocardiography was performed with mdx mice and then ADM was transplanted. Second echocardiography was performed with the same mice 10 weeks after ADM transplantation, and then mice were euthanized with dilated cardiomyopathy.
Table 5. Echocardiography in comparison of pre- and post ADM transplantation (Mdx)

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<th>Post-injection</th>
<th>P-value</th>
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<td>Mdx HBSS (n=4)</td>
<td>Mdx ADM (n=4)</td>
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<td>IVSd (cm)</td>
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1. <sup>a</sup>, <sup>b</sup>, <sup>c</sup> Within a row, means without a common superscript differ in each type of mice (P < 0.05).
2. PSEM= Pooled SEM.
3. T*M= Interaction between time and mice
Table 5. Echocardiography in comparison of pre- and post- ADM transplantation in mdx mice. WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. IVSd: Interventricular septum at diastole, LVIDd: Left ventricular internal dimension at diastole, LVPWd: Left ventricular posterior wall at diastole, IVSs: Interventricular septum at systole, LVIDs: Left ventricular internal dimension at systole, LVPWs: Left ventricular posterior wall at systole, EDV: End-diastolic volume, ESV: End-systolic volume, EF: Ejection fraction, FS: Fractional shortening, SV: Stroke volume, HR: Heart rate.
Figure 33. Left ventricular internal dimension at diastole (LVIDd) in mdx mice

Left ventricular internal dimension at diastole (LVIDd) in mdx mice. WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. *: $p<0.05$. 
Figure 34. Left ventricular posterior wall thickness at diastole (LVPWd) in mdx mice

Left ventricular posterior wall thickness at diastole (LVPWd) in mdx mice.

WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. *: $p<0.05$. 
Figure 35. Ejection fraction (EF) in mdx mice

Ejection fraction (EF) in mdx mice. WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. *: $p<0.05$. 
Figure 36. Fractional shortening (FS) in mdx mice

Fractional shortening (FS) in mdx mice. WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. *: $p<0.05$. 
Figure 37. End diastolic volume (EDV) in mdx mice

End diastolic volume (EDV) in mdx mice. WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. *: p<0.05.
Figure 38. Collagen I expression in hearts

Collagen I expression in hearts. Wild type mice with saline injection (WT/HBSS) (A), mdx mice with saline injection (Mdx/HBSS) (B) and mdx mice with ADM injection (Mdx/ADM) (C). Collagen I: green, 20x magnification
Figure 39. Quantitative data of collagen I expression in hearts.

Quantitative data of collagen I expression in hearts. Average collagen I positive areas of graph (A). WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of collagen I positive area (arrow) (B, 20x magnification).
Figure 40. DDR2 expression in hearts

DDR2 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx mice with saline injection (Mdx/HBSS) (C and D) and mdx mice with ADM injection (Mdx/ADM) (E and F). A, C and E: DDR2 (green), B, D and F: DDR2 (green) and DAPI (blue), 20x magnification
Figure 41. Quantitative data of DDR2 expressing in hearts

Quantitative data of DDR2 expressing cardiac fibroblasts in hearts. Average DDR2 positive cell number of graph (A), WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of DDR2 positive cells (arrow) (B, 20x magnification).
Figure 42. Ki-67 expression in hearts

Ki-67 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx mice with saline injection (Mdx/HBSS) (C and D) and mdx mice with ADM injection (Mdx/ADM) (E and F). A, C and E: Ki-67 (green), B, D and F: Ki-67 (green) and DAPI (blue), 20x magnification
Figure 43. Quantitative analysis of Ki-67 expression in hearts

Quantitative data of Ki-67 expression in hearts. Average Ki-67 positive cell number of graph (A), WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of Ki-67 positive cells (arrow) (B, 20x magnification).
Figure 44. CD31 expression in hearts

