Towards Stem Cell Treatment for Duchenne Muscular Dystrophy-Related Cardiomyopathy

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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TOWARDS STEM CELL TREATMENT FOR DUCHENNE MUSCULAR DYSTROPHY-RELATED CARDIOMYOPATHY

(Thesis format: Monograph)

by

Bogdan Andrei Bondoc

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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London, Ontario, Canada

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Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscular degenerative disease affecting 1 in 3,500 boys. Cardiomyopathy is observed in 95% of patients aged 18 and older, accounting for 30% of deaths. While innovative treatments have been employed to curb symptoms in skeletal muscle, few show success in the heart, and no cure for DMD exists. Recently, regenerative therapy using cardiac stem cells (CSCs) has shown very promising results, but efficacy in DMD remains undetermined. This dissertation sought to provide preliminary insight into the effects of murine Sca-1+ CSC therapy. Three-dimensional echocardiography was validated and used to gauge cardiomyopathy in the mdx:utrn−/− mouse model. CSCs were isolated from healthy mice and displayed differentiation potential. They were implanted into mdx:utrn−/− mice under ultrasound guidance, and showed preliminary trends towards functional improvement. These results support the notion that stem cells may be an excellent avenue of therapy that should be further investigated.

Keywords

Duchenne Muscular Dystrophy, Dilated Cardiomyopathy, Regenerative Therapy, Sca-1+ Cardiac Stem Cells, mdx:utrn−/− Mice, 3D Echocardiography, M-mode Echocardiography, Gated Micro-CT, Ultrasound-Guided Injection.
Co-Authorship Statement

The work described herein related to assessing cardiomyopathy in the \textit{mdx:utrn}\textsuperscript{-/-} mouse model of Duchenne muscular dystrophy via three-dimensional echocardiography and micro-CT was adapted from the following published article:


BAB performed all echocardiography-related work, including image acquisition and analysis, as well as mouse care and handling, histological analysis, statistical analyses, background research, figure preparation, and manuscript writing and correction. SD and JDB performed micro-CT image acquisition and analyses, and wrote methods section regarding this work. KMG performed histological sectioning and image analysis, statistical analysis, and wrote methods section regarding this work. LL performed histological image analysis. AK and SH performed histological image acquisition. RM provided help with statistical analysis. JH provided help with animal handling and placed tail vein catheters for intravenous injections of contrast agent for micro-CT. MD and LMH designed the study, provided supervisory and financial support, background research, scientific input, and manuscript writing and correction. All authors reviewed and edited the manuscript prior to submission.

Portions of this dissertation were adapted from this article, and several figures were used, with permission from the publisher Elsevier; the acknowledgement of permission can be found in Appendix A.
Acknowledgments

Firstly, I would like to acknowledge the tremendous support and guidance of my supervisor, Dr. Lisa Hoffman. Ever since I started working in her lab as an undergraduate student, she has always been patient, helpful and encouraging. She has also always kept an open door and aided me in approaching all of my (many) setbacks with a positive mind and a healthy attitude towards finding solutions. I consider my master’s degree to be the greatest learning experience I have ever had the privilege of receiving, and Lisa allowed that to happen; I am grateful for her mentorship and friendship throughout my years at Lawson.

Secondly, I would like to acknowledge my advisors Dr. Maria Drangova, Dr. Savita Dhanvantari, and Dr. Tianqing Peng for their endless guidance throughout my project. Whether our meetings were formal or impromptu, I always felt like you were truly interested in and cared about my work, while also wanting me to succeed. This meant a lot, and I greatly appreciate it.

I would further like to send a sincere thank-you to my fellow lab members: Becky McGirr, Kelly Gutpell, and Linshan Liu. Without your constant support and advice, my thesis would definitely not have been possible. I’ve learned a great deal from our scientific conversations, and our non-scientific conversations really helped keep my head up (while also being very entertaining).

Thank-you also to our collaborators, Dr. Maria Drangova, Dr. Sarah Detombe, and Joy Dunmore-Buyze for their enormous help with the three-dimensional echocardiography/micro-CT study. Their guidance and contribution were indispensable, and our conversations were riveting, giving me the drive to explore new possibilities with imaging and research in general. I would also like to sincerely thank Jennifer Hadway and Lise Desjardins for offering their skilful support with tail vein catheters and IV injections.

Furthermore, I would like to thank a number of people without whose help I would not have been able to carry out any of my work. Thank-you to Dr. Kristin Chadwick and the London Regional Flow Cytometry Facility for helping me obtain my (very specific) population of stem cells, and for putting up with my crazy schedules. Thank-you to Yin Liu for teaching me the ropes on the Vevo ultrasound scanner, and for getting me started on my journey with
echocardiography. Thank-you also to Adem Hadj Boussaad for providing training with the ultrasound injection arm and for our early trial implant experiments, and to Dr. James Lacefield and Matthew Lowerison for helping me obtain the ultrasound contrast agent that was crucial for my injections. Also, thank-you to Dr. Yves Bureau for his excellent advice regarding my data analysis and for a great stats course, and to the Lawson Animal Care Facility for taking care of my very sick mice and putting up with me through the years.

Last but certainly not least, I would like to send a huge thank-you to my parents, Dan and Ruxandra Bondoc, and my girlfriend, Alex Carrillo-Hayley, who have been there for me throughout all my ups and downs and were a rock for me during my lowest points. I will be forever grateful for your endless support, and I would not be where I am today if it weren’t for you. Thank-you.

I would like to acknowledge funding support from the CIHR Strategic Training Program in Vascular Research, the Western Collaborative Program in Molecular Imaging, and the Western Graduate Research Scholarship. This research was funded by The Heart and Stroke Foundation of Ontario, and through a Lawson Health Research Institute Internal Research Fund.
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List of Abbreviations and Symbols

3DE ................................................................. Three-Dimensional Echocardiography
5-aza ............................................................................... 5-Azacytidine
7-AAD ........................................................................... 7-Aminoactinomycin D
ANCOVA ............................................................... Analysis of Covariance
ANOVA ................................................................... Analysis of Variance
AON ................................................................. Antisense Oligonucleotide
BMD ................................................................. Becker Muscular Dystrophy
BSA ................................................................. Bovine Serum Albumin
CIHR ............................................................. Canadian Institutes of Health Research
c-Kit ................................................................. Tyrosine Protein Kinase Kit
CSC ................................................................. Cardiac Stem Cell
CT ................................................................. X-Ray Computed Tomography
cTnT .............................................................. Cardiac Troponin T
CVD ............................................................. Cardiovascular Disease
Cx43 .............................................................. Connexin 43
cxmd ..................................................................... Canine X-Linked Muscular Dystrophy
DAG ........................................................ Dystrophin-Associated Glycoprotein Complex
DCE-CT ........................................................ Dynamic Contrast-Enhanced Computed Tomography
DMD ........................................................ Duchenne Muscular Dystrophy
DMSO ........................................................ Dimethyl Sulfoxide
DNA ........................................................... Deoxyribonucleic Acid
ECG ............................................................ Electrocardiogram
EDV ............................................................ End-Diastolic Volume
EF ................................................................. Ejection Fraction
ESC ............................................................ Embryonic Stem Cell
ESV ............................................................ End-Systolic Volume
FACS .......................................................... Fluorescence-Activated Cell Sorting
FBS .............................................................. Fetal Bovine Serum
FITC .......................................................... Fluorescein Isothiocyanate
FS ................................................................. Fractional Shortening
GFP ........................................................ Green Fluorescent Protein
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>hfmd</td>
<td>Hypertrophic Feline Muscular Dystrophy</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>ICC</td>
<td>Intra-Class Correlation Coefficient</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor 1</td>
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<tr>
<td>IMC</td>
<td>Intra-Myocardial</td>
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<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<td>IV</td>
<td>Intravenous</td>
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<td>LVID</td>
<td>Left Ventricular Internal Diameter</td>
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<td>LVIDd</td>
<td>Left Ventricular Internal Diameter at Diastole</td>
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<td>mdx</td>
<td>X Chromosome-Linked Muscular Dystrophy Mouse</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PE-Cy7</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>ROI</td>
<td>Region of Interest</td>
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<td>Sinoatrial Node</td>
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<td>Wild-Type</td>
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<td>αMHC</td>
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1 Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked neuromuscular disorder occurring in about 1 in 3500 boys. It is caused by genetic mutations in the dystrophin gene, which encodes a cytoplasmic protein connecting the cytoskeleton of a muscle fibre to the surrounding extracellular matrix, providing muscle cell membrane stability. In the absence of functional dystrophin, skeletal muscle progressively deteriorates, resulting in a weakening of voluntary movement. Patients are confined to a wheelchair, subsequently experiencing reduced strength and endurance in the advanced stages of DMD; they eventually succumb due to asphyxia brought on by loss of diaphragm function.

Aside from its effects on skeletal muscle, DMD also manifests itself in heart muscle tissue as a condition called dilated cardiomyopathy. Although clinical advances and therapeutic successes have greatly improved the quality of life for patients struggling with this disease, most are focused on managing the skeletal muscle involvement, and few directly tackle the problems that occur within the heart; as such, patient death as a result of cardiomyopathy alone accounts for over 30% of all DMD deaths. To date, no cure for DMD exists.

These statistics represent a clear and unmet need for novel and innovative treatment strategies to combat DMD-related cardiomyopathy. This dissertation will provide background to DMD, related research and therapeutic strategies, and will present the rationale and preliminary pre-clinical evidence supporting a novel stem cell-based therapeutic approach.

1.1 The Heart

The circulatory system is a complex group of organs that functions to circulate blood around the body, delivering necessary oxygen and nutrients, as well as hormones and immune cells, to the tissues of the body. Importantly, it also plays a crucial role in maintaining homeostasis, or the physiological balance of an organism. At the centre of this system is the heart—a four-chambered pump that provides the force to drive the blood volume through blood vessels.
The heart is a fist-sized organ that is divided into the ‘right heart’, which drives circulation of deoxygenated blood to the lungs, and the ‘left heart’, which drives oxygenated blood arriving from the heart to the rest of the body. Each of the two halves is comprised of two chambers—an atrium and a ventricle—which function to pump blood through rhythmic contraction and relaxation; the former is known as systole, and the latter as diastole. The chambers are separated by a set of valves, which serve to maintain unidirectional movement of blood through systole and diastole. Deoxygenated blood arrives into the right atrium through the superior and anterior vena cava, and is pumped by the right atrium into the right ventricle through the tricuspid valve during atrial systole. The right ventricle, in turn, pumps blood through the pulmonary valve to the lungs in ventricular systole. Blood becomes oxygenated in the lungs, after which it is brought back to the left atrium of the heart through the pulmonary veins. In a similar fashion as the right atrium, the left atrium pumps blood to the left ventricle through the mitral valve during atrial systole. The left ventricle is the largest and strongest chamber of the heart, as it drives oxygenated blood throughout the entire body. The muscle of the left ventricle is arranged in a ‘swirling’ pattern that allows it to pump blood very effectively through the aortic valve and to the rest of the body during ventricular systole.

The dynamics of systolic-diastolic cardiac contraction are controlled by a nervous region within the heart known as the sinoatrial node (SA node), or the pacemaker. It generates electrical impulses called action potentials that travel quickly through the muscles of the heart; the rate at which these are generated will ultimately determine heart rate. The SA node is located close to the atria, and as such causes them to contract first, in unison, resulting in atrial systole. Action potentials are then transmitted to a relay point known as the atrioventricular node, where they are delayed by a matter of milliseconds before being transmitted through Purkinje fibres, a special type of muscle tissue, to the apex of the heart and to the ventricular tissue. This results in the ventricles contracting in unison after the atria, and ensures proper unidirectional blood flow through the heart.

### 1.1.1 Cardiovascular Disease (CVD)

As the heart is an important and central organ in the body, diseases affecting it are very debilitating. Despite significant advancements in clinical treatment and patient care,
cardiovascular disease (CVD) remains the leading cause of death worldwide. Though its prevalence is higher in developing countries, the effects of CVD in Western countries remain substantial. In Canada, it is responsible for 19.7% of total death yearly, second only to cancer. As such, it places a heavy burden on the healthcare system and costs the Canadian economy an estimated $29 billion annually. As the incidence of CVD continues to increase, the global death toll is expected to rise to 23.6 million by 2030.

A wide array of conditions are classified under CVD, and these affect the cardiovascular system in many different ways. They can range from diseases of the blood vessels (e.g. hypertension, atherosclerosis), which also include conditions affecting the coronary arteries that supply blood to the heart muscle (coronary artery disease), to diseases of the heart itself, such as dysregulation of the heartbeat (arrhythmia), inflammatory heart disease, and cardiomyopathy, or disease of the heart muscle. Each condition is complex and requires innovative approaches for diagnosis and treatment.

1.1.2 Cardiac Muscle and Cardiomyopathy

Although coronary artery disease remains the single largest contributor to CVD, conditions affecting the heart muscle, collectively known as the cardiomyopathies, are also very clinically devastating. The myocardium, or muscle of the heart, is an involuntary type of striated muscle that is comprised of cells called cardiomyocytes. Unlike skeletal muscle, these cells are short and branched, with one cardiomyocyte connecting to many neighbours through junctions called intercalated disks. This forms a network of muscle cells which contract together in order to produce the strong pumping force necessary to move blood around the body. As with skeletal muscle cells, cardiomyocytes are surrounded by a specialized cell membrane called the sarcolemma. Action potentials are transmitted through cardiac muscle by special structures in the sarcolemma called gap junctions; these are found between neighbouring cells at the intercalated disks. The sarcolemma contains many ‘L-type’ calcium channels, which allow calcium ions from the extracellular space to enter the cell and depolarize it, generating and maintaining action potentials. Despite structural differences at the cellular level, cardiac muscle contracts much in the same way skeletal muscle does, and as such, can be described by the actin/myosin sliding filament model. Individual cardiomyocytes
are surrounded by a layer of connective tissue called the endomysium, which is made up of fibroblasts and extracellular matrix proteins such as collagen and laminin. Together, the cells form the myocardium, which itself is lined by two layers: the facet which makes up the chambers is lined with endocardium, while the outer facet is covered by the dual-walled pericardium. Two major coronary arteries run along either side of the heart, which supply fresh oxygenated blood to the myocardium.

Cardiomyopathies are defined as structural and functional abnormalities of the myocardium associated with dysfunction. They present serious clinical concern due to their prevalence and high likelihood for patients to experience heart failure in later stages as a result of arrhythmia and sudden cardiac death. The diseases classified as cardiomyopathies are wide-ranging and heterogeneous, involving organs other than the heart, and have many intrinsic and extrinsic contributing factors. In fact, their complexity necessitates a distinct nosology and nomenclature. However, for the past 50 years, they have been divided into five general categories: dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic cardiomyopathy, and left ventricular non-compaction.

### 1.1.3 Dilated Cardiomyopathy

Dilated cardiomyopathy is the most common type of cardiomyopathy. It may be caused by viral infection and autoimmunity, but in most cases, it is genetically inherited from the parents. The typical age of occurrence is 20 to 60, and prevalence is different among the sexes, with men more likely to have it than women. Prototypically, it involves a combined thinning and stretching (dilatation) of the myocardium that mainly affects the left ventricle; this causes the chamber to enlarge, and results in substantially impaired contractile function. This leaves patients at high risk of heart failure in advanced stages, especially as a localized disease can spread to the other chambers of the heart over time. Some of the symptoms include swelling in the lower extremities, fatigue, shortness of breath, and patients with dilated cardiomyopathy may also exhibit arrhythmia and heart valve problems. Due to its prevalence and involvement with other diseases, it has received much research interest in the last decade.
1.2 The Muscular Dystrophies

The muscular dystrophies are a family of neuromuscular diseases characterized by progressive muscular degeneration leading to weakness in voluntary and involuntary contraction. Over 40 different types of muscular dystrophy have been classified to date\textsuperscript{14}, and these can affect the skeletal muscle, diaphragm muscle, eyes, and brain\textsuperscript{15}. While the term is used to classify a wide range of conditions affecting multiple parts of the body, the most common and clinically relevant forms of muscular dystrophy share a similar molecular basis, namely the dystrophin protein. Widely-known as one of the largest human proteins at 427 kilodaltons\textsuperscript{16}, dystrophin is a cytoplasmic protein located at the muscular sarcolemmal membrane. It is the archetype in a family of related muscle-associated proteins known as the dystrophin-associated glycoproteins; these include the dystroglycans, dystrobrevins, sarcoglycans, and syntrophins\textsuperscript{17}. Together, these proteins form a complex at the sarcolemma known as the dystrophin-associated glycoprotein complex (DAG) or the costamere. This crucial membrane-spanning complex serves to link the cytoskeletal actin filaments of a muscle fibre to collagen in the fibrous endomysium outside the sarcolemma, providing stability, deformability, and muscle integrity during contraction\textsuperscript{1,18}. Many of the proteins also have roles in maintaining the integrity of neuromuscular junctions. Dystrophin itself is found in the cytoplasmic compartment, and serves as the linkage between actin and the intercellular domain of the dystroglycan complex (Figure 1.1).
Dystrophin serves to bind the F-actin cytoskeleton to the extracellular matrix (basal lamina) through the DAG. Many proteins, including dystroglycans, dystrobrevins, sarcoglycans, and syntrophins, are associated with and offer support to the DAG; for ease of interpretation, they are omitted here. The dystrophin protein has three domains: the actin-binding domain (1), the rod/spring domain (2), and the C-terminal domain (3) which binds dystroglycan.

It is evident that dystrophin forms an integral part of the complex; indeed, changes in its protein structure ultimately lead to a loss of cellular membrane integrity. A lack of functional linkage between the sarcolemma and the actin cytoskeleton leads to shearing during muscle contraction. Sarcolemmal ion channels are compromised, causing an abnormal influx of extracellular calcium into myocytes, and overproduction of nitric oxide. This results in the activation of calpain proteases which degrade troponin I, compromising contractile ability. Furthermore, it causes an influx of water into mitochondria, which eventually shear; the cellular stress resulting from this causes more calcium ions to flow into the myocyte. It also results in an increased production of reactive oxygen species which stimulate the NF-κB signalling pathway responsible for the expression of pro-inflammatory cytokines, stimulating an immune response at the site. Macrophages infiltrate afflicted tissue, resulting in inflammation, further sarcolemmal instability, and loss of muscle fibres as the rate of necrosis exceeds that of regeneration. Although cardiac myoblasts regenerate cardiomyocytes efficiently in the very early stages of the disease, continued shearing and inflammation soon cause the demand for new cardiomyocytes to surpass the regenerative capacity of the tissue. A
large number of fibroblasts and myofibroblasts are recruited along with macrophages to the injured site, where they begin to produce collagenous scar tissue to fill the void left by dead cardiomyocytes\textsuperscript{25,26}. The persistence of fibroblasts results in the production of a large quantity of extracellular matrix, which unlike the myocardium, is not contractile. A combination of increased wall stress, increased demand for oxygen, continued myocyte death, and replacement fibrosis, results in a vicious cycle of muscular deterioration. As the muscle is continuously and progressively damaged over time, excess fibrosis results in loss of contractile function, the hallmark of the muscular dystrophies\textsuperscript{27}.

The bases of dystrophin protein malformations lie at the genetic level, and are caused by inherited mutations. The gene encoding dystrophin is the largest gene within the human genome—found at locus Xp21 on the X-chromosome, it spans over 2.5 megabases and is comprised of 79 exons, or coding regions of DNA\textsuperscript{18}. Its large size makes it prone to genetic mutation; the mutated allele of dystrophin is recessive, meaning that a healthy or dominant allele on the opposite chromosome will be preferentially expressed. Its X-linkage explains why females are most likely to be carriers, as a functional dystrophin allele on one X chromosome will compensate for a mutated allele on the other. However, if a mother passes the mutant allele on to her son, he will be affected as males only have a single copy of the X-chromosome, termed hemizygosity.

Mutations affecting dystrophin vary in type and extent. Generally, the causes of these mutations are deletions within the internal regions of the gene, which code for the central, spring-like domain of the protein\textsuperscript{28}. When these deletions are ‘in-frame’, meaning that downstream regions of the coding sequence are unaffected, a mostly working dystrophin is generated. Although it is shortened, this protein still has the ability to function due to the unaffected binding domains found on either side of the protein—they will still link the cytoskeleton to the extracellular matrix, albeit with reduced efficiency. This results in a condition known as Becker muscular dystrophy (BMD). It is the second-most common form of muscular dystrophy, wherein patients exhibit a relatively mild phenotype with late onset and long life expectancy. Patients with BMD are largely ambulatory and often live into their fifties and sixties\textsuperscript{1}.
1.2.1 Duchenne Muscular Dystrophy (DMD)

The most common and severe form of muscular dystrophy is known as Duchenne muscular dystrophy (DMD). It affects around 1 in 3500 boys, with onset in early childhood. As opposed to the mild phenotype of BMD, DMD is characterized by a progressive and severe muscular degeneration. Patients require walking supports very early in life, are confined to a wheelchair by age 12, and subsequently experience debilitating reductions in strength and endurance in the advanced stages of the disease; they eventually succumb to asphyxia brought on by loss of diaphragm function.

Whereas in BMD the dystrophin protein is merely truncated, DMD is caused by a completely non-functional dystrophin. A nonsense mutation, or a mutation that introduces a premature stop codon in the sequence, halts dystrophin protein synthesis prematurely—ribosomes are essentially unable to read past this stop signal. The result is a cut-off dystrophin protein containing only a single binding domain; the absence of either the C-terminal or N-terminal domain effectively abolishes the possibility for linkage between the cytoskeleton and the extracellular matrix within muscle cells.

As muscle becomes damaged, it releases creatine kinase, an enzyme found in low levels in healthy individuals, into the bloodstream; this has been the classic diagnostic indication of DMD-related muscle degeneration. If left untreated, patients have a life expectancy of under 30 years. This is largely caused by DMD’s involvement in all muscle tissue types of the body, including skeletal, cardiac, and smooth muscle. It also has significant osseous involvement, characterized by disuse osteopenia.

1.2.2 Cardiac Muscle Manifestations of DMD

Aside from its effects in skeletal muscle, DMD has substantial involvement in the heart, where it manifests itself as dilated cardiomyopathy. While DMD has classically been considered a skeletal muscle pathology that also includes the diaphragm musculature, improvements in palliative care now extend the lifespan of patients such that clinically relevant cardiomyopathy is observed in over 95% of affected individuals. Around 25% of children under the age of 6 suffering from DMD exhibit discernible cardiac involvement, which becomes a clinically relevant deterioration of cardiac function.
around 10 years of age; by 18 years of age, nearly all patients exhibit cardiomyopathy\textsuperscript{33,34}. In the clinic, cardiac involvement is most often detected through electrocardiography (the analysis of the electrical activity within the heart) and echocardiography (the assessment of heart function by ultrasound imaging), but may also be diagnosed through x-ray computed tomography (CT), cardiac magnetic resonance imaging (MRI), and radionuclide imaging\textsuperscript{25,34–37}.

The fibrotic region characteristic of DMD gradually expands along the walls of the heart, causing them to become thinner and lose contractility. This results in a dilated cardiomyopathy characterized by an enlarged, dysfunctional left ventricle, compromised systolic function, and mitral valve regurgitation; this causes decreased cardiac output and hemodynamic decompensation\textsuperscript{25,28}. Cardiomyopathy is also be accompanied by conduction abnormalities, arrhythmias, and tachycardia when patients enter their late twenties\textsuperscript{37}. Unfortunately, the cardiac aspect of DMD is largely asymptomatic until heart function is severely hindered\textsuperscript{38}.

Research over the last decade has largely and appropriately focused on developing treatments to address the skeletal muscle manifestations of DMD, allowing patients to experience a much longer life expectancy and improved quality of life than ever before\textsuperscript{32}. However, DMD-related cardiomyopathy has received less clinical interest, and mortality due to cardiomyopathy continues to represent over 30\% of all DMD-related deaths, causing serious concern\textsuperscript{39}.

As it stands, the current paradigm of DMD is that a combination of ischemia, inflammation, and fibrosis, all acting together and building upon the effects of the other, are responsible for the devastating condition seen in patients\textsuperscript{40,41}. There is a clear and unmet need for therapies to curb the effects of this vicious cycle, and these need to be explored first in a pre-clinical model, before an effective treatment can be brought to clinical trial.
1.3 Pre-Clinical Models of DMD

Animal models have played a crucial and indispensable role in pre-clinical therapeutic research. They provide an accurate and dynamic model of human disorders, and can offer an insight into the mechanics of disease that is simply unattainable with standard in vitro techniques. The optimal animal model is one that closely resembles the human condition, and which will also respond in the same way as humans to potential treatments. Further factors, such as size, gestation period, life expectancy, and cost of upkeep, must also be taken into account.

For the purposes of DMD research, several mammalian models have been used. Perhaps most appropriate are the canine and feline models. The former, termed canine X-linked muscular dystrophy (cxmd), contains a premature stop codon much like in humans, and exhibits a rapidly progressing muscular phenotype, as well as significant left ventricular dilation and decreased systolic function\(^4\). However, aside from their large size and costly upkeep, the cxmd dogs show high variation in disease severity among littermates, and cardiac involvement appears only after several years\(^3\). The second large animal model is the hypertrophic feline muscular dystrophy (hfmd) cat, which possesses mutations in the dystrophin gene and elevated creatine kinase levels. Nevertheless, this model is largely characterized by a hypertrophic muscle phenotype, and shows little sign of fibrosis and cardiac involvement\(^1,8,28,30\). This is thought to arise as a result of the feline physiology, and as such, the hfmd cat provides a relatively poor representation of the human condition. Furthermore, the large size of both of these models presents limitations in terms of obtaining and administering sufficient amounts of therapeutic agents under study, which is particularly problematic for research using biological agents.

1.3.1 Mouse Models of DMD

Considerable research employs rodent models, specifically mice, due to the availability of a wide range of inbred, genetically modified, and transgenic strains that accurately represent many human diseases\(^4\). As such, DMD has largely been characterized in mice due to their short life cycle, high breeding rate, and similarity to humans in regards to genetic orthology and disease symptoms\(^3,8\). Moreover, their susceptibility to effective
genetic modification has allowed the generation of a multitude of different single- and double-knockout DMD strains, including knockouts for dystrophin (mdx), utrophin (mdx:utron–/–), MyoD (mdx:myod–/–), dystrobrevin (mdx:adbn–/–), α7-integrin (mdx:itga7–/–), parvalbumin (mdx:pv–/–), and desmin (mdx:des–/–)30,45,46.

By far, the most widely used model of DMD is the dystrophin-knockout mouse known as ‘X chromosome-linked muscular dystrophy’ or mdx mouse, which genotypically mirrors the human condition. Much like in patients, this is induced by a nonsense mutation in the dystrophin gene on the X chromosome, halting protein synthesis prematurely47,48. Thus, mdx mice have found great applicability in skeletal muscle studies. Aged mdx mice have also been shown to exhibit features of cardiomyopathy observed in patients, including compromised growth factor response41. However, this model only shows mild, non-progressive skeletal pathology, with no signs of cardiomyopathy before 10 months of age49, at which point it varies widely in severity between animals50. These factors limit this strain’s applicability to time-sensitive cardiac functional studies. The relatively mild phenotype of mdx mice is attributed to up-regulation of utrophin, a protein homolog of dystrophin which can substitute, at least in part, for dystrophin in the dystrophin-associated glycoprotein complex51. In comparison, the dystrophin/utrophin double-knockout mouse model (mdx:utron–/–) exhibits severe progressive skeletal muscle degeneration as well as cardiac pathology between 8-10 weeks of age52. Specifically, these mice exhibit a characteristic crescent-shaped region of fibrosis in the epicardium of the left ventricle, which is also observed in the human condition53; this abnormal fibrosis is not localized and affects all regions of the ventricle similarly49. The mice also exhibit a short stature, abnormal gait, severe kyphosis, joint contracture, hind limb weakness, labored breathing, abnormal electrocardiogram (ECG) patterns, tachycardia, and premature death before 20 weeks of age54. Recent evidence revealed that mdx:utron–/– mice show dysregulation in matrix metalloproteinases (MMPs) which in turn leads to cardiac involvement53. Other degenerative changes in bone and cartilage make them an effective phenocopy of the human condition, which is evident in Figure 1.2.
**Figure 1.2**  DMD mouse models. Representative image showing both a) the *mdx* dystrophin-knockout mouse model of DMD, and b) the *mdx:utrn*−/− dystrophin/utrophin double-knockout model. Note the abnormal stature, kyphosis, posture, and muscle contraction in the double-knockout mouse compared to the *mdx* mouse, which appears relatively unaffected. Photo courtesy of Kelly Gutpell.

Thus, the *mdx:utrn*−/− mouse proves to be more representative of true DMD than the ubiquitous *mdx* model for pre-clinical investigation of DMD-related cardiomyopathy and novel therapies. Importantly, no other single- or double-knockout mouse shows significant changes in cardiac involvement or fibrosis in the same manner as *mdx:utrn*−/− mice30. Longitudinal assessment of cardiac function in this strain is thus crucial to quantitatively determine the progression of cardiomyopathy, which would further our fundamental understanding of murine dystrophy, and would also be essential in optimizing *in vivo* therapies. Recent research employing non-invasive, longitudinal techniques in the mildly-affected *mdx* mouse has elucidated valuable information about its cardiac function49,54–59. Nevertheless, the double-knockout model has not received the same degree of research interest, and most of the current knowledge of its cardiac manifestations are based on terminal techniques. For instance, early functional studies in the *mdx:utrn*−/− mouse determined contractile dysfunction in the heart via cardiac muscle preparation52. However, this study was performed using muscle fibres from the right ventricle, and in order to obtain these, mice had to be euthanized, eliminating the
possibility of longitudinal cardiomyopathy progression studies. Few groups have focused on characterizing cardiomyopathy in the dystrophin- and utrophin-deficient \textit{mdx:utrn}\textsuperscript{−/−} mouse non-invasively. Two studies employed cardiac MRI\textsuperscript{16,59}, which is an expensive and time-consuming procedure. Another study employed ultrasound, namely motion-mode (M-mode) echocardiography\textsuperscript{38}. While it is a cost-effective alternative, M-mode is inherently very prone to inaccurate measurements (discussed in detail below). This emphasizes the need for an alternative method of non-invasively assessing the extent and progression of cardiomyopathy in the \textit{mdx:utrn}\textsuperscript{−/−} mouse that is cheap, quick, and relatively easy to perform, which would be a great asset for therapeutic studies.

1.4 Treatment Strategies for DMD

To date, no cure for DMD exists, and even with clinical intervention, the disease remains invariably fatal before 30 years of age\textsuperscript{60}. Historically, treatments have been largely palliative, which has indeed afforded patients longer life expectancy and quality of life\textsuperscript{32}. In the past twenty years, advances in walking and ventilation support have eased the maintenance of the disease even more. In terms of therapy, the current clinical standard is a glucocorticoid called prednisolone, which slows the progression of the disease by reducing the inflammatory response\textsuperscript{31,61}. However, responses to this therapy have been mixed—improvements in muscle strength and function are generally seen, but they are short-term, lasting from six months to two years. Many adverse effects have also been observed as a direct result of this therapy, and no true long-term data on glucocorticoid treatment exists to validate its efficacy\textsuperscript{62}. Furthermore, steroid therapy poses a very serious risk of osteoporosis when used over a long period of time\textsuperscript{31}. Given the osseous involvement in DMD, such a therapy is not feasible.

In recent years, novel and innovative approaches have been investigated to tackle DMD; these can be divided into four overarching categories: gene replacement therapy, utrophin up-regulation, exon skipping, and cell therapy\textsuperscript{1}. The first involves the delivery of a truncated-but-functional dystrophin gene, termed a ‘micro-dystrophin’, through an adenoviral vector to the patient. The viral vector was used as it delivers the genetic material to cells ubiquitously through transduction. Indeed, studies using micro-dystrophin have shown excellent results in animal studies\textsuperscript{63}, which led to a clinical trial.
However, it showed limited success—the therapy did not restore adequate dystrophin expression and was plagued by host rejection, wherein an immune response was produced against myocytes that novelly expressed the protein\textsuperscript{64}. Moreover, gene therapy has shown little beneficiary effects on the heart in animal studies\textsuperscript{65}.

Utrophin up-regulation, as its name implies, involves using high levels of the analog protein in humans. In adults, utrophin is 80\% homologous to dystrophin and is primarily found at neuromuscular junctions, with little found along the sarcolemma post-partum\textsuperscript{16,66,67}. Nevertheless, it has the ability to localize there and confer function, particularly after exercise\textsuperscript{68}. Experiments using \textit{mdx} mice have shown that up-regulation of utrophin can reduce blood creatine kinase levels by 25\%\textsuperscript{9}. Treatment strategies in adult tissue work at multiple levels, with drugs administered to up-regulate expression of the utrophin gene, while others up-regulate chaperone proteins to bring utrophin to the sarcolemma. Unfortunately, none of these approaches have resulted in high enough up-regulation, and \textit{mdx} mice retained the DMD phenotype after treatment\textsuperscript{1}.

Exon skipping is a technique by which a faulty region of a gene is ‘skipped’ when generating protein. It uses small pieces of RNA called antisense oligonucleotides (AONs) that either block splice sequences or alter host mRNA folding\textsuperscript{69,70}. DMD involves a stop mutation, and thus skipping the faulty stop codon renders a mostly functional protein that ameliorates symptoms, giving rise to a more BMD-like phenotype\textsuperscript{71}. Indeed, several clinical trials have taken place using exon skipping. Nevertheless, these showed poor uptake of AONs into cells, fast clearance from the bloodstream, and variable efficiency depending on the specific dystrophin mutation—the treatments were all targeted to exon 51. Importantly, the clinical trials showed little efficacy in the heart; if exon skipping therapy were to become widely-adopted, it would cause cardiomyopathy to become much more prevalent in patients treated with AONs\textsuperscript{1,50,59}. Moreover, the resulting increase in skeletal muscle function in these patients may cause them to be more active, putting a greater strain on their weak heart\textsuperscript{59}; the same problem also applies for micro-dystrophin gene therapy.
1.4.1 Stem Cell Therapy

The key to slowing or attenuating DMD-associated cardiomyopathy may be an approach that promotes the regeneration of injured tissue. In this respect, a good candidate is exogenous stem cell therapy. When administered to injured tissues, stem cells have been reported to improve cardiac function through multiple mechanisms, including direct replacement of cardiomyocytes through cellular differentiation of implanted cells, fusion with endogenous cardiac cells, and also through paracrine effects, i.e. stimulating the endogenous host cells to regenerate injured tissue. The replacement of old, diseased tissue with new, healthy, and fully functional one may confer a much more substantial benefit to patients and could improve clinical outcome on a long-term basis.