CD31 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx mice with saline injection (Mdx/HBSS) (C and D) and mdx mice with ADM injection (Mdx/ADM) (E and F). A, C and E: CD31 (green), B, D and F: CD31 (green) and DAPI (blue), 20x magnification
Quantitative analysis of CD31 expression in hearts. Average CD31 positive cell number of graph (A), WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice (P < 0.05). An example of CD31 positive cells (arrow) (B, 20x magnification).
Figure 46. Connexin 43 expression in hearts

Connexin 43 expression in hearts. Wild type mice with saline injection (WT/HBSS) (A and B), mdx mice with saline injection (Mdx/HBSS) (C and D) and mdx mice with ADM injection (Mdx/ADM) (E and F). A, C and E: Connexin 43 (green), B, D and F: Connexin 43 (green) and DAPI (blue), 20x magnification
Figure 47. Quantitative analysis of connexin 43 expression in hearts

Quantitative data of connexin 43 expression in hearts. Average connexin 43 positive cell number of graph (A), WT/HBSS: wild type mice with saline injection, Mdx/HBSS: mdx mice with saline injection and Mdx/ADM: mdx mice with ADM injection. a, and b indicate a statistically significant difference in each group of mice. (P < 0.05). An example of connexin positive cells (arrow) (B,20x magnification).
Figure 48. Restored dystrophin expression in mdx/utrn<sup>−/−</sup> mice with ADM
Figure 48. Restored dystrophin expression. Wild type control mice with saline injection (A and B), mdx/utron\(^{-/}\) mice with ADM injection (C-F), mdx mice with ADM injection (G and H). A, C, E and G: Dystrophin (green), B, D, F and H: Dystrophin (green) and DAPI (blue). Mdx/utron\(^{-/}\) mice with ADM injection showed weaker dystrophin positive signal (C and D) and stronger dystrophin positive signals (E and F). Mdx mice with ADM injection showed no restored dystrophin expression (G and H). 20x magnification.
Figure 49. Restored dystrophin expression in mdx/utrn<sup>−/−</sup> mice with ADM

No dystrophin detected by western blot in mdx/utrn<sup>−/−</sup> mice with ADM injection.

A, B, C and D: wild type mice with HBSS injection, E, F, G and H: mdx/utrn<sup>−/−</sup> mice with HBSS injection, I, J, K, L and M: mdx/utrn<sup>−/−</sup> mice with ADM injection. Dystrophin protein is Dp427 kDa and GAPDH is loading control.
Chapter V

Summary and conclusion

The focus of this dissertation was to evaluate the capacity of ADM multipotent adult stem cells for the treatment of DCM in DMD. The absence of dystrophin in DMD causes skeletal and cardiac muscle defects [Hoffman et al. 1987, Oldfors et al. 1994]. There have been many studies to improve skeletal muscle defects including respiratory system that brought prolonged life to patients with DMD [Eagle et al. 2007]. Unfortunately, extended lifespan leads to worsening of cardiac muscle condition [Danialou et al. 2001, Fiona et al. 2005, Townsand et al. 2008 and Kamogawa et al. 2009]. There are increasing numbers of studies and clinical trials to understand cardiac involvement [Townsend et al. 2010, Spurney et al. 2011] and how it might be addressed, but there is still no long-term cure for cardiac pathology in DMD [Das et al. 2009, Bostick et al. 2009].

For the treatment of DCM in DMD it is important to replace damaged/dead cardiomyocytes to prevent further cardiac dysfunction. Stem cell therapy may be a good strategy for treating cardiac involvement in DMD because of their ability to proliferate and differentiate to cardiomyocytes for regeneration. Multipotent ADM differentiate skeletal muscle fibers and restore both healthy muscle fibers and dystrophin in mdx/utrn–/– mice [Berry et al. 2007]. Here I demonstrated that ADM may be useful for therapy for not only skeletal but also cardiac muscle in DMD. Three points were addressed in this study to achieve this conclusion: 1) characterization of DCM in the phenotypic animal model for DMD to establish its relevance for
use in studies with ADM treatment for DCM in DMD, 2) differentiation capacity of ADM into cardiomyocytes, and 3) the effect(s) of ADM transplantation in dystrophic hearts.