Stem cells are categorized into two major subtypes: embryonic stem cells (ESCs), or cells derived from the inner mass of the blastocyst, and adult stem cells, which are resident in developed organs and function to replenish tissues throughout postnatal, childhood, and adult life. The former have received great scientific attention in recent years as they readily self-renew to generate large numbers of cells for regenerative purposes; their characteristic pluripotency allows them to differentiate and regenerate many tissues of the body. Indeed, ESCs have been shown to be effective at regenerating myocardium, and evidence has shown that these cells are capable of restoring dystrophin. However, they suffer from a large and significant caveat—ethical concerns over obtaining cells from human embryos drastically hinder their future translational applicability. An ingenious solution to this problem is to use induced pluripotent stem cells (iPSCs), or adult cells that are reverted into a pluripotent, ESC-like state. While these cells may circumvent the aforementioned ethical issues, the field of iPSC therapy is still in its infancy, and much research is needed to optimize their mechanism of reversion before extensive regeneration experiments can be performed.

Adult stem cells have also received a great deal of research interest, and are now being very actively investigated for CVD treatment. The earliest studies used bone marrow-derived stem cells, which were shown to be capable of alleviating the effects of myocardial infarct, or heart attack. However, further investigation into this stem cell type showed that they ultimately demonstrate poor long-term engraftment potential,
while not being reflective of true myocardial regeneration. Another type of stem cell that has been investigated is the skeletal myoblast. While these cells showed excellent potential for treating DMD in initial mdx mouse studies, a subsequent clinical trial in skeletal muscle revealed a very poor regenerative capacity, with novel myogenesis in only 1 of 12 patients. Importantly, skeletal myoblast therapy was investigated in the heart, and while the transplanted cells indeed engrafted into the host tissue, they also produced dangerous arrhythmias. This may have come as a result of these types of cells’ inability to form functional and active electromechanical coupling with the host tissue. Mesenchymal stem cells have also been explored, as they have been shown to be able to differentiate into mesodermal cells, couple with host tissue, and elicit paracrine stimuli to endogenous cells. Studies with mesenchymal stem cells in rats showed they are able to differentiate into cardiac muscle and can confer some improvement in function in non-ischemic cardiomyopathy. They have also been used in DMD therapeutic research, but unfortunately did not produce an adequate degree of contractile improvement, which is crucial to treating such a condition.

Thus, none of the aforementioned cell types have been determined to be an optimal candidate for DMD cardiac therapy. Importantly, none of these cells constitute true myocardial regeneration. Due to the limitations of these cell types and the ethical concerns over ESCs, an approach using cardiac-specific autologous or histocompatible stem cells may be the best option for treating heart disease and DMD cardiomyopathy.

1.4.2 Cardiac Stem Cells (CSCs)

Until roughly the last decade, the general consensus among scientists was that the heart was a terminally-developed organ without the capability for self-renewal or regeneration. However, research has uncovered a new population of multipotent stem/progenitor cells resident in the adult heart; these are known as cardiac stem cells (CSCs). CSCs were first discovered in rats by Beltrami et al.; they isolated a population of cells that were able to self-renew, and were found to have the potential to differentiate into all three cell types present in the heart—cardiomyocytes, endothelial cells, and smooth muscle cells. Human CSCs are characterized by their expression of tyrosine protein kinase kit (c-Kit), the receptor for a cytokine known as stem cell factor-kit ligand.
They also lack expression of common hematopoietic lineage surface markers such as CD34 and CD45, and have also recently been found to express stage-specific embryonic antigen 4 (SSEA-4), a well-known marker of stemness and pluripotency in ESCs. Importantly, CSCs are well-documented to have a high capacity for cardiomyocyte regeneration, and preferentially differentiate along a cardiomyocyte lineage as evidenced by high levels of cardiac transcription factors such as Nkx2.5, myocyte enhancer factor 2C (MEF2C), and GATA4. Studies indicate that, on average, about 50% of adult cardiomyocytes are replaced during a healthy human life span, while in mice, novel cardiomyocyte formation occurs in the adult heart following injury; these are thought to arise as a result of CSCs.

Thus, these stem cells may be an excellent candidate for therapy due to their being enriched for stem/progenitor cell activity, and their ability to successfully differentiate into cardiomyocytes in vitro. Since their initial discovery, multiple subsets of CSCs have been identified in both the human and mouse heart, including c-Kit+ cells, side population (SP) cells, cardiosphere-derived cells, and stem cell antigen 1-expressing (Sca-1+) cells. The first, c-Kit+ cells, are arguably the most relevant CSC population in humans; they can be cultured for extended periods of time to generate clinically relevant numbers, which have been shown to have up-regulated expression of cardiac transcription factors such as GATA4. Indeed, these cells have shown a striking ability to regenerate functional cardiomyocytes and prevent remodeling in diseased hearts, while also conferring functional improvement; this led to their inclusion in the SCiPIO clinical trial, which showed promising initial results. Nevertheless, they are a rare population of cells in the mouse, and very recent contending evidence has shown they largely generate endothelial tissue in mice. Cardiac SP cells, notably characterized by their exclusion of Hoechst 33345 dye, have been described as much more heterogeneous, expressing any number of surface markers, including CD31, CD34, CD45, c-Kit, and Sca-1. SP cells were shown to be tissue-specific and cardiogenic, expressing high levels of both Nkx-2.5 and cardiac troponin T (cTnT). However, they are a curiously rare population in the murine heart, and to date, their therapeutic potential remains in question. Moreover, the expression of hematopoietic and bone marrow-specific cell markers may lead to certain limitations; for example, CD34+ cells have been shown to
preferentially differentiate into endothelial cells and hematopoietic cells\textsuperscript{101}, while CD31\textsuperscript{+} cells do not show cardiomyogenic potential in the same manner as CD31\textsuperscript{-} cells do\textsuperscript{102}. More recently, cardiosphere-derived cells, which are directly grown from human biopsy specimens, have been investigated. Cardiospheres were first described by Messina et al.\textsuperscript{86}, who showed that a population of cells obtained from explant culture readily formed multicellular clusters, or spheres, in culture. Cells derived from these cardiospheres have been shown to engraft, differentiate, and secrete angiogenic/anti-fibrotic cytokines in the injured heart that ultimately conferred functional benefit\textsuperscript{103}; indeed, successful pre-clinical results led to these cells’ use in the CADUCEUS clinical trial, which presented promising improvements in cardiac wall thickening and contractility. However, further research needs to evaluate these cells over the long term, as they did not show significant improvements in left ventricular ejection fraction (EF), the gold-standard measurement of cardiac systolic function. Moreover, mixed populations of cardiosphere-derived cells express CD31 and CD34, and some reports have contested their efficacy as being due to retained cardiomyocytes and their stemness as falsely attributed to hematological contamination\textsuperscript{104}.

When discussing pre-clinical stem cell research, specifically as it relates to DMD, certain aspects must be considered. While the above cell types are relevant CSC populations in humans, they have been shown to be relatively rare in the adult murine heart\textsuperscript{7,98,105}, and thus, would not be ideal cell populations to use for investigating regenerative therapies. In humans, these cells must be cultured and expanded in order to get clinically relevant numbers. Even after expansion, one may not be able to obtain enough cells to elicit a regenerative effect. Thus, they are not an ideal choice for time-sensitive applications in the severely dystrophic \textit{mdx: utrn}\textsuperscript{-/-} mouse. Furthermore, it remains unclear whether these subtypes are distinct, or are different developmental manifestations of a single CSC type\textsuperscript{106}. In these respects, the Sca-1\textsuperscript{+} subtype of CSCs may be key. These murine cells, which also lack expression of c-Kit and hematopoietic markers\textsuperscript{107,108}, have been found to be capable of self-renewal and clonogenesis, while also being largely dedicated to the cardiomyocyte lineage\textsuperscript{105,109–111}. Specifically, some groups have shown that Sca-1\textsuperscript{+} cells can generate beating cardiomyocytes in culture with 80-90\% efficiency\textsuperscript{112}. Sca-1-expressing cells were able to partially restore function after transplant to injured hearts,
and have been shown to up-regulate paracrine effectors inducing angiogenesis, while also supporting the expansion of other CSCs in the injured heart\textsuperscript{106,108}. Sca-1 knockout mice exhibited severe cardiomyopathy, stressing its importance to cardiac development\textsuperscript{113,114}. Although a definitive human equivalent to the murine Sca-1\textsuperscript{+} cell has not yet been discovered, several functional orthologs exist, and Sca-1 has been used as an alternative marker to identify stem cells\textsuperscript{7,113}. Research using the Sca-1\textsuperscript{+} population may uncover the subtle details that characterize them as a distinct subtype of CSC, and may also help investigate CSC delineation and the conditions needed for regenerative CSC therapies. As such, results from this research may have translational value when investigating CSC therapies in humans.

Importantly, a stem cell therapeutic approach using CSCs may aid in DMD tissue repair not only by regenerating damaged muscle tissue, but also indirectly, by combatting the three major hindrances to endogenous repair: ischemia, inflammation, and fibrosis\textsuperscript{40}. Autologous CSCs are known to stimulate stem cells in the host, including endogenous CSCs and endothelial progenitor cells\textsuperscript{115}, and may further produce angiogenesis and neovascularization through paracrine secretion of vascular endothelial growth factor (VEGF)\textsuperscript{116}. In this way, CSCs may be able to ameliorate the defective secretion of pro-angiogenic growth factors that has been evidenced in pre-clinical models of DMD-related cardiomyopathy\textsuperscript{41,115}. They have been shown to reduce inflammation and modulate the immune response to suppress the fibrotic scars evident in diseases such as DMD, which may promote an adaptive healing process\textsuperscript{115}. Furthermore, by regenerating functional myocardium, CSCs may also prevent the recruitment of fibroblasts, further ameliorating fibrosis.

1.5 Non-Invasive Pre-Clinical Imaging

The necessity of reliable, non-invasive methods of assessing disease quickly becomes clear when trying to develop methods to assess both degenerative changes over time in DMD, and also the efficacy of therapeutic applications. Medical and molecular imaging techniques targeting biomarkers and reporter proteins have become complementary to \textit{in vitro} and \textit{ex vivo} methods in studying cardiomyopathy and cell therapy. The advent of new imaging technologies has allowed unprecedented amounts of information to be
obtained in great detail without the need for invasive procedures, in both mice and humans. A number of imaging techniques, such as ultrasound, have been applied to study DMD-related cardiomyopathy in the clinic\textsuperscript{36}, and imaging may be an indispensable tool when studying the disease at the pre-clinical level.

1.5.1 Ultrasound

Ultrasound is an imaging method that uses high-frequency acoustic waves that are beyond the range of human hearing to examine tissues within the body. The ultrasound probe, or transducer, emits pulsed ultrasonic waves through a conducting gel into the body. These sounds are reflected wherever changes in density and compressibility exist, which correspond to changes in acoustic impedance; this occurs between tissue boundaries, and produces ‘echoes’ which are then picked up by the transducer. The signals are processed by the scanner, which generates images based on the strength of the echoes and time it takes them to return to the transducer. There is a trade-off between imaging depth and resolution, depending on the frequency of the emitted sound—lower-frequency ultrasonic waves produce deep tissue penetration with low resolution, whereas higher-frequency ultrasound produces very detailed images, albeit with low tissue depth. Ultrasound has been very popular in the clinic due to its low cost, portability, and ability to produce images in real-time; as such, it has found clinical application in such fields as cardiology, obstetrics, and surgical guidance. It can also take advantage of the Doppler Effect to provide blood flow analysis, important in the imaging of heart valve dysfunction.

Over the last decade, this method has found widespread popularity in studying heart disease in small animals. Technological advances in the manufacture of piezoelectric crystals and multi-element ultrasound transducers has permitted the use of high resolution ultrasound to generate images of striking quality in rodents\textsuperscript{117}. This technology enables systolic function to be measured non-invasively in mice using small animal scanners, which produce images at a frequency range of 20-60MHz. Transthoracic micro-echocardiography, or the imaging of the heart via ultrasound, offers a powerful non-invasive alternative to gauge morphometry and function in the living animal\textsuperscript{118}. As opposed to other tomographic imaging methods, echocardiography is quick, cheap, high
resolution (as fine as 30µm)

However, it is operator-dependent, with a high chance of inter-
sonographer variability. Furthermore, artifacts in the image are introduced by hair and
ribs, which may block certain regions of the heart depending on the orientation of the
transducer and the transmitted frequency. Despite these shortcomings, the absence of
injectable contrast agent, as well as cost- and time-effectiveness, make echocardiography
ideal for the assessment of cardiac function in frail mouse models of disease, which may
not handle contrast agents like their wild-type (WT) counterparts.

1.5.1.1 Limitations of 2D Echocardiography

A common method of determining cardiac systolic function and cardiomyopathy is by
measuring cardiac output. This can be described by stroke volume (SV), or the volume of
blood pumped by the heart with each heartbeat, obtained by subtracting end systolic
volume (ESV) from end diastolic volume (EDV) for the left ventricle. It can also be
described by ejection fraction (EF), which is equal to SV/EDV, and represents the
fraction of blood pumped out of the ventricle with each heartbeat. Until recently, the
use of ultrasonic methods was hampered by the small size and fast heart rate (around
400-700bpm) of mice, and few systems were able to produce information of enough
quality to develop quantitative results. For this reason, and likely due to its relative
simplicity, the most popular means of determining SV and EF has been through one-
dimensional motion-mode echocardiography (M-mode). This involves placing
the ultrasonic transducer over the left parasternal line and obtaining either a long or short
axis view through the centre of the heart; an M-mode image with high temporal
resolution is obtained, from which the one-dimensional value for left ventricular internal
diameter (LVID) at both diastole and systole (LVID and LVID, respectively) is
determined by measuring the distance from the anterior to posterior facets of the
endocardium. Subsequently, a correction formula known as the Teichholz’ formula (Vol
= 7/(2.4 + LVID) + LVID^3) is applied to these linear measurements in order to estimate
the values for EDV and ESV. Another measurement, known as fractional shortening
(FS), is calculated from LVID and LVID, through the formula FS = (LVID – LVID) / LVID^3.
FS is preferred by some as an alternative to EF, as a greater cavity
obliteration in mice produces a much larger EF value than seen in humans\textsuperscript{126}. The M-mode method is relatively quick and provides rapid EF and FS calculations by applying the assumption of an overall geometric model to the heart\textsuperscript{125}.

However, M-mode is prone to inter- and intra-user variability\textsuperscript{129}, and may be inaccurate due to the inherent assumptions made in order to convert a linear value into a three-dimensional volume\textsuperscript{130,131}. Indeed, reports of inter- and intra-user variability and bias with this methodology are well noted—patient studies show that the Teichholz method produced the widest limits of agreement compared to CT\textsuperscript{132}, with EDV values showing the weakest correlation, often resulting in a significantly over- or under-estimated EF\textsuperscript{129,133}. Furthermore, the assumption of shape inherent in the Teichholz method may cause even greater discrepancies when studying diseased hearts such as those affected by dilated cardiomyopathy, as these specimens may not have the same geometry as healthy hearts\textsuperscript{128,131,134}. The presence of regional wall motion abnormalities such as those present in heavily fibrotic hearts may call for an entirely different approach altogether\textsuperscript{126,128}. Indeed, the practice of using the Teichholz’ formula to measure EF in the clinic has been discouraged for some time\textsuperscript{130}, but in pre-clinical models, it arguably remains the most widely-used method of assessing systolic function.

1.5.1.2 Three-Dimensional Echocardiography (3DE)

With the advent of very high-frequency ultrasound scanners, three-dimensional echocardiography (3DE) has become possible in mice with the help of dedicated 3D motors and physiological monitoring—the motor functions to sweep the transducer in the z-plane to produce slices, while physiological monitoring permits prospective ECG and respiratory acquisition gating, allowing sonographers to generate stitched 3D volumes of the heart at systole and diastole\textsuperscript{135,136}. Although gated 3DE requires more scan time (around 30 minutes total time per scan), it proves to be more accurate than M-mode, and is much cheaper and quicker than the standard MRI method for 3D measurement of cardiac function, while producing similar results\textsuperscript{137}. Nevertheless, 3DE produces gating artifacts which are apparent in views perpendicular to the sweep axis\textsuperscript{136}. 
1.5.1.3 Echocardiographic Evaluation of DMD Mouse Models

Although cardiomyopathy in *mdx* mice has been described longitudinally in great detail through non-invasive imaging\(^49,54-59\), *mdx:utrn\(^{−/−}\) mice have not received as much research interest\(^{16,59}\). The *mdx:utrn\(^{−/−}\)* dystrophic mouse is one of very few small animal models available to date with which to study the dilated, non-ischemic form of cardiomyopathy—the others being hamsters\(^{44}\). Thus, quantitative evaluation of cardiac ventricular sizes, ventricular function, and left ventricular mass, first in *mdx:utrn\(^{−/−}\)* dystrophic mice, is important for prognosis and management of the disease in patients. To this end, Chun et al.\(^{38}\) have recently characterized DMD-related cardiomyopathy in *mdx:utrn\(^{−/−}\)* mice using M-mode echocardiography. In this study, the authors found that by 15 weeks of age, dystrophin/utrophin-deficient mice exhibit a decrease in left ventricular EF and FS, and an increase in EDV, left ventricle dilation, and thinning of the ventricular wall and septum, relative to healthy WT mice. Nevertheless, a limitation of this study is the M-mode procedure used to measure changes in cardiac output/ventricular function. Aside from the aforementioned errors arising from geometrical volume assumptions, the M-mode method may not be sensitive enough to detect early changes in cardiomyopathy. As such, the subtle effects that may come as a result of therapies can go unnoticed. No studies to date have investigated the progressive changes in cardiac function caused by DMD-related cardiomyopathy in *mdx:utrn\(^{−/−}\)* mice using 3DE.

1.5.2 Micro-CT

X-ray computed tomography (CT) also plays a steadily increasing role in the field of cardiac imaging. This imaging technique uses high-energy electromagnetic radiation to produce images of the internal organs of the body. Briefly, x-rays are emitted from an energized source on a moving gantry that rotates 360° in a single axis around the patient. A detector located opposite the source measures the attenuation of the emitted x-rays by tissues at multiple points along the 360° rotation of the gantry, providing this information to a computer. Through an algorithmic process known as filtered back projection, the computer reconstructs cross-sectional tomographic images through the body of a patient, which are isotropic and can be rendered in three dimensions for interpretation.
Image contrast is dependent on the degree of x-ray attenuation by different matter in tissues, measured in Hounsfield units. Since the soft tissues of the body are largely composed of water, their inner details are generally not discernable on CT images. For this reason, contrast agents based on iodine and barium are administered through various routes in order to improve the interpretability of the images, and also to provide functional and dynamic information (e.g. blood flow and perfusion). This allows CT to provide insight into the body, which is a great asset in the clinic. Despite the delivery of a discernible dose of ionizing radiation, CT is widely used in studying bones, lungs, abdominal conditions, cancer, and CVD.

In vivo cardiac micro-computed tomography (micro-CT) technology has also recently evolved to permit quantitative measurement of cardiac structure, function, volume, and mass in small animal models of heart disease; it has proven to be very reliable and accurate, while also being faster and cheaper than other tomographic methods such as MRI. Potential advantages of micro-CT over traditional methods, such as M-mode echocardiography, include 3D volumetric assessment of cardiac chambers without the need for geometric assumptions. This method employs a vascular, iodinated contrast agent in order to differentiate the myocardium from the blood pool. ECG and respiration are monitored, and using these parameters, retrospective gating can be employed to produce images at systole and diastole, respectively. Micro-CT produces high-resolution images that provide an incredible amount of anatomical and structural detail; as such, it is generally considered to be one of the gold standard tomographic techniques for assessing systolic function. The injection of contrast agent may inadvertently have negative impacts on severely diseased mouse models. Despite this shortcoming, it is highly appealing as a reference point for other imaging technologies, such as 3DE.

1.5.3 Non-Invasive, Echocardiography-Guided Delivery of Therapeutics

One of the most important aspects of a therapy is its method of delivery. A number of groups investigating cardiac therapies have approached the problem by delivering the therapeutic agent systemically via intravenous (IV) administration. However this may be
disadvantageous, as the therapeutic agent is not being directed wholly to the affected site, resulting in nonspecific delivery; as such, large quantities of an agent may be necessary to see any effect\textsuperscript{143}. For this reason, groups have begun to use direct intra-myocardial (IMC) injections to deliver treatment directly to the injured heart\textsuperscript{43}. Unfortunately, the small size of the murine heart hinders the accuracy of the injection into the affected site in the left ventricular wall. To ensure optimal injections, labs have very often employed highly-invasive thoracotomy procedures which require a skilled surgeon, and even in the best cases, may lead to long-term complications in the mouse. These types of procedures are also not ideal for repeated injections and time-sensitive experiments.

A technique that is quickly gaining ground involves delivering therapeutic agents directly to the affected site under ultrasound guidance. Recently, several reports have surfaced exploring the feasibility and accuracy of closed-chest thoracic injections into mice using ultrasound-guided needles\textsuperscript{43,143–147}. These describe success in the delivery of a bolus within the left ventricle, into the myocardium, and into the pericardial space under ultrasound guidance. Springer et al.\textsuperscript{43} were the first group documented to perform an IMC injection under ultrasound guidance. They tested the method against a typical open-chest procedure, and further used it to deliver both a fluorescent tracer and skeletal myoblasts into an infarct model. Despite their pioneering success with this technique, no therapeutic assessment was carried out in this study. Nevertheless, later research from this group showed improvements in heart function in mouse models of myocardial infarct; these came as a result of IMC injection of extracts from bone marrow and mononuclear cells\textsuperscript{144–146}, as well as from the implant of human ESC-derived cardiomyocytes. Thus far, they are the only group to actively perform IMC injections under ultrasound guidance for therapeutic research. Laakmann et al.\textsuperscript{143} showed success injecting a viral vector into the pericardial space under ultrasound guidance, targeted towards the right atrium; however, this was largely performed in wild-type mice. Moreover, despite not being a therapeutic study, Zhou and Zhao\textsuperscript{147} recently published a methodological article outlining ultrasound-guided injection of breast cancer cells into the left ventricle. This method of producing breast cancer brain metastases has been adopted by other groups as well\textsuperscript{148}. Evidently, all of these procedures varied in terms of delivered agent and route of administration, and the therapeutic studies have been largely focussed on mouse models of myocardial
infarct. Thus, the methodology remains to be explored in a model of dilated cardiomyopathy.

Despite their differences, these groups have established that IMC implant is an accurate and valid delivery method that is more effective than traditional systemic alternatives. It is fast, circumvents the need for highly trained veterinary surgeons, and avoids the stringent requirements and complications of thoracotomies, while maintaining the ability to deliver both drugs and biological agents in a targeted manner. This method is especially appealing when considering dystrophic \( mdx: utrn^-\) mice, where surgery would be further complicated by their severe kyphosis and bone abnormalities. Importantly, a thoracotomy would greatly aggravate the already-present inflammation and fibrosis present in these mice. Thus, the implementation of a closed-chest, ultrasound-guided IMC injection method has great merit.

### 1.6 Rationale, Objectives, and Hypotheses

While new and promising experimental strategies are being developed to ameliorate the skeletal muscle manifestations of DMD, few have been shown to have any efficacy in the myocardium. This stresses the need to develop a novel, effective CSC therapy for DMD-related cardiomyopathy. Furthermore, such a therapy needs to be investigated first in an appropriate pre-clinical model of DMD, with well-characterized and effectively delivered cells, while also requiring an accurate and sensitive method to gauge small changes in cardiac function longitudinally. **The objectives of this dissertation are as follows:**

1) To implement a three-dimensional echocardiography (3DE) technique to measure systolic function in \( mdx: utrn^-\) mice, validate its accuracy against current gold standard micro-CT, compare its inter-/intra-user variability against traditional M-mode echocardiography, and use it to determine the progression of dilated cardiomyopathy in the mouse model.

2) To isolate and characterize a subtype of Sca-1\(^+\) cardiac stem cells (CSCs) from the hearts of healthy mice as a potential avenue for regenerative therapy, and determine their ability to differentiate into a cardiac lineage \textit{in vitro}. 
3) To explore the accuracy and feasibility of a minimally-invasive, closed-chest, ultrasound-guided injection technique to deliver therapeutic agents directly to the left ventricular myocardium in vivo.

4) Given the previous objectives, to combine these techniques to determine any regenerative effects that freshly-sorted Sca1+CSCs may have on mdx:utrn⁻/⁻ systolic function.

It was hypothesized that the use of 3DE would correlate well with micro-CT analyses of cardiac volume, and importantly, would provide greater accuracy with less bias and variability than standard M-mode. Furthermore, 3DE was expected to be a feasible and useful tool for determining cardiomyopathy in mdx:utrn⁻/⁻ mice, in which a decrease in cardiac systolic function was hypothesized to occur in dystrophic animals as compared to healthy controls. Sca-1+CSCs isolated from healthy mice were expected to grow readily in culture, and after differentiation, express markers of adult cardiac tissue. The proposed method of implanting these cells under ultrasound guidance was speculated to cause very little harm to the animal, and post-mortem evaluation was expected to confirm its accuracy and targeting ability. Lastly, transplanted CSCs were hypothesized to have a beneficial effect on mdx:utrn⁻/⁻ cardiomyopathy at 5-10 weeks post-implant, manifested as a reduced deterioration of functional parameters compared to untreated mice.
2 Materials and Methods

For the sake of clarity and brevity, the methodology used for each objective is outlined first, followed by a detailed description of the methods used herein.

Objective 1: To validate three-dimensional echocardiography (3DE) and use it to assess cardiac function, power analysis was first conducted. mdx:utrn⁻/⁻ (n = 5) and wild-type (WT; n = 8) mice were then imaged via motion-mode echocardiography (M-mode), 3DE, and micro-computed tomography (micro-CT) at 6-8 weeks and 15-17 weeks. The accuracy of each echocardiographic method was determined by comparing its measurements to micro-CT via Bland-Altman analysis. Variability within measurements obtained with either echocardiographic modality was determined via intra-class correlation analysis. Next, differences in the progression of cardiomyopathy between and within groups was assessed from 3DE measurements via two-way mixed-design multivariate analysis of variance (ANOVA) with Tukey’s post-hoc test. Findings were confirmed histologically via Masson’s trichrome and hematoxylin/eosin staining; a two-way ANOVA with Tukey’s post-hoc test was used to compare measurements (all groups n = 3 for histology).

Objective 2: In order to isolate and characterize a novel population of Sca-1⁺ cardiac stem cells (CSCs) as a potential therapeutic agent, 6-12-week-old wild-type mice were used. Cells from their hearts were obtained via fluorescence-activated cell sorting (FACS). They were propagated in culture, differentiated, and their cardiomyogenic potential was assessed via fluorescence immunocytochemistry.

Objective 3: To determine the accuracy and feasibility of an ultrasound-guided injection technique to deliver therapeutic agents directly to the myocardium, various test experiments were carried out. The accuracy of the injection was examined in WT mice (n

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in real-time using a microbubble contrast agent; furthermore, the targeting ability of
the technique was subsequently confirmed after excision of the hearts through the use of
a blue dye in the bolus. The safeness of the procedure was confirmed by monitoring
saline-injected WT mice (n = 3) over 2 weeks.

**Objective 4:** Lastly, to determine what therapeutic effects they may have *in vivo*, CSCs
were isolated via FACS and implanted under ultrasound guidance into *mdx:utrn*+/− mice (n = 2). Cardiomyopathy was assessed before implant and 5 weeks post-implant via 3DE,
and trends in the data were analyzed by comparing the implanted mice to the untreated
*mdx:utrn*+/− mice used in Objective 1.

### 2.1 Mice and Anaesthesia

All animal protocols were approved by the Institutional Animal Use Subcommittee,
(Western University, London, ON, Canada) and conducted according to guidelines set by
the Canadian Council on Animal Care (CCAC); ethics approval documentation can be
found in Appendix B. *mdx:utrn*+/− mice were produced by breeding pairs of *mdx* mice
heterozygous for the utrophin gene (*mdx:utrn*+/−; generously supplied by Dr. Robert
Grange, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA; strain
originally created by Drs. Mark Grady and Joshua Sanes, Washington University, St.
Louis, MO, USA[140]). The offspring were genotyped by PCR; these mice were fed a diet
of soft food and monitored every other day to ensure proper health. Mice that declined in
health, characterized by a loss of 15% body mass, lethargy, dehydration, labored
breathing, and lack of appetite, were euthanized to prevent suffering. As controls and to
obtain healthy Sca-1+ CSCs, wild-type C57BL/6 mice (6-8 weeks of age) were purchased
from Charles River Laboratories (Wilmington, MA, USA). *mdx:utrn*+/− mice were again
used as recipients to determine the effects of Sca-1+ cell implant, with untreated
*mdx:utrn*+/− mice used as controls.

For all imaging studies, mice were individually placed in an induction chamber receiving
3% isoflurane in 100% oxygen at a constant flow rate of 1L/min. Once anaesthetized,
mice were removed from the induction chamber and weighed; anesthesia was
subsequently maintained with 1-1.5% isoflurane in 100% oxygen at a flow rate of
1L/min. A rectal thermocouple was used to monitor body temperature. Images were only acquired when heart rate of the mouse was 500 ± 100bpm and temperature was 37 ± 0.5°C, in order to ensure heart function was not affected by the anaesthetic regime\textsuperscript{150,151}. Anaesthesia and heating were adjusted accordingly to achieve this. In contrast, throughout the implant process, anaesthesia was maintained with 2% or higher isoflurane to ensure mice were not sentient during the injection; this was confirmed using the pedal withdrawal reflex test.

### 2.2 Echocardiography

All images were acquired using a Vevo 2100 (VisualSonics, Toronto, ON, Canada) high-frequency ultrasound scanner by a method similar to Pistner et al. (2010). Anaesthetized mice were placed in the supine position on the pre-heated ultrasound stage. Electrode gel was applied to the paws, which were taped down onto the ECG electrodes of the scanner, and corneal lubricant was applied to the eyes. Depilatory cream was applied to the chest region for 1-2min, after which the hair was removed with wet gauze. An MS-400 18-38MHz linear array transducer producing 50μm axial by 110μm lateral resolution was attached to a 3D motor (VisualSonics) that allowed sweeping of the transducer along the z-axis. Pre-warmed ultrasound gel was placed on the chest of the mouse, after which the transducer was placed 10°-20° from the longitudinal axis along the coronal plane of the mouse in order to obtain a long axis view of the left ventricle. Images were acquired at 30 MHz with 27dB gain.

#### 2.2.1 M-mode Imaging

To obtain M-mode images, a long axis view through the centre of the left ventricle was established just out of view of the papillary muscles using brightness mode (B-mode), and a line of reference for M-mode was drawn close to the base of the heart; 4.95s cine images were acquired (Figure 2.1). To simulate user variability, test-retest was performed by completely lifting the transducer off of the animal, replacing the ultrasound gel, and rotating the mouse stage in various increments along the transverse and coronal planes. The transducer was then replaced and images obtained as before; this was repeated to produce n = 3 for M-mode measurements for each mouse.
2.2.2 3DE Imaging

For three-dimensional echocardiography (3DE) scans, respiratory and electrocardiogram (ECG) prospective gating was achieved through the electrodes of the ultrasound system. Prior to scanning, the respiratory gate was set for end-exhalation, and the ECG trigger was set at the R-wave for end-diastolic volume (EDV, the volume of the heart when it is highest at the end of the diastolic phase), and halfway in between two consecutive R-waves for End-Systolic Volume (ESV, the volume of the heart when it is lowest at the end of the systolic phase). The ESV gate typically occurred at about 45-65ms after the R wave, given the heart rate of each animal at the time of scan. Furthermore, the gating parameters were confirmed at the time of scanning by qualitative evaluation of real-time gated B-mode images produced by the Vevo 2100 system. Parallel long axis B-mode slices covering the full volume of the heart were obtained by sweeping the transducer along the short axis in 50μm steps. Long axis slices were used because they have been shown to be more accurate than short axis views in this type of acquisition. 3D maps were stitched and constructed automatically by the built-in software of the scanner (Figure 2.2). The transducer was then replaced on the mouse as described previously in order to obtain n = 3 for both EDV and ESV measurements for each mouse. For comparison with M-mode measurements, 3DE scans were taken immediately following M-mode scans.

Figure 2.1 Representative M-mode image. a) A brightness mode (B-mode) view of the heart was attained, after which a line of interest (blue dotted line) was placed through the centre of the heart. b) Time-lapse cine images were acquired along this line to generate motion mode (M-mode) images. Ordinate: depth; Abscissa: time (s).
Figure 2.2  3D map generated from a 3DE scan. A series of adjacent short axis 250μm-thick slices captured at end-diastole in a 16-week-old wild-type mouse using three-dimensional echocardiography (3DE). Images are displayed in the superior-inferior direction from the base of the heart (top left) to the apex (bottom right). The scanner software constructed the maps from long axis slices acquired under electrocardiogram and respiration gating; minor breathing artifacts are evident in this view (arrowhead), and shadows cast by the ribs can also be seen (arrow). Bar = 4mm.
2.2.3 Echocardiography Image Analysis

All qualitative and quantitative analyses of the images were made using the Vevo 2100 workstation software, version 1.5.0 (VisualSonics). For M-mode cine images, the values for left ventricular internal diameter at diastole and systole (LVID$_d$ and LVID$_s$, respectively) were determined by measuring the distance between the anterior and posterior endocardium (Figure 2.1b). All myocardial wall thickness and internal diameter measurements were repeated at least 5 times in different cardiac cycles on the M-mode image, and the mean for each parameter was obtained. EDV and ESV were subsequently calculated by using the Teichholz’ formula:

\[
EDV = \frac{7 \times LVID_d^3}{2.4 + LVID_d}
\]

\[
ESV = \frac{7 \times LVID_s^3}{2.4 + LVID_s}
\]

Stroke volume (SV), the volume of blood pumped by the heart in one cycle, was calculated as:

\[
SV = EDV - ESV
\]

Ejection fraction (EF) was calculated as:

\[
EF = \frac{SV}{EDV} \times 100\%
\]

With 3DE, the values for EDV and ESV were directly measured from the stitched 3D images at diastole and systole by manual contouring of the endocardium (Figure 2.3a) in every fifth parallel long axis slice, giving a 250μm distance between contours. If high motion artifact was present in a particular slice, an adjacent slice was used. After the entire endocardium was delineated, the software automatically produced a volume measurement, and also rendered the volume as an overlay to the data in three-dimensional space (Figure 2.3b). The values for SV and EF were calculated as before, using equations (3) and (4).
2.3 Cardiac Micro-CT

Retrospectively ECG-gated cardiac micro-computed tomography (micro-CT) was performed using techniques previously described in detail\textsuperscript{138,152}. In brief, eXIA 160XL (Binitio Biomedical, Ottawa, ON, Canada), a blood-pool contrast agent, was injected intravenously into anaesthetized mice at a volume of 5µL/g body weight. Clinical neonatal electrodes were attached to the paws to record ECG, enabling retrospective gating. Scans were acquired using a slip-ring gantry micro-CT scanner (eXplore Locus Ultra, GE Healthcare, London, ON, Canada) at 80kVp and 50mA over 10 rotations (5s per rotation, 416 projections per rotation, total scan time of 50s), with a field of view of 14cm (transaxial) x 5.4cm (longitudinal).