There are two transgenic mice which have been used as animal models for DMD. Mdx mice have a single dystrophin mutation identical to that of many DMD patients that makes them genotypic animal model for DMD. Despite this, mdx mice have mild skeletal muscle defect with relatively normal lifespan unlike DMD patients [Bulfield et al. 1984]. Mdx/utrn<sup>−/−</sup> mice have dystrophin and utrophin mutations with severe, progressive skeletal muscle defects similar to DMD patients [Grady et al. 1997] and are therefore a good phenotypic model for DMD. Mdx mice develop DCM in close to the end of their lifespan [Quinlan et al. 2004] However, mdx/utrn<sup>−/−</sup> mice had not previously been reported to develop DCM. The mdx/utrn<sup>−/−</sup> mice were examined to determine whether they develop DCM like patients with DMD [Chun et al. 2012].

Echocardiography was used to demonstrate that mdx/utrn<sup>−/−</sup> mice gradually develop DCM. At 5 weeks of age no decreased in cardiac function was detected in mdx/utrn<sup>−/−</sup> mice. At the age of 10 weeks thinning heart walls and an enlarged left ventricular chamber were present, and at 15 weeks of age decreased EF, FS and EDV with excessive fibrosis formation was detected. In addition mdx/utrn<sup>−/−</sup> mice develop DCM with a similar progression and time frame to DMD patients. These data, collectively, confirm that mdx/utrn<sup>−/−</sup> mice develop DCM like DMD patients, and are a good disease model for pre-clinical studies to treat DCM in DMD.

As stem cell therapy for cardiomyopathy in DMD, ADM must have the capacity to differentiate into cardiomyocytes and restore dystrophin. Here I have reported that ADM were induced to differentiate into cardiomyocytes, using one of three different supplements in vitro. 97.8% of ADM in N2B27 differentiation medium exhibited a change in morphology similar to that of cardiomyocytes, and 60% of them expressed cTm protein. ADM also expressed the
cardiac markers Nkx2.5, cTnI, cTm and α-actinin, as well as connexin 43, expressed at 14 days in cardiomyocytes. After transplantation to hearts DiI-labeled ADM were colocalized with the cardiac marker cTn. Moreover restored dystrophin was detected in the heart of mdx mice after ADM transplantation. As a result, we concluded that ADM likely differentiate into cardiomyocytes and even restore dystrophin in the heart of mdx mice.

The effect of ADM transplantation on DCM in DMD was tested in two different experimental groups. First, 14~16 month old mdx mice were injected with ADM following development of severe DCM to study whether ADM alleviated DCM in DMD. Using echocardiography no functional improvement was detected after ADM transplantation. Likewise, no decrease in pathology was detected by immunohistochemistry, and no restored dystrophin was detected. Next, ADM were transplanted into the hearts of 5 week old mdx/utrn−/− mice, prior to the development of DCM to determine whether ADM prevent or delay onset of DCM. Echocardiography was used to determine that mdx/utrn−/− mice do not exhibit ventricular dilation and in fact have similar heart function to age matched wild type mice. There were also endogenous regenerative activities in the heart of mdx/utrn−/− mice injected with ADM. include increased CD31 expression, indicating new vessel formation, and a trend towards increased numbers of Ki-67 positive cells (p<0.07), indicating that there was an increase in proliferating cells in cardiac muscle injected with ADM. Interestingly restored dystrophin was detected in the heart of mdx/utrn−/− mice by immunohistochemistry, but not by western blot, potentially because the amount of restored dystrophin may be too small to be detected by western blot. Altogether, these data indicate that ADM transplantation prior to the onset of cardiac pathology and dysfunction prevented or delayed development of DCM in mdx/utrn−/− mice but transplantation after development of pathology and dysfunction did not improve existing DCM in mdx mice.
Thus, I conclude that ADM could be a good source of stem cell for therapeutic purposes that prevent/delay development of DCM in DMD.
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