2.3.1 Micro-CT Image Analysis

Cardiac-gated reconstructions were performed by sorting the projection images of each scan based on the cardiac phase during which they were acquired, thereby allowing for reconstruction of 3D images at 12ms intervals. In the present study, 9-12 3D images were reconstructed per cardiac cycle, depending on heart rate. All images were reconstructed with 150µm isotropic voxels. Images were converted to Hounsfield units prior to analysis. To calculate left ventricular volume, a threshold level to separate the myocardium from blood was determined automatically, and done separately for each scan, as contrast level in the blood changed over the course of the study. A region-
growing approach was used to automatically segment the left ventricular chamber from the myocardium. Left ventricular volume was calculated for all the images reconstructed per cardiac cycle, and the minimum and maximum values for left ventricular volume were used as ESV and EDV, respectively. EF was calculated using equation (4). Sample images from each mouse group obtained with each modality can be found in Figure 2.4.

![Figure 2.4](image)

**Figure 2.4** Representative images from each mouse group with each imaging modality. Hearts from a healthy wild-type (WT) mouse and an mdx:utrn\(^{-/-}\) mouse are shown in diastole. a-d) Cine M-mode images representing a full cardiac cycle. e-h) 3DE long axis slices (50\(\mu\)m thick) with left ventricular contours shown in blue. i-l) micro-CT coronal slices (150\(\mu\)m thick) from 3D reconstructions. Bars = 4mm.

### 2.4 Histology

After euthanasia, the chest cavities of the mice were opened and hearts flushed with cold PBS. Hearts were then extracted and fixed in 10% formalin for 48h, cut into two equally sized axial sections, and embedded in paraffin. Embedded tissue was sliced in 5\(\mu\)m-thick serial sections, with representative sections taken at 6 different points along the long axis of the entire heart. Masson’s trichrome staining was performed in order to gauge the
progression of fibrosis, and hematoxylin and eosin staining was performed in order to assess cardiomyocyte hypertrophy\textsuperscript{153,154}. Quantification was conducted by taking five representative fields of view in each of the four walls of the left ventricle (anterior, posterior, lateral and septal) using a Zeiss Axioskop microscope (Oberkochen, Germany) with Northern Eclipse software v7.0 (Empix Imaging, Mississauga, ON, Canada), which were subsequently analyzed with NIS-Elements BR software v3.1 (Nikon Instruments, Melville, NY, USA). Fibrosis was assessed by a blinded observer; regions of collagen content stained in blue were delineated and represented as percentage fibrosis area from total tissue area. Cardiomyocyte hypertrophy was also assessed by a blinded observer, where at least 30 cardiomyocytes were delineated in each cardiac wall at each of the 6 points along the long axis, and the average cardiomyocyte area was calculated.

2.5 Fluorescence-Activated Cell Sorting of CSCs

Cardiac stem cells (CSCs) were sourced from 6-12-week-old healthy mice, as previous studies have shown that CSCs taken from this age group have the most potential for \textit{in vivo} proliferation and tissue regeneration\textsuperscript{155}. For each experiment, 4-5 mice were euthanized and their hearts were excised, followed by manual mincing in a biological safety cabinet and incubation in a collagenase/dispase solution [1% collagenase B (Roche Diagnostics, Laval, QC, Canada), 0.25% dispase (Gibco, Carlsbad, CA, USA), and 2.5mM CaCl\textsubscript{2} in phosphate-buffered saline (PBS)] for 30min at 37°C in order to generate a homogeneous suspension of single cells. Following filtration through a 100μm filter and centrifugation at 530×g for 5min, cells were re-suspended in fluorescence-activated cell sorting (FACS) buffer [5% fetal bovine serum (FBS; Gibco) in PBS] for staining with fluorescent antibodies. An aliquot of approximately $5 \times 10^4$ cells was collected as an unstained control, and multiple aliquots of $5 \times 10^4$ cells were collected for antibody compensation and fluorescence-minus-one controls. The remaining cell population was utilized as a source for sorting, using a stringent protocol to isolate Sca-1\textsuperscript{+} CSCs. 7-Aminoactinomycin D (7-AAD; BD Pharmingen, San Diego, CA, USA), a nuclear dye taken up by non-viable cells, was used to exclude dead cells. The population was further depleted by using a series of fluorescein isothiocyanate (FITC)-labelled antibodies (BD Pharmingen) to exclude cells expressing the cell surface markers CD11b, CD31, and
CD45. These are largely expressed on hematopoietic stem cells and their derivatives\textsuperscript{156–158}, and stem cells expressing them show little cardiomyogenic potential\textsuperscript{102}. Cells expressing CD34, another hematopoietic cell antigen\textsuperscript{159,160}, were also excluded using an Alexa Fluor 700-conjugated antibody (eBioscience, San Diego, CA, USA). c-Kit was selected against using a phycoerythrin-cyanine 7 (PE-Cy7)-labelled antibody (BD Pharmingen). To enrich for the endogenous population of Sca-1\textsuperscript{+} CSCs, a violet 450 (V450)-labelled antibody against Sca-1 (BD Horizon, San Diego, CA, USA) was used. Live cells were collected at a rate of up to 3000 cell events/s on a three-laser FACSaria III cell sorter (BD Bioscience, San Diego, CA, USA) with kind help from Dr. Kristin Chadwick and the London Regional Flow Cytometry Facility. Forward and side light scatter were used to differentiate cells from debris based on size.

### 2.6 CSC Differentiation and Live Cell Microscopy

Immediately following FACS, the isolated Sca-1\textsuperscript{+} CSC population was pelleted at 530×g for 5min, re-suspended in growth medium [DMEM/F12 (1:1) + 10% FBS + 100U/mL Penicillin + 100μg/mL Streptomycin (Gibco)] and seeded onto 0.2% gelatin-coated Falcon 12-well culture plates (Corning, Tewksbury, MA, USA) at a density of around 1×10\textsuperscript{4} cells/well, depending on FACS yield. The growth medium was replenished every 2-3 days, and the cells were grown to around 90% confluency. At this point, they were placed in differentiation medium [DMEM/F12 + 2% FBS + 100U/mL Penicillin + 100μg/mL Streptomycin + 1% DMSO]. CSCs were monitored daily and differentiation medium was replenished every 2-3 days. Cells were differentiated for up to 10 days. Live CSCs were monitored using an Eclipse TE2000-S inverted fluorescence microscope with a Digital Sight DS-Qi1Mc digital camera and NIS-Elements BR 3.00 software (Nikon Instruments). Images were processed using Fiji 2.0.0 (National Institutes of Health, Bethesda, MD, USA) and Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

### 2.7 Fluorescence Immunocytochemistry

To determine their potential to differentiate into cardiomyocytes, Sca-1\textsuperscript{+} CSCs at days 4 and 10 post-differentiation were fixed in 2% paraformaldehyde for 20min, after which they were permeabilized with 0.25% Triton-X-100 for 5min. Following a series of
washes (3×10min) in PBS, slides were incubated in blocking buffer [1% bovine serum albumin (BSA) and 10% normal donkey serum in PBS] for 1h at room temperature. Primary antibodies against cardiac troponin T (cTnT, responsible for regulating contraction; 20μg/mL; Abcam, Cambridge, UK) and Connexin 43 (Cx43, main gap junction protein in cardiac muscle; 5μg/mL; Sigma-Aldrich, St. Louis, MO, USA), or against myosin heavy chain alpha (αMHC, part of cardiac myosin contractile complex; 5μg/mL; Santa Cruz Biotechnology, Dallas, TX, USA) and Cx43, were diluted in blocking buffer and slides were incubated with one of the two solutions overnight at 4°C. Respective secondary antibodies against mouse conjugated with Alexa Fluor 647, goat conjugated with Alexa Fluor 594, and rabbit conjugated with Alexa Fluor 488 (all 4μg/mL; Molecular Probes, Carlsbad, CA, USA) were applied for 2h at room temperature. Slides at day 4 were also stained with Hoechst 33258 (Sigma-Aldrich) for nucleus visualization. Cover slips were then mounted onto slides using ProLong Gold anti-fade mountant (Molecular Probes). Cells were visualised with an IX81 motorized inverted fluorescence microscope (Olympus, Tokyo, Japan) with a CoolSnap HQ2 digital camera (Photometrics, Tucson, AZ, USA) and InVivo 3.2.2 software (Media Cybernetics, Rockville, MD, USA). Ten optical sections per region of interest (ROI) were collected in 0.2μm steps along the z-axis using a 60× oil immersion objective lens. The z-stacks were processed for spherical aberrations and deconvolved using the 3D blind algorithm in Image-Pro Plus 6.2.1 software (Media Cybernetics). Images were further processed using Fiji 2.0.0 (National Institutes of Health) and Photoshop 7.0 (Adobe Systems).

### 2.8 Ultrasound-Guided Injection

The methods described here are roughly based on Springer et al. The Vevo 2100 system described herein was used in largely the same manner for cell implants, with some slight modifications. An injection arm (VisualSonics, catalog number VS-11282) was mounted on the rail system of the Vevo imaging station. A 10μL Gastight 1801 syringe (Figure 2.5a) with a custom sickle-shaped needle (Figure 2.5b; Hamilton Company, Reno, NV, USA) was used to draw up the solution to be injected, and was subsequently mounted onto the injection arm.
Figure 2.5  Intra-myocardial injection system. a) The 10μL Hamilton syringe used for ultrasound-guided injections is presented here (1) alongside a traditional 300μL insulin syringe (2; BD Medical, Franklin Lakes, NJ, USA). Bar = 1cm. b) A transverse histological section of a mouse heart stained with hematoxylin/eosin is presented along with the custom sickle-shaped Hamilton needle (3) used in ultrasound-guided injections and a regular 30 gauge needle (4, BD Medical). Bar = 1mm. c) Overview of the entire injection system, with major components denoted: 5) mouse; 6,7) stage controls; 8) transducer control arm; 9) syringe control arm; 10) physiological monitor for temperature, heart rate, and controlling stage temperature; 11) 3D motor for z-axis scanning; 12) isoflurane vaporizer; 13) anesthesia induction chamber; 14) heat lamp. Note the entire injection system is contained within a biological safety cabinet. d) The mouse (15) was placed in the supine position on the heated ultrasound stage (16); it was anesthetized with 2% isoflurane (17) and maintained at 36-37°C (18). The ultrasound transducer (19) was moved slightly inferior from its usual imaging window in order to visualize the needle (20), which was oriented 20-30° from the stage.
The sickle-shaped point style was selected as its shape was expected to help contain the injected bolus within the beating myocardial wall; its parameters were: small RN-style hub, 30 gauge, 12.7mm length, point style #2. The intended injection target was the mid-anterior/septal wall of the heart, as it showed the least motion on the sonogram. A sub-sternal, sub-diaphragmatic entry point was established as this route would allow for the least soft tissue penetration while also avoiding the ribcage. The transducer was oriented parallel to the needle to be able to visualize its angle and trajectory, and the mouse was rotated 5-10° clockwise along the sagittal axis to obtain a longitudinal view of the heart. For the initial penetration of the substernum/diaphragm, the needle was oriented at a 20-30° angle from the mouse stage, and the transducer was moved 5-10mm inferior to guide the needle (Figure 2.5c-d), which was then positioned into the field of view (Figure 2.6a). This angle was chosen as it was more perpendicular to the mouse and would require less force on the needle to penetrate the skin. Using real-time image guidance, the needle was advanced into the thorax of the mouse, and once there, its trajectory was re-oriented towards the anterior/septal wall (Figure 2.6b). The needle was then carefully advanced into the myocardium, with some manipulation of the transducer to ensure the needle tip was within the heart wall. Once this was confirmed, the 10μL bolus was injected very slowly to prevent leakage into the ventricle or thorax. As a real-time confirmation of successful implant, the injected volume contained 10% MicroMarker non-targeted microbubble contrast agent (VisualSonics), which was expected to be visible as a bright, hyperechoic bolus on the sonogram. The transducer was manipulated by hand during the injection to ensure there was no leakage; if any was detected, the needle tip was adjusted accordingly. After injection, the needle was retracted and the mouse was recovered. For Sca-1+ CSC implants, immediately following FACS, CSCs were pelleted at 530×g and resuspended in growth medium + 10% MicroMarker at a concentration of 1×10^4 cells/10μL. The cell suspension was warmed to 37°C to reduce temperature-induced injury to the recipient mice, and was implanted as described above.
Figure 2.6   **Needle positioning under ultrasound guidance.** a) The ultrasound transducer was adjusted to visualize the needle tip (1), which was placed at a 10-20° angle from the mouse stage and brought into the ultrasound field of view. For initial penetration into the thorax, an entry point directly under the sternum (2) was selected, with the needle’s trajectory (*dotted line*) aimed posterior to the heart. The needle was then carefully advanced into the thoracic cavity through the substernum and the diaphragm. b) The view was adjusted and the trajectory of the needle was then changed so that it aimed towards its intended target, the mid-lateral/septal wall of the heart (3). Note the bevel of the needle, visible in a), which was intended to help keep the bolus within the myocardial wall, and the bright reverberation artifacts present below the needle. Bar = 4mm.

### 2.9 Statistical Analysis

To determine optimal sample sizes, power analysis was conducted according to Charan and Kantharia\(^{161}\). Briefly, an effect size of at least 15% was chosen to be considered significant for each of the parameters of systolic cardiac function. Given previous work in mice\(^{138}\), a standard deviation of 12% was used. As such, the minimum number of mice required to detect this difference, via imaging, at the 95% confidence level with a power of 0.8 is 5 per group.

Statistical analyses were performed using IBM SPSS Statistics 22 (Armonk, NY) and plotted using GraphPad Prism 5.03 (San Diego, CA). The accuracies of M-mode and 3DE respectively were determined by Bland-Altman analysis in order to calculate agreement with and bias against micro-CT; cardiac function measurements in all groups were pooled and plotted together as percent difference between echocardiography/micro-CT measurements vs. average echocardiography/micro-CT measurement. To determine user variability within measurements made by M-mode and 3DE, test-retest was
performed by taking three scans for each mouse, at each time point, with each method as
described previously in order to simulate different imaging sessions. The data were
analyzed using two-way random-effects intra-class correlation analysis; this was
performed to assess absolute agreement between measurements. Significant ($p < 0.05$)
intra-class correlation coefficients (ICC) $\geq 0.80$ were considered to indicate good
agreement. To evaluate the progression of cardiomyopathy in $mdx:utrn^{-/-}$ mice from 3DE
images, a two-way mixed-design multivariate analysis of variance (ANOVA) with
Tukey’s post-hoc test was used to determine significant differences ($p < 0.05$) between
measurements in $mdx:utrn^{-/-}$ mice and wild-type controls at baseline (6-8 weeks) and
endpoint (15-17 weeks). For histological analyses, a two-way ANOVA followed by
Tukey’s post-hoc test was conducted to determine significant differences ($p < 0.05$) in
fibrosis and cardiomyocyte size between baseline and endpoint as well as between wild
type and $mdx:utrn^{-/-}$ mice.
3 Results

3.1 Assessment of Cardiac Function in \textit{mdx:utrn}^{-/-} Mice via 3D Echocardiography

The first goal was to show that a three-dimensional echocardiography (3DE) method was not only more accurate than the widely-employed motion-mode (M-mode) echocardiography standard, but also that it was less variable in terms of its measurements. As a control, gated cardiac micro-CT was used as a gold standard of non-invasive imaging. After its optimization, the ability of 3DE to detect changes in cardiac function in severely-affected \textit{mdx:utrn}^{-/-} mice was assessed$^\text{ii}$.

3.1.1 Accuracy of 3DE and M-mode

One mouse died before its 15-17 week CT scan, and as such, was excluded from this analysis, resulting in a final sample of \( n = 24 \). As illustrated in Figure 3.1, Bland-Altman analysis revealed that M-mode echocardiographic measurements of end-diastolic volume (EDV; Figure 3.1a), end-systolic volume (ESV; Figure 3.1c), stroke volume (SV; Figure 3.1e) and ejection fraction (EF; Figure 3.1g) showed considerable bias (overestimation of EDV, ESV, and SV, underestimation of EF) and wide confidence intervals. In contrast, far less bias and tighter confidence intervals were found for 3DE measurements of the same parameters (Figure 3.1b, d, f, h).

Figure 3.1  Accuracy of M-mode and 3DE. Bland-Altman plots of functional values measured via echocardiography vs. micro-CT for each mouse (n = 24). a,c,e,g) M-mode; b,d,f,h) 3DE. Abscissa: mean of echocardiography and CT measurements; ordinate: % difference between the two; black line: bias; large dotted lines: 95% confidence intervals; small dotted line: zero reference. Bias is denoted as % ± SD.
3.1.2 Variability of 3DE and M-mode

The next goal was to examine the variability present in M-mode and 3DE echocardiographic measurements, and determine if 3DE measurements showed less bias. For this purpose, repeated measurements were carried out with either modality as described above and intra-class correlation analysis was performed to determine absolute agreement between the three measurements taken within the same mouse. The results of the analysis are presented in Figure 3.2. Evidently, values obtained through M-mode had poor agreement (ICC < 0.80), illustrated by large differences of individual scan measurements from their mean, whereas 3DE values showed very good agreement (ICC ≥ 0.80), with smaller differences of individual measurements from their mean. Note also that M-mode measurements showed more variability in mdx:utrn⁻/⁻ mice than in wild-type mice.
Figure 3.2  Variability of M-mode and 3DE. Intra-class correlation plots illustrating variability in measurements obtained via a,c,e,g) M-mode and b,d,f,h) 3DE. Each mouse (abscissa) was scanned three times; all the values obtained are presented as % difference between each measurement and the mean within each mouse (ordinate). Dotted line: zero reference. Significant ($p < 0.05$) intra-class correlation coefficients (ICC) are noted.
3.1.3 Evaluation of Ventricular Function in \textit{mdx:utrn}⁻/⁻ Mice

After its accuracy and precision were established to be higher than M-mode, 3DE was used to assess changes in cardiac function in \textit{mdx:utrn}⁻/⁻ mice. The findings in the present study confirm those recently reported by Chun et al.\textsuperscript{38}, namely that \textit{mdx:utrn}⁻/⁻ mice develop dilated cardiomyopathy as early as 15 weeks, as detected by M-mode. Specifically, in this study, both traditional M-mode and 3DE measurements of EDV, ESV, SV and EF in healthy C57BL/6 mice relative to \textit{mdx:utrn}⁻/⁻ dystrophic mice were directly compared to those obtained with micro-CT; the pooled mean values for each group, along with standard deviations, are summarized in Table 3.1. Note that n = 4 for aged micro-CT \textit{mdx:utrn}⁻/⁻ mice as one mouse died before its scheduled 15-17 week CT scan and was thus excluded from statistical analyses. Of particular interest is the higher standard deviation of measurements acquired by M-mode echocardiography than by either 3DE or micro-CT. It is for this reason that further statistical analyses were conducted on 3DE alone; these results can be found in Figure 3.3.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6-8 Weeks</th>
<th>15-17 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 8)</td>
<td>\textit{mdx:utrn}⁻/⁻ (n = 5)</td>
</tr>
<tr>
<td></td>
<td>M-mode</td>
<td>3DE</td>
</tr>
<tr>
<td>EDV (mm(^3))</td>
<td>63.20 ± 15.04</td>
<td>44.12 ± 10.37\textsuperscript{†}</td>
</tr>
<tr>
<td>ESV (mm(^3))</td>
<td>26.68 ± 10.42</td>
<td>17.95 ± 6.95</td>
</tr>
<tr>
<td>SV (mm(^3))</td>
<td>36.52 ± 6.15</td>
<td>26.16 ± 6.14</td>
</tr>
<tr>
<td>EF (%)</td>
<td>59.09 ± 7.76</td>
<td>59.58 ± 8.74</td>
</tr>
</tbody>
</table>

EDV = end-diastolic volume; ESV = end-systolic volume; SV = stroke volume; EF = ejection fraction.
All values are expressed as the mean ± standard deviation.
\textsuperscript{*}significant increase (p < 0.05) within group; \textsuperscript{†}significant increase (p < 0.05) between groups; \textsuperscript{‡}significant increase (p < 0.005) within group and also between groups at the same time point; \textsuperscript{§}significant decrease (p < 0.005) within group
Note that for micro-CT measurements, n = 4 for \textit{mdx:utrn}⁻/⁻ mice as one mouse died before its scheduled 15-17 week scan and as such was excluded from analyses

Table 3.1 Summary of M-mode, 3DE, and micro-CT measurements of cardiac functional parameters.
Figure 3.3  **Progression of cardiomyopathy in mdx:utrn⁻/⁻ mice.** Cardiac functional parameters were measured in wild-type and mdx:utrn⁻/⁻ mice using 3DE and micro-CT. Abscissa: time point; ordinate: mean measurement ± SD. Values measured through micro-CT are shown for reference only. According to 3DE measurements, EDV and ESV were significantly higher between 6-8 weeks and 15-17 weeks in both wild-type and mdx:utrn⁻/⁻ mice (*p < 0.05). EDV was significantly higher in wild-type mice at both time points (**p < 0.05). Moreover, healthy mice had a significantly higher SV (†p < 0.005) at 15-17 weeks compared to baseline. These mice also had a significantly higher SV (†p < 0.005) than aged mdx:utrn⁻/⁻ mice. The 15-17 week old mdx:utrn⁻/⁻ mice also showed a significantly decreased EF (†p < 0.005) compared to baseline.

Upon analysis of 3DE measurements, EDV and ESV were found to be significantly increased (average EDV by 40.32%, average ESV by 54.47%, p < 0.05) by 15-17 weeks in both wild-type and mdx:utrn⁻/⁻ mice. Furthermore, overall, EDV was found to be significantly higher (37.41%, p < 0.05) in wild-type mice than in mdx:utrn⁻/⁻ mice; this is likely correlated to the increased size and weight of wild-type animals compared to dystrophic mice. More importantly, a significant interaction (p < 0.05) was found between age and disease state for SV and EF. Post-hoc analysis revealed that healthy mice had a significantly higher SV (47.68%, p < 0.005) at the 15-17 week time point...
compared to the 6-8 week time point. These mice also had a significantly higher SV (58.81%, \( p < 0.005 \)) than \textit{mdx:utrn\( ^{-/-} \)} mice of the same age. No other significant differences in SV were found. This indicates that stroke volume in \textit{mdx:utrn\( ^{-/-} \)} mice does not increase as the mice age, compared to healthy mice where the SV does increase. The post-hoc analysis also revealed a significant decrease in EF (22.70%, \( p < 0.005 \)) for 15-17 week old \textit{mdx:utrn\( ^{-/-} \)} mice as compared to 6-8 week old \textit{mdx:utrn\( ^{-/-} \)} mice. No other groups were significantly different. This shows that as \textit{mdx:utrn\( ^{-/-} \)} mice age, they experience a decrease in ejection fraction, while this is not observed in wild-type mice.

3.1.4 Fibrosis and Cardiomyocyte Hypertrophy in \textit{mdx:utrn\( ^{-/-} \)} Mice

Lastly, cardiomyopathy was confirmed histologically. While control mice showed little presence of fibrosis at either time point (0.01% and 0.9%, respectively), \textit{mdx:utrn\( ^{-/-} \)} mice displayed an increase in fibrotic tissue from 1.1% of total area to 2.5% over the course of the study \( (p < 0.05; \text{Figure 3.4}) \).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fibrosis.png}
\caption{Fibrosis in the \textit{mdx:utrn\( ^{-/-} \)} myocardium. a) Quantification of collagen staining is presented as % fibrosis of total myocardium + SEM. Aged \textit{mdx:utrn\( ^{-/-} \)} mice showed significantly higher fibrosis than all other groups \( (*p < 0.05) \). b) Masson’s trichrome staining in wild-type (WT) and \textit{mdx:utrn\( ^{-/-} \)} heart tissue at baseline and termination. Collagen is indicated by areas stained in blue (arrows). Bar = 50\( \mu \text{m} \).
}
\end{figure}

Furthermore, the average cardiomyocyte area was significantly different between young and aged mice (17.05% increase in size by 15-17 weeks, \( p < 0.01; \text{Figure 3.5a}) \), and \textit{mdx:utrn\( ^{-/-} \)} mice showed a significantly higher cardiomyocyte area than wild-type mice at
both time points (21.23%, \( p < 0.005 \)), indicating the presence of cardiomyocyte hypertrophy (Figure 3.5b).

![Figure 3.5](image)

**Figure 3.5** Hypertrophy in the \textit{mdx:utrn}^{-/-} myocardium. a) Average cardiomyocyte area is presented in \( \mu m^2 \). Both mice showed significantly increased cardiomyocyte size at 15-17 weeks of age, and \textit{mdx:utrn}^{-/-} mice showed larger cells than wild-type mice. \(^{†}\)significant difference between age groups (\( p < 0.01 \)); \(^{‡}\)significant difference between \textit{mdx:utrn}^{-/-} and wild-type mice (\( p < 0.005 \)). b) Hematoxylin and eosin staining of cardiac tissue. Note the enlarged cardiomyocytes in the aged \textit{mdx:utrn}^{-/-} mouse. Bar = 50\( \mu m \).

### 3.2 Characterization of Sca-1\(^+\) Cardiac Stem Cells

The next goal was to isolate and culture a population of Sca-1\(^+\) CSCs from healthy donor mice via fluorescence-activated cell sorting (FACS), and subsequently characterize them \textit{in vitro} in order to determine their growth and differentiation capacity.

#### 3.2.1 Cardiac Marker Expression in Sca-1\(^+\) CSCs Post-Differentiation

On average, FACS yield was \( 4 \times 10^4 \) Sca-1\(^+\) cells, from 4-5 hearts, per sort. Upon plating, the CSCs took around 3-5 days in culture to adhere to the bottom of the growth chamber (Figure 3.6a). Cells reached confluency by 2-3 weeks (Figure 3.6b); at this point, the cells were placed in low serum, dimethyl sulfoxide (DMSO)-based differentiation medium and observed. No substantial differences in shape or viability were initially seen. However, cell growth decreased noticeably, and after a few days, their morphology began to change, with some cells appearing to take on a rod-shaped cytoplasm (Figure 3.6c). This trend continued as the cells progressed through day 10 post-differentiation (Figure 3.6d).
Figure 3.6  **CSC microscopy.** Representative phase contrast micrographs of live cardiac stem cells (CSCs) in culture. Images were obtained at 20× magnification. a) Early-stage adherent CSCs (4 days in culture). b) Upon contact with one another, the cells grew more readily and their morphology became more heterogeneous. Confluency was reached around 2-3 weeks in culture. c) CSCs at 4 days post-differentiation. Although the morphology was still heterogeneous, the cells began to form extended, rod-like shapes (*arrows*). d) After 10 days in differentiation medium, more cells took on the elongated shape. Bar = 50μm.

Fluorescence immunocytochemistry was then performed. At day 4, differentiating CSCs began to show signs of αMHC and cTnT expression, albeit in an unorganized and diffuse manner (Figure 3.7a,c). Cx43 was also expressed to some extent just outside of the nucleus, but also within some of the appendages extending from the cells (Figure 3.7e). By day 10, as differentiation progressed, the proteins seemed to take on a more organized appearance; αMHC was found localized in long cells and appendages (Figure 3.7b,f), and while Cx43 was still expressed outside the nucleus, it was found to organize along the cellular membrane. Overall, about 5-10% of cells expressed cardiac markers by day 10.
Figure 3.7  Cardiac marker expression in differentiating CSCs. Fluorescence immunocytochemistry analysis shows the expression patterns of connexin 43 (Cx43), myosin heavy chain alpha (αMHC), and cardiac troponin T (cTnT) in cardiac stem cells (CSCs) at a,c,e) day 4, and b,d,f) day 10 post-differentiation. Markers are indicated on each micrograph in colour; nuclei of day 4 cells were stained with Hoechst. At day 4, some cells begin to show diffuse expression of αMHC (a) and cTnT (c), while Cx43 starts to appear in some of the cellular appendages (e, arrowheads). However, by day 10, cells show evidence of Cx43 expression along the cellular membrane (arrowheads), indicating possible gap junction formation. Some organization of αMHC is evident in the cellular appendages (arrows). Bar = 10 μm.
3.3 Closed-Chest Injections into the Left Ventricular Myocardium In Vivo

In order to make a cell implant study feasible, an effective method of delivering the cells to the myocardium was needed. To this end, a minimally-invasive closed-chest, ultrasound-guided injection method was implemented. Pilot studies were conducted in order to develop the basis of the technique that would ultimately be used to deliver stem cells to the \( mdx:utrn^{+/−} \) left ventricular myocardium.

3.3.1 Accuracy, Effectiveness, and Safeness of Ultrasound-Guided Injection

To determine the effectiveness of method at delivering injectables to the target site, healthy C57BL/6 mice (\( n = 3 \)) were injected with 10\( \mu \)L of a saline solution containing 0.1% bromophenol blue dye and MicroMarker. Representative real-time ultrasound images depicting the injection can be found in Figure 3.8. Furthermore, to ease interpretation of the images and provide a truly detailed illustration, a video clip depicting the injection is provided in Supplementary Material 3.1. The microbubble contrast agent evidently helped confirm the accurate injection of the bolus in real-time and also served to alert the sonographer of any leakage into the ventricle or thorax. After the needle was retracted from the myocardium, the bolus remained within the myocardium, appearing as a bright accumulation on the sonogram.
Figure 3.8  **Real-time confirmation of ultrasound-guided injection.** Representative images of ultrasound guidance process and the use of MicroMarker microbubble contrast agent to confirm injection in real-time. The site of injection is indicated with a *blue arrow*. a) The needle tip (*blue arrowhead*) was aimed towards its target, the mid-anterior/septal myocardium; b) once aligned, it was carefully advanced into the heart wall. c) The bolus was then slowly injected; it appeared as a bright mass around the needle tip on the sonogram. d) After injection, the needle was retracted from the myocardium. The bolus is still visible within the beating heart wall. The ventricle remained dark, indicating no leakage of the solution into the circulating blood. Bar = 2mm. For a better illustration of the injection, please see Supplementary Material 3.1.

Immediately after the injection was confirmed in real time, the mice were euthanized under anaesthesia and their chest cavities were carefully opened. Visual inspection revealed no substantial acute trauma to the liver, lungs, or diaphragm as a result of the injections (data not shown). The hearts were then excised and examined for accumulation of blue dye. As is evident from Figure 3.9, all three implants were successful, targeted the mid-anterior/septal wall, and lacked any major indication of dye leakage from the myocardium.
Figure 3.9 Ultrasound-guided test injections of dye into the left ventricular myocardium. 10μL of a 0.1% bromophenol blue solution was injected into the left ventricular myocardia of three mice under ultrasound guidance. Hearts were subsequently excised and analyzed for the presence of dye. a) The heart excised from Mouse A shows clear evidence of a successful injection into the heart wall (arrowhead). The point of entry of the needle is also evident in the pericardium (arrow). The heart chambers are denoted (RA: right atrium; RV: right ventricle; LA: left atrium; LV: left ventricle). b) While an entry point is visible in the heart from Mouse B (arrow), no distinct accumulation of bromophenol blue is evident; c) however, when the heart was cut transversely, the bolus became visible (arrowhead) in both the apical and basal halves. d) The heart from Mouse C shows both a clear entry point (arrow) and a streak of dye (arrowheads), possibly indicating a needle track; e) upon transverse sectioning, the bolus (arrowhead) indicates the injection correctly targeted the mid-anterior/septal wall. No substantial signs of acute leakage are present in any of the hearts. Bar = 1mm.
To ensure animals were not adversely affected by the injection procedure proper, healthy 7-week-old C57BL/6 mice (n = 3) were injected with 10% MicroMarker in saline. Mice A and B received a single 10μL bolus at a single location within the heart wall, while Mouse C received four consecutive injections of 10μL each at different locations of the heart. The mice were monitored and weighed prior to the injections, the day after, and subsequently every other day for two weeks post-injection to assess physiological effects. The results are presented in Figure 3.10. A very slight drop in weight occurred in all three mice at Day 1. However, while mouse weights fluctuated from day-to-day, no substantial, continuous drops in weight were evident. In fact, all of the mice grew in size and weight by the end of the two week window. Surprisingly, Mouse C, which received four injections, showed the greatest increase in weight from baseline (2.7g). Apart from weight, the mice appeared healthy and active even on Day 1 post-injection, despite the small drop in mass. No signs of lethargy, piloerection, lack of appetite, or lack of thirst were observed.

![Graph showing weight gain for mice A, B, and C over 15 days post-injection.](image)

**Figure 3.10  Effect of closed-chest injection on mouse thrivability.** Three healthy mice were injected with contrast solution in order to gauge whether the ultrasound-guided injection procedure was harmful to them. The mice were weighed and monitored every other day for two weeks post-injection. Their weights are presented above, which each mouse represented by a separate line. Abscissa: day of weighing; ordinate: weight. As is evident from the graph, while the weights of the mice fluctuated to some degree, all three mice consistently gained weight as they grew and thrived post-injection. Mouse C, which received four injections, showed the greatest weight gain from baseline.
3.4 Effects of CSC Implant on Systolic Function in \textit{mdx:utrn}^{/-} Mice

The ultimate goal of this project was to determine what effects, if any, transplanted cardiac stem cells (CSCs) may have on cardiac function. For this purpose, the methodologies described in the previous sections were jointly used.

3.4.1 Cardiac Function in Response to Sca-1\(^{+}\) CSCs

A pilot study was conducted to shed light on the possible effects of Sca-1\(^{+}\) CSC treatment on systolic function in dystrophic mice. The cells were FACS-isolated as before, and were implanted into \textit{mdx:utrn}^{/-} mice via ultrasound-guided injection. 3DE was then used to assess cardiac function. The optimal control for this type of experiment would have been sham-injected mice; however, in the current situation, assessing such a treatment was not a possibility. While great effort was put forth to obtain a sample size of at least \(n = 5\) for each of the cell-injected and sham-injected groups, due to breeding difficulties, small litter sizes, and the inherent fragility of these animals leading to premature death, only 2 \textit{mdx:utrn}^{/-} mice successfully completed this study. Both were assigned to the CSC treatment group. As such, to determine any possible therapeutic effects derived from stem cell treatment, the measurements obtained from implanted mice were directly compared to those from the cohort of untreated \textit{mdx:utrn}^{/-} mice used in the 3DE/micro-CT study (4.1.2). Despite not receiving sham injections, it was reasoned that these mice would be the best available control for the current early-stage study.

The \textit{mdx:utrn}^{/-} mice in the treatment group (\(n = 2\)) each received a cell dose of \(1 \times 10^4\) CSCs. This therapeutic dosage is in the low range of cell numbers administered relative to previous studies\(^{50,75,162–166}\), a smaller dosage was chosen based on the yield of CSCs from FACS isolation, and because it was expected to be less harsh on the fragile dystrophic mice. Treated Mouse A received cell injection at 7 weeks of age, while Mouse B received injection at 4 weeks of age. Baseline 3DE scans were performed immediately prior to cell injection while endpoint scans were performed at least 5 weeks post-implant, as this is considered the minimum time required for stem cells to have a regenerative effect\(^{93}\). Endpoint was ultimately determined by the onset of substantial skeletal/smooth
muscle deterioration, manifested as lethargy and breathing difficulty. In Mouse A, this occurred at 16 weeks, whereas in Mouse B, it occurred at 9 weeks. The results from both of these mice are presented against untreated controls in Figure 3.11. No statistical analysis could be performed on the data due to the small sample size, and as such, no definitive effects can be inferred. Nevertheless, certain trends can be observed from the data. In Mouse A, which was larger in size and lived to an older age than Mouse B, EDV increased by endpoint much in the same way it did for controls. However, while ESV showed a substantial increase in untreated mice, the change was not as drastic in Mouse A. This translated to an increase in SV by endpoint, which led to smaller changes in ejection fraction compared to controls. On the other hand, the smaller Mouse B showed little change in both EDV and ESV compared to untreated animals. This meant that SV changed very little by endpoint. As such, ejection fraction in this mouse again did not change as drastically as it did in the controls.

Figure 3.11 Preliminary systolic function data from CSC-treated dystrophic mice. Cardiac stem cells (CSCs) were implanted into *mdx:utrn*^-/-^ mice and cardiac function was monitored before implant and at least 5 weeks post-implant. Treated mice (n = 2) are respectively shown in gray and black. By endpoint, treated Mouse A exhibited a smaller increase in ESV compared to controls, which ultimately translated to an increased SV and only slightly decreased EF by endpoint. Mouse B showed little change EDV, ESV, and SV, and as such, EF did not drastically decrease as it did in the controls. The high degree of variability in the two mice can be explained by their varied size and weight.
4 Discussion and Future Directions

Duchenne muscular dystrophy (DMD) is a serious genetic neuromuscular disease which has a devastating impact on many young patients. Although clinical intervention has allowed the management of the skeletal muscle and diaphragmatic manifestations of DMD, treatments targeting the characteristic dilated cardiomyopathy have been unsuccessful, and no cure exists. This dissertation sought to investigate a novel subtype of stem cell antigen 1 (Sca-1)-expressing cardiac stem cells (CSCs), and determine what regenerative effects they have in a mouse model of DMD. Briefly, the main objectives were: 1) to validate and implement a three-dimensional echocardiography (3DE) method to assess cardiac systolic function in the \( \text{mdx:utrn}^{-/-} \) double-knockout model of DMD; 2) to isolate a population of Sca-1\(^+\) CSCs from healthy mice and investigate their potential to differentiate down the cardiomyocyte lineage; 3) to implement a closed-chest ultrasound-guided injection method of delivering therapeutic agents directly to the injured myocardium and corroborate previous evidence indicating its usefulness; and 4) after accomplishing the aforementioned goals, to implant Sca-1\(^+\) CSCs into the heart walls of young \( \text{mdx:utrn}^{-/-} \) mice and examine any changes that may have occurred in terms of cardiac function.

4.1 Assessment of Cardiac Function in \( \text{mdx:utrn}^{-/-} \) Mice via 3D Echocardiography

In this study, the ability of non-invasive, prospectively-gated 3DE to quantitatively assess the development of dilated cardiomyopathy in the \( \text{mdx:utrn}^{-/-} \) mouse model of DMD, and its accuracy in doing so, were investigated. This was proposed as an alternative to the current standard echocardiographic method of assessing cardiac function in mice, which involves taking motion mode (M-mode) images and applying the Teichholz correction formula to linear measurements; this practice may be problematic because of the inherent and necessary assumptions for heart shape, which may lead to inaccuracies in the data.

4.1.1 3DE is More Accurate and Less Variable than M-mode

The results of this study support the validity of 3DE as a reliable method of assessing cardiomyopathy in \( \text{mdx:utrn}^{-/-} \) mice and as a quick and cost-effective alternative to M-
mode. Compared with a gold-standard method, retrospectively gated cardiac micro-computed tomography (micro-CT), 3DE showed less bias and greater agreement with micro-CT measurements, suggesting it is more accurate than M-mode. It also showed less variability between multiple measurements than did the M-Mode method, which may translate to less user variability. The high reproducibility and small variability offered by 3DE over M-mode echocardiography will allow the use of the method for longitudinal studies, in which highly accurate measurements of cardiac function can be easily taken at multiple time points throughout the life of the dystrophic mouse.

The present study is the first of its kind to correlate cardiac volumes and ventricular function in dilated cardiomyopathy acquired using 3DE with those acquired using another 3D imaging technology, namely, retrospectively gated micro-CT. Although 3DE necessitates more time and user input for scanning and analysis (around 30 minutes total time per scan, 15 minutes for volume segmentation), it proves to be more accurate than traditional M-mode echocardiography and circumvents the use of radiation and contrast agents, which can be potentially dangerous to the fragile mdx:utrn\(^{-/-}\) mice and stem cells. 3DE also requires less training on the part of the researcher, and it shares the cost-effectiveness of M-mode while providing a truly volumetric method of evaluation.

4.1.2 Systolic Function is Reduced in mdx:utrn\(^{-/-}\) Mice

Once the validity of 3DE was determined, it was then used to assess cardiac function and the development of dilated cardiomyopathy in the mdx:utrn\(^{-/-}\) mouse model of DMD. Although the data show that both healthy and dystrophic mice exhibit larger end-diastolic and end-systolic volumes as they age (further evident in Figure 2.4), healthy mice have a significantly higher stroke volume at 15-17 weeks of age, which translates into a larger volume of blood pumped out per heartbeat. This is to be expected, because as the mice age, the heart will have to pump a higher volume of blood around the body. In contrast, mdx:utrn\(^{-/-}\) dystrophic mice exhibited no increase in stroke volume as they age. This ultimately translated to a significantly decreased ejection fraction compared to 6-8-week-old mdx:utrn\(^{-/-}\) mice, indicating that cardiac function in these mice was greatly hindered over time. These findings corresponded to histological analysis of cardiac tissue from both healthy and dystrophic mice, wherein Masson’s trichrome staining revealed
significant fibrosis in hearts of 15-17-week-old \textit{mdx:utrn}^{−/−} mice as compared to all other groups. Indeed, it is also evident from Figure 3.4 that young \textit{mdx:utrn}^{−/−} mice exhibit roughly the same degree of fibrosis as aged controls. These results further corroborate the idea that the double-knockout model provides an apt representation of the fibrotic progression present in DMD\textsuperscript{40}. Furthermore, although both mouse groups exhibited increased cardiomyocyte size by 15-17 weeks, the dystrophic mice had significantly larger cardiomyocytes compared to controls at both time points, illustrating the hypertrophy and increased muscle mass characteristic of their phenotype\textsuperscript{59}. Together, these observations support the notion that the \textit{mdx:utrn}^{−/−} mouse develops a clinically relevant form of cardiomyopathy in the later stages of its life. Because cardiomyopathy arises much earlier in these mice than in the ubiquitous \textit{mdx} model, they are much more valuable to cardiac function studies, and as such, are a reliable model of DMD and dilated cardiomyopathy.

Understanding the cardiomyopathy process first in pre-clinical small animal models of DMD, and subsequently in young patients before the development of overt cardiomyopathy, may later provide us with unique opportunities for the early detection of heart disease in other patient populations—for instance, in idiopathic populations, wherein the development of dilated cardiomyopathy is not certain. Many groups now report the use of echocardiography to measure changes in cardiac output/ventricular function in mice; nonetheless, most of these studies assess such changes in models of ischemic cardiomyopathy\textsuperscript{123,134,167}. To date, DMD animal models, including the \textit{mdx} and \textit{mdx:utrn}^{−/−} mouse models, are some of the only models of dilated, non-ischemic cardiomyopathy\textsuperscript{44}; hence, there is a great need for the characterization of disease development/progression in these mice. This study suggests that the use of 3DE over standard M-mode will allow for increased sensitivity to detect subtle early changes in the development of dilated cardiomyopathy that occur before gross changes are evident. Moreover, 3DE will be useful to accurately assess the effects of treatments in mice.

\textbf{4.2 Characterization of Sca-1\textsuperscript{+} Cardiac Stem Cells}

After establishing the accuracy and utility of 3DE in determining small changes in systolic function, the effects of CSCs on DMD-related cardiomyopathy were sought to be
investigated. For this purpose, a population of Sca-1$^+$ CSCs needed to be characterized; these cells were expected to successfully grow and differentiate \textit{in vitro}, expressing protein markers typical of cardiomyocytes.

4.2.1 Isolation of Sca-1$^+$ CSCs

While it is also a marker of multipotent hematopoietic stem cells in mice, the phosphatidylinositol-anchored protein Sca-1 has been used to enrich for stem and progenitor cell populations in other non-hematopoietic tissues\textsuperscript{168}. In this study, fluorescence-activated cell sorting (FACS) was used to purify a population of CSCs that expressed Sca-1. The isolated Sca-1$^+$ CSC population was negative for other endothelial and hematopoietic progenitor cell markers, postulating the notion of a purified CSC subtype\textsuperscript{109}. Overall, cell yield was around $4 \times 10^4$ per sort, obtained from 4-5 mice. This was not unexpected, as the population is relatively rare under normal conditions in mice\textsuperscript{105}, and so is the orthologous Sca-1$^+$ CSC population in humans\textsuperscript{112}. As a point of comparison, the remainder of non-Sca-1$^+$ cells counted during the sorts was generally in the order of $2 \times 10^6$ cells. This was likely composed of cardiac myocytes, cardiac myoblasts, and other subtypes of CSCs. Thus, the Sca-1$^+$ population represented about 2\% of the total cells, which is on par with previous reports\textsuperscript{106,107,109,111}.

\textit{In vitro} primary cardiac myocyte/myoblast culture has been a very fruitful undertaking in modern labs as it complements mouse models of cardiovascular disease; it is particularly useful for studying changes in CSCs at the molecular level, especially in ion homeostasis and excitation-contraction coupling\textsuperscript{109,169}. Although cells have been sourced from both humans and animals of varying ages, most groups study neonatal cardiomyocytes largely derived from rats\textsuperscript{170–173}. The isolation process generally involves excising the heart from euthanized animals, digesting the connective tissue by a combination of physical and enzymatic means, performing a crude enrichment for cardiomyocytes with different growth media, and finally propagating and studying the cells\textsuperscript{86,106,110,155,174}. However, neonatal cardiomyocytes lack many structural and functional characteristics of adult cardiomyocytes, and disease models are much more readily available in mice, which may make them more appealing than rats as a source for primary cardiomyocytes\textsuperscript{169}. In this study, the process by which adult cardiac stem cells of murine origin were obtained is
superior to more commonly used methods; stringent selection based on an array of surface markers allows for the isolation of specific populations with high purity, without the need for timely media cycling.

As the Sca-1+ population studied here was very specific and requires high purity, the FACS approach was ideal. However, the main compromise and disadvantage to this method was that cell yield was relatively low compared to what would have been obtained with other, less-specific isolation procedures. This has important connotations when considering the stage at which CSCs should be implanted: freshly sorted, or propagated in culture. Indeed, there is much debate on this topic, and the protocols of various labs differ on this issue7,107,110,163–165. Although the optimal number of CSCs required to elicit an effect is still uncertain7, one may not be implanting sufficient numbers of cells if using the freshly-sorted approach. Future studies should investigate and determine at which of the two stages Sca-1+ CSCs exhibit the greatest degree of functional improvement post-implant.

4.2.2 Evidence of Cardiac Marker Expression in Sca-1+ CSCs Post-Differentiation

After isolation, the Sca-1+ CSCs were seeded onto tissue culture plates, to which the cells noticeably adhered after 3-5 days; this was again to be expected, as primary cardiac stem cells are documented to require upwards of 12-24 hours to become adherent171. They were then successfully expanded in culture, reaching confluency after 2-3 weeks. Their growth rate in culture increased with cell density, which is on par with previous reports of CSC culture98. After reaching confluency, the cells were cultured in differentiation medium containing dimethyl sulfoxide (DMSO) for up to 10 days.

At days 4 and 10 post-differentiation, representative samples of cells were probed for three proteins: myosin heavy chain alpha (αMHC), one of six subunits in the myosin contractile complex within the cardiomyocyte sarcomere; cardiac troponin T (cTnT), one of three proteins in the troponin complex responsible for regulating contraction; and connexin 43 (Cx43), the main gap junction protein in ventricular myocardium, which ultimately allows for intercellular communication and the transmission of action
potentials. Differentiating CSCs began to show expression of all three markers as early as day 4. However, their expression patterns were unorganized and diffuse—all proteins were found largely unstructured in the cytoplasm. This may indicate that, at this stage of differentiation, the cells were just beginning to commit to the cardiac lineage and beginning to express the proteins, which were observed in an immature state. Some Cx43 expression was found within cellular appendages, which may indicate it is being transported to its final location, the cellular membrane. This is further supported by cells probed at day 10, which show expression of Cx43 organized along the membrane. Although expression is relatively low, it could point to the formation of early gap junctions. Interestingly, Cx43 was found to be localized around the nucleus in all of the cells tested. It was proposed that this is indicative of Cx43 being transcribed and processed within the endoplasmic reticulum; indeed, other groups probing stem cells with the same antibody used here have observed similar results. Moreover, at day 10, αMHC began to take on a more organized appearance, appearing to congregate within the appendages observed in differentiating cells. This further supports the notion that the appendages may later produce troponin- and myosin-rich striations.

Taken together, the results provided here support the hypothesis that cardiac stem cells can be isolated from adult murine heart. Nevertheless, at the two stages of differentiation analyzed, all markers appeared relatively unorganized and immature. Even so, only around 5-10% of total cells expressed cardiac markers by day 10, and no beating phenotype was observed. This low efficiency of differentiation may have come as a result of day 10 being too early along the timeline of differentiation for these cells. Moreover, the in vitro differentiation system used here inherently does not capture the subtle nuances of paracrine signalling that are characteristic of in vivo differentiation. DMSO was chosen as a differentiation agent due to its DNA-demethylating characteristics and ease of use; this agent is widely reported to unmask seldom-expressed genes and promote differentiation in embryonic stem cells (ESCs) and mouse embryocarcinoma cells, where it produces a cardiomyocyte-like beating phenotype and expression of cardiac markers, including GATA4 and Nkx-2.5. However, DMSO-mediated differentiation of cells into cardiomyocytes is known to be relatively inefficient. A simple alternative is the use of 5-azacytidine (5-aza), another demethylation agent. It
has also been widely documented to induce differentiation in both ESCs and CSCs, being highly efficient at producing beating cardiomyocytes\textsuperscript{77,107,112,179,182}. Nevertheless, the use of such an agent is still not an accurate reflection of \textit{in vivo} paracrine signalling. Future studies focusing on emulating this type of environment would be very interesting, and may help produce a truly accurate representation of these cells’ cardiomyogenic potential.

Previous reports have indicated that adult ventricular cardiomyocytes are resistant to cell cycle re-entry due to their lack of telomerase activity; for this reason, it has been suggested that these adult cells do not play a large role in repair during cardiac tissue injury\textsuperscript{187}. Sca-1\textsuperscript{+} CSCs are a potential source of regeneration during injury, but their ability for endogenous cardiac repair is largely unknown. Thus, in the present study, a population of Sca-1\textsuperscript{+} CSCs was isolated and evidenced to differentiate along a cardiac-specific lineage.

4.3 Closed-Chest Injections into the Left Ventricular Myocardium \textit{In Vivo}

After characterizing the Sca-1\textsuperscript{+} CSCs, the next goal of this dissertation was to implement a closed-chest method of implant to deliver them to the \textit{mdx:utrn} \textsuperscript{-/-} myocardia. For this purpose, test studies were carried out using a minimally-invasive, ultrasound-guided needle technique to deliver therapeutic agents directly into the live left ventricular myocardium of mice. This method circumvents the need for complicated thoracotomy procedures, while also being a timely and highly repeatable procedure well-suited for fragile \textit{mdx:utrn} \textsuperscript{-/-} mice. The method was expected to be accurate at targeting regions in the left ventricular myocardium and effective at maintaining the injected bolus there.

4.3.1 Ultrasound-Guided Injection is Accurate, Effective, and Safe

Test injections were carried out using healthy mice as recipients of the injections. These mice were chosen due to their being readily available and more able to withstand possible complications that may have arisen from the injection process. Real-time analysis of the sonogram allowed for the confirmation of the injection into the myocardium by way of bright contrast visualization. This highlighted the two main advantages of this technique: firstly, that visualization of the needle within the thoracic cavity allows one to target
specific regions of the myocardium for injection; and secondly, that usage of hyperechoic contrast helps to assess the injection of the bolus within the myocardium, immediately alerting the sonographer to any leakage into the ventricle or into the thoracic cavity. As a control, very early tests showed that deliberate injection directly into the ventricle caused the blood pool to immediately appear bright on the sonogram, even with minuscule amounts of contrast agents. The ability of this technique to show leakage into the ventricle will be key to troubleshoot future experiments in real-time. This is impossible using classic thoracotomy, as the person delivering the injection is somewhat blind to the depth of the needle tip once it is in the myocardium, and the thickness of the heart wall (1-2mm) makes it easy to over- or under-shoot. Furthermore, the only way to determine the occurrence of leakage in this case is post-mortem.

After injections were confirmed in real-time, mice were sacrificed and their hearts excised. In all three hearts examined, the bolus was clearly contained within the myocardial wall as indicated by localized concentration of bromophenol blue dye, and showed no signs of leakage on either facet of the myocardial wall. If leakage had indeed occurred, clear blue areas would have been visible. Moreover, examination of the bolus location provided definitive evidence that the technique was accurate in targeting the injection to the middle of the anterior-septal myocardium, providing proof-of-concept for ultrasound targeting.

A study using healthy mice was performed to fully ensure the guided injection itself produced no substantial harm to the animals. The method again proved exceptional in this respect, with young mice gaining healthy weight over the two weeks following implant. It came as a surprise that Mouse C, which received a total of four injections, had the greatest degree of weight gain; this is very promising for future studies wherein cells may need to be delivered to multiple regions of the myocardium. Indeed, these results were to be expected, as this method is far less invasive than common thoracotomy procedures.

The next logical step for future validation studies is to perform test implants using cells: firstly, to determine whether they are detectable histologically post-implant, and based on this, to determine the degree of cell retention within the myocardium at various time
points post-implant. For these studies, the cells should be labelled with a fluorescent marker (e.g. DiI), or should express some type of reporter [(e.g. green fluorescent protein (GFP)], which will allow their subsequent detection by immunohistological analysis. Implanted cells are also expected to be located as a concentrated amalgamation of nuclei within the tissue, while healthy cardiomyocytes should be readily distinguishable from the host tissue via their expression of dystrophin. Moreover, as is evident in Figure 3.9, the injection method kept the bolus tightly concentrated within the myocardial wall. As such, to avoid superfluous tissue sectioning in the future, the bolus should contain some type of visual marker (e.g. coloured dye or microbeads), which is safe for the mouse in the long term, and will also allow for gross localization of the injectate during embedding and sectioning. This will greatly help in orienting the histologist to the site of the injection, and will ensure that this region is aptly probed for the presence of cells. Another interesting strategy is to engineer the cells to express a protein that sequesters a certain substrate. Subsequently, a given time after injection, one may inject the substrate systemically and perform biodistribution analysis to evaluate the relative quantities of cells within the injected site, and perhaps determine how much of the bolus was lost from the target. This may also be applicable in future stem cell studies to determine the amount of neo-angiogenesis and migration ability of cells.

These results show great promise for the ultrasound-guided injection method. However, as with any procedure, this method is not without its limitations. There will be an inherent learning curve for any untrained sonographer starting to use the method, and moreover, a steady hand is required to maneuver the needle using the injection arm. Scientists wishing to implement this technique in their research should take due time to train themselves in using it prior to any attempting any experiments. Furthermore, one of the key players in the success seen here was the sickle-shaped needle. Preliminary injections were performed using regularly-bevelled needles, which were found to easily leak excessive injectate into the ventricle as a result of piercing through the endocardium. The advantages of the special tip are thus clear, but come at the compromise of cost. Despite these limitations, overall, the results presented here are on-par with findings from the only other group reported to perform intra-myocardial (IMC) injections under ultrasound guidance in mice\textsuperscript{43,145,146}, which is testament to the strengths of the
methodology. Through the use of a higher-resolution ultrasound scanner, specialized needle, and a preclinical contrast agent, the method used here presents advances that will greatly benefit future therapeutic experiments.

4.4 Effects of CSC therapy on Systolic Function in mdx:utrn⁻/⁻ Mice

Once the ultrasound-guided injection method was shown to be successful at delivering and maintaining a bolus locally within the myocardium in vivo, it was deemed suitable for use in preliminary Sca-1⁺ CSC studies with mdx:utrn⁻/⁻ mice. Importantly, this procedure was not only expected to produce accurate delivery of the stem cells, but also to be less strenuous on the fragile mdx:utrn⁻/⁻ mice. These animals were expected to show a smaller decrease in systolic functions than untreated controls.

4.4.1 Preliminary Evidence of Functional Improvement

Despite the small sample size, the results presented here indicate functional improvement in the CSC-treated mdx:utrn⁻/⁻ mice, as evidenced by 3DE. Specifically, while EDV and ESV increased in both groups by endpoint, the increase in CSC-treated mice was less severe than in untreated controls. This may be indicative of the treatment ameliorating the dilated phenotype in these animals. SV also showed an increase in Treated Mouse A by endpoint, which is reflective of the situation in healthy animals. Perhaps most intriguingly, neither of the treated mice showed the substantial decline in EF characteristic of the mdx:utrn⁻/⁻ strain and exhibited by the controls. These results may indicate that Sca-1⁺ CSC treatment potentially slowed the progression of dilated cardiomyopathy in these animals.

Nevertheless, as this was a very preliminary study, certain limitations have to be considered. Firstly, the sample size obtained for the treatment group was insufficient, meaning true statistical analysis was not possible. Furthermore, the optimal negative control for cell injection would have been a sham-treated group, which was not possible for the aforementioned reason. Ideally, and with due time, sufficient animals will be acquired for both groups, and the data obtained from them will be analyzed using a two-way mixed-design multivariate analysis of variance with Tukey’s post-hoc test.
Furthermore, as is evident from Figure 3.11, the two treated animals differed greatly in the measured parameters. This came as a result of their differing sizes and weights: Treated Mouse A was larger and heavier than Treated Mouse B. In the future, once optimal sample sizes are obtained, variability between the animals should be accounted for and statistically removed from the data. One way to achieve this is through analysis of covariance (ANCOVA), wherein mouse weight will be considered a confounding variable and its effects on the measurements removed. This may further help to elucidate small but significant differences in the data that would otherwise go unnoticed due to variability. In addition, as mentioned previously, Treated Mouse A was scanned at an endpoint of 9 weeks post-injection, whereas Treated Mouse B was scanned at 5 weeks post-injection due to substantial weight loss. This supports the notion that the disease can affect the animals differently based on their anatomy and physiology. Here, Mouse B exhibited more severe kyphosis than Mouse A; as kyphosis is characteristic of this mouse strain, it can serve as a rough indicator of disease progression and diaphragm function. As is the case for patients, mdx:utrn\textsuperscript{-/-} mice largely die as a result of severe respiratory dysfunction, and perhaps this was the case for Mouse B. In future experiments, three defined time points will be used to assess function: 5 weeks (baseline), 10 weeks (intermediate), and 15 weeks (endpoint). This will provide a more robust basis for statistical analysis and will allow for easier exclusion of mice that die prematurely.

Another aspect to consider in such a study is the number of cells implanted. As mentioned previously, the cell dosage delivered here is relatively low compared to previous reports\textsuperscript{50,75,107,162–166,188} due to the Sca-1\textsuperscript{+} CSC yield, and also to cells being implanted immediately following isolation. It can be argued that the number of cells implanted here may have been too low to elicit an optimal response. Previous work showed that stem cells have a low retention once inside target tissues\textsuperscript{74,165}; this may come as a result of any number of factors, including cell death due to shear stress from the needle, death due to injection-induced inflammation in the target site, and bolus leakage. Thus, only a fraction of the total number of implanted cells may have been truly able to elicit a regenerative effect. As discussed in 4.2.1, there is still debate in the field regarding whether to implant freshly-sorted cells or cells propagated in culture. Given a high enough sample size, future studies should investigate both of these possibilities, as
the regenerative capacity of CSCs may differ depending on preparation. Once the minimum number of implanted cells detectable by histology is confirmed, it will provide a better indication as to the number of Sca-1+ CSCs that should be implanted into dystrophic mice for therapeutic studies.

With this in mind, immunohistochemical analysis should also be performed in future experiments to determine the presence of implanted cells, the degree of tissue regeneration, the expression of cardiac markers, as well as any changes in fibrosis and hypertrophy. Furthermore, the mechanism of Sca-1+/mediated regeneration should be determined. Stem cells are generally known to migrate poorly post-implant into muscle, and as such it is theorized that the direct effect of cell transplantation is local. Evidence also shows that endogenous Sca-1+ CSCs may have a reduced rate of regeneration in diseased states, but that autologous, transplanted Sca-1+ cells stimulate endogenous CSCs and also promote neovascularization. The current study did not investigate whether the observed improvement was due to myocardial regeneration, paracrine stimulation, or neovascularization; as such, future work should also determine the paracrine or pro-angiogenic effects that Sca-1+ may have in mdx:utrn−/− myocardia. These analyses will ultimately elucidate the true effect that these cells are having within the injured mouse myocardium.

Ultimately, although statistical inference could not be incurred using the current data, it presents promising results that provide impetus to continue exploring Sca-1+ CSC therapy in future studies. As of yet, only a single study has been published investigating any stem cell regenerative therapy for cardiomyopathy in mdx:utrn−/− mice; Chun et al. describe improvements in systolic function and expression of dystrophin as a result of aorta-derived mesoangioblast treatment. These findings are very encouraging and have important potential for future therapeutic application. However, as with any disease, it should be approached from multiple angles, and autologous Sca-1+ CSCs may provide certain advantages over non-cardiac-derived stem cells, which should be explored further.
4.5 Future Directions

Importantly, the current study helped to produce valuable tools that will be indispensable to pre-clinical DMD-related cardiomyopathy research, and also the delivery of treatment in such studies. It would now be of particular interest to employ 3DE in assessing the progression of cardiomyopathy in utrophin-heterozygous mice (mdx:utrn+/−). Due to their genotype, they may prove to be a more accurate representation of DMD-related cardiomyopathy than mdx mice, while also being more resilient than severely-affected mdx:utrn−/− mice. Aside from 3D imaging, the Vevo 2100 system also allows for high-quality Colour Doppler imaging, which can be used to assess blood flow directionality in vessels and through valves—this may have the potential to determine the presence of mitral valve regurgitation in mdx and mdx:utrn−/− mice, a phenomenon which is observed in DMD patients25. One can also perform regional strain analysis using the Vevo system to determine regional myocardial dysfunction121; this may elucidate valuable information on regional fibrosis in these mice, which can help in targeting CSC treatment to the most severely-affected areas of the heart. Indeed, echocardiography is actively used in the clinic with DMD patients37, and this encourages further studies in mice using this methodology.

Previous reports using nuclear medicine techniques in DMD patients have also uncovered a reduction in myocardial perfusion, along with an increase in glucose metabolism within cardiac muscle35,36. This was speculated to arise as a result of ischemia, one of the main hallmarks of DMD40. In order to further elucidate changes in myocardial ischemia in mdx:utrn−/− mice, it would be interesting to gauge these two functional parameters. Previous work by our group has shown that non-invasive imaging using dynamic contrast-enhanced computed tomography (DCE-CT) and micro-positron emission tomography (μPET) can reliably detect changes in perfusion and glucose uptake/inflammation in the skeletal muscle of mdx:utrn−/− mice15. Specifically, while initial increases in both parameters were observed at 8 weeks of age, both significantly decreased by 20 weeks compared to baseline and age-matched controls. Importantly, these changes were reported to occur prior to a significant reduction in muscle contractile function, and thus may predict end-stage DMD. As such, assessing myocardial perfusion
and glucose metabolism non-invasively may also provide an important insight into the mechanism of cardiomyopathy within mdx: utrn−/− mice, and to date, no studies have explored this. For this purpose, myocardial perfusion will be assessed by DCE-CT in mdx: utrn−/− mice, while myocardial metabolism and/or inflammation will be determined by μPET imaging of the uptake of a radiolabelled glucose analog. Furthermore, if these methods prove to be feasible, it would also be interesting to investigate these functional parameters in cell-treated mice to ascertain the local growth and regenerative capacity of the transplanted CSCs, and whether they have an anti-ischemic effect.

Furthermore, prospective studies may investigate the effects of co-administration of certain factors with CSCs. For instance, a previous study has shown that c-Kit cells can be stimulated to regenerate myocardium by treatment with insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), and others indicate that co-transplant of CSCs with circulatory angiogenic stem cells may have a greater combined therapeutic effect, as they act synergistically: one regenerates myocardium while the other regenerates the vasculature. These are intriguing ideas, since this kind of therapy may be able to target DMD-related ischemia, inflammation, and fibrosis concomitantly, and so far no studies have investigated Sca-1+ cells in this context.

4.6 Conclusions and Significance

In summary, this dissertation reported that 3DE correlates well with cardiac micro-CT in the analysis of cardiac volume and cardiac output/ventricular function in mdx: utrn−/− mice and, importantly, provides greater accuracy with less user bias/variability than standard M-mode echocardiography. 3DE was then used to demonstrate the progression of cardiomyopathy in mdx: utrn−/− mice. Subsequently, the ability of purified cardiac stem cells to differentiate into cardiomyocytes in vitro was shown, as indicated by the expression of cardiac-specific markers. The accuracy and attractiveness of closed-chest ultrasound-guided injections were then demonstrated, and preliminary evidence of Sca-1+ CSC-mediated improvement of cardiac function was presented. These studies were conducted in the order presented above and all built on each other; when used together, they proved to be an indispensable set of tools for assessing cardiac function in, and delivering treatment to, the dystrophic mouse heart.
Although it is still largely in its infancy, regenerative stem cell therapy has the potential to change the clinical landscape and significantly improve treatment outcome for patients struggling with DMD and other cardiovascular diseases. However, before this can occur, the exact processes behind their differentiation and mechanism of repair need to be understood. Nevertheless, cell replacement therapy presents an appealing and sustainable alternative to drug administration, and in the future, may curb the need for heart replacement in patients with end-stage heart failure. Furthermore, it may prove to be an excellent candidate for DMD patients, and may be the missing piece of the puzzle in providing truly effective therapy for DMD-related cardiomyopathy. It is our hope that stem cell therapy will abolish the inefficient treatments currently standard in clinics, while greatly improving DMD survivability and quality of life.
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Please confirm in writing or by email that these arrangements meet with your approval.

Sincerely,
Andrew Bondoc

MSc Candidate 2014, BMSc 2012
Western University Canada

Graduate Research Assistant
Appendix B. Ethics Approval for the Use of Animal Subjects

-------- Original Message --------
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2008-067::5
Date: 2014-10-20 15:23
From: AUSPC
To: "Lisa Hoffman"

2008-067::5:
AUP NUMBER: 2008-067
AUP TITLE: Non-Invasive Imaging of Therapeutics in Mouse Models of DMD

YEARY RENEWAL DATE: 12/01/2013

THE YEARLY RENEWAL TO ANIMAL USE PROTOCOL (AUP) 2008-067 HAS BEEN APPROVED, AND WILL BE APPROVED FOR ONE YEAR FOLLOWING THE ABOVE REVIEW DATE.

* This AUP number must be indicated when ordering animals for this project.
* Animals for other projects may not be ordered under this AUP number.
* Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
**Curriculum Vitae**

**NAME**

Bogdan Andrei Bondoc

**EDUCATION**

09/2012 – 12/2014 **Candidate, Master of Science (M.Sc.)**
Department of Medical Biophysics
Western University, London, Ontario, Canada
Thesis: “Stem cell treatment for Duchenne muscular dystrophy-related cardiomyopathy”
Supervisor: Lisa Hoffman, Ph.D.

09/2008 – 04/2012 **Honors Bachelor of Medical Science (B.M.Sc.)**
Department of Microbiology & Immunology
University of Western Ontario, London, Ontario, Canada
Honors Thesis: “Molecularly characterizing the expression of a myocardial transgenic reporter gene in a novel murine line”

**AWARDS, HONOURS, AND DISTINCTIONS**

06/2014 **Poster Prize, 2nd Place ($50)**
20th Annual Canadian Connective Tissue Conference, London, ON
“Imaging cardiac stem cells to treat Duchenne muscular dystrophy-related cardiomyopathy”

03/2014 **Poster Prize, 3rd Place ($100)**
Ontario Consortium in Imaging for Cardiovascular Therapeutics
12th Annual Imaging Network Ontario Symposium, Toronto, ON
“Application of 3D echocardiography and gated micro-CT to assess cardiomyopathy in a Duchenne muscular dystrophy mouse model”

09/2013 – 12/2014 **Strategic Training Fellowship in Vascular Research ($16,000)**
Canadian Institutes of Health Research

09/2012 – 08/2014 **Western Graduate Research Scholarship ($9000)**
Western University, London, Ontario, Canada

03/2013 **Certificate of Completion in Career Development**
Western University, London, Ontario, Canada

12/2012 **Certificate of Academic Engagement**
Western University, London, Ontario, Canada
04/2012  Dean’s Honor List  
University of Western Ontario, London, Ontario, Canada

09/2008  Undergraduate Scholarship of Distinction ($1500)  
University of Western Ontario, London, Ontario, Canada

09/2008  Entrance Scholarship ($2000; declined)  
Guelph University, Guelph, Ontario, Canada

09/2008  Entrance Scholarship ($2000; declined)  
York University, Toronto, Ontario, Canada

PROFESSIONAL EXPERIENCE

09/2012 – 12/2014  Graduate Research Assistant  
Imaging Program  
Lawson Health Research Institute, London, Ontario, Canada

07/2012 – 08/2012  Summer Research Assistant  
Imaging Program  
Lawson Health Research Institute, London, Ontario, Canada

06/2011 – 08/2011  Student Laboratory Assistant  
Public Health Laboratory  
Public Health Ontario, Toronto, Ontario, Canada

06/2010 – 08/2010  Internal Communications Junior Coordinator  
Assistant Deputy Minister’s Office  
Ministry of Health and Long-Term Care, Toronto, Ontario, Canada

VOLUNTEER AND EXTRA-CURRICULAR EXPERIENCE

01/2013 – 04/2014  Graduate Student Representative  
Academic Administrative Committee  
London Health Research Day, London, Ontario, Canada

09/2012  Teaching Assistant Training Program  
Teaching Support Services  
Western University, London, Ontario, Canada

01/2006 – 10/2006  Volunteer, CT Patient Services  
Diagnostic Imaging, Peel Memorial Hospital, Brampton, Ontario, Canada
PEER-REVIEWED PUBLICATIONS

Published or In Press


In Preparation


Conference Proceedings


PEER-REVIEWED PRESENTATIONS

Podium


Poster


9. Bondoc BA, Moazami H, and Hoffman LM. 2012. Use of a luciferase reporter transgenic murine line to target cardiac stem cells following implantation into mouse


PUBLISHED ABSTRACTS


