The Role of Cardiac-Resident Mesenchymal Progenitors in the Development of Fibrosis in the Heart

by

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Abstract

Fibrosis is a response to injury that involves cell proliferation and deposition of extracellular matrix; pathological fibrosis can be considered to be imperfect wound healing that impairs organ function. Our understanding of the role and source of cardiac fibroblasts (CFs) in the development of pathological fibrosis is severely hampered by a lack of robust in vivo markers and understanding of the heterogeneity within this vaguely defined population. Here we demonstrate the existence of cardiac-resident mesenchymal progenitor cells (cMSCs, identified as CD31-:CD45-:PDGFRα+:Sca-1+) and show that their in vivo pharmacological modulation with the receptor tyrosine kinase inhibitors nilotinib or imatinib reduces fibrosis and improves cardiac function following acute or chronic cardiac injury. We observed in vivo the initial expansion of Sca-1+ cMSCs following acute cardiac injury contributes to mature collagen-producing PDGFRα+:Sca-1- CFs. In two models of acute injury, isoproterenol treatment and ligation of the left anterior descending artery (LAD), treatment with nilotinib led to reduced proliferation of both cMSCs and CFs but an increased relative prevalence of cMSCs, suggesting a blockade in the fibrogenic differentiation of these cells. Extending these observations into mdx mice, a model of chronic myocardial dystrophy displaying cardiac fibrosis after one year of age, we investigated the effects of long-term treatment with imatinib as a potential pharmacologic therapy. mdx mice treated with imatinib for 15 months displayed improved cardiac function and reduced cardiac fibrosis. These improvements were matched by a reduced quantity of cMSCs within treated myocardial tissue. Finally, given the well-known role of PDGFRα+ Sca-1+ mesenchymal progenitors in the development of fibrofatty infiltration in skeletal muscle, we postulated a role for phenotypically similar cMSCs in the fibrofatty infiltrate observed in
arrhythmogenic cardiomyopathy. Using a genetic model that dysregulates quiescence in mesenchymal populations, we observed hyperproliferation of cMSCs and spontaneous generation of adipose deposits within the myocardium, supporting our hypothesis that cMSCs are the cellular source of the fibrofatty infiltrate. In their entirety, our findings demonstrate the importance of differing subsets of mesenchymal cell populations within the heart and potential therapeutic benefit of targeting them pharmacologically following acute ischemic damage or during chronic dystrophic injury.
Preface

Chapter 2 is modified from a manuscript which is being prepared for publication: Paylor B, Lemos D, Low M, Soliman H, Lee C, Fiore D, Macleod K, Rossi M. 2014. Nilotinib attenuates the development of cardiac fibrosis by inhibiting the fibrogenic differentiation of cardiac-resident mesenchymal progenitors. My contributions include: 1) developing the concept, 2) performing the majority of experiments and 3) writing the entire manuscript.

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List of Abbreviations

AC – Arrhythmogenic cardiomyopathy
ARVC – Arrhythmogenic right ventricular cardiomyopathy
bHLH – Box helix-loop-helix
BMP2 – Bone morphogenic protein two
BSA – Bovine serum albumin
CD – Cluster of differentiation
CF – Cardiac fibroblast
CFU-f – Colony forming unit fibroblast
cMSCs – Cardiac-resident mesenchymal progenitor cells
CPC – Cardiac progenitor cell
CTGF – Connective tissue growth factor, an extracellular-matrix associated protein
cVSMC – Coronary vascular smooth muscle cells
DDR – Domain discoidin receptor, a tyrosine kinase receptor that binds collagen as its ligand and is involved in numerous disease including cancer and fibrosis
DMD – Duchenne muscular dystrophy
DMEM – Dulbecco’s modified eagle medium
DPI – Days post infarction
ECM – Extracellular matrix
EDV – End diastolic volume
EF – Ejection fraction
EMT – Epithelial-to-mesenchymal transition
EndMT – Endothelial-to-mesenchymal transition
FS – Fractional shortening
FSP1 – Fibroblast specific protein one, a calcium binding protein that has classically been used to identify fibroblasts in various tissues
GFP – Green fluorescent protein
GMP – Good manufacturing practice
IHC – Immunohistochemistry
iPSC – Induced pluripotent stem cell
ISCT – International Society for Cellular Therapy
LAD – Left anterior descending artery
LV – Left ventricle
MI – Myocardial infarction
miR / miRNA – Micro ribonucleic acid
MMP – Matrix metalloproteinase
MPI – Myocardial performance index
MSC – Mesenchymal stromal cell
NGS – Normal goat serum
OCT – Optimal cutting temperature formulation
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PDGFRα – Platelet-derived growth factor receptor alpha, a cell surface tyrosine kinase receptor that is known to act as a mitogen for cells of mesenchymal origin
PFA – Paraformaldehyde
RTK – Receptor tyrosine kinase

Sca-1 – Stem cell antigen one, a commonly used stem cell marker first used to identify hematopoietic stem cells in mice

SM – Skeletal muscle

TAC – Transaortic constriction

Tbx – T-box transcription factor

TGF-β1 – Transforming growth factor beta one, a secreted pro-fibrotic protein involved in many cell functions including proliferation, differentiation and apoptosis

WT – Wild-type

αSMA – Alpha smooth muscle actin, a highly conserved protein involved in cell motility, structure and integrity
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Dedication

Dedicated to all of the mice that were sacrificed to complete this thesis.
Chapter 1: Introduction

1.1 Role of fibrous connective tissue in the heart

The heart’s extracellular matrix is a highly regulated and dynamic component that can become discomposed in situations of disease (Bowers et al., 2010). Similar to the loosely defined cardiac fibroblasts and myofibroblasts that regulate it, the extracellular matrix is increasingly recognized to play an important role in pathologies outside of just providing organ structure. The ECM scaffold – primarily composed of fibrillar collagens and elastins; adhesive proteins such as laminin and fibronectin, antiadhesive proteins including tenascin and thrombospondin; factors regulating intercellular signaling such as proteoglycans; and enzymes involved in the remodeling of the ECM such as matrix metalloproteinases (MMPs) (Espir and Czubryt, 2009) – facilitates efficient force transduction for the mechanical functions of the heart while also being a vital component of intercellular communication and metabolic processes. In situations of injury, such as an acute ischemic event, a highly choreographed fibrogenic process occurs that is typically divided into three overlapping phases: 1) early injury response that involves infiltration of inflammatory cells; 2) proliferative phase involving expansion of fibroblasts and their differentiation into collagen-secreting myofibroblasts as well as expansion of endothelial cells leading to the formation of new vascular tissue through angiogenesis; and finally, 3) maturation phase in which continued collagen deposition and cross-linking leads to an effective scar (Li et al., 2014). This entire process can take up to 1 month in rodents and over 2 months in larger mammals (Dobaczewski et al., 2010). Although by definition fibrosis is framed as a deleterious consequence of excessive deposition of ECM, the development and maturation of a functional
scar after ischemia is a crucial process that, if perturbed, can lead to mortal consequences such as ventricular free wall rupture (Khalil et al., 2001). Several studies (Garcia et al., 2007; Shimazaki et al., 2008) have demonstrated that the timely development of a mature scar following a myocardial infarction (MI) is necessary for survival and that modulation of collagen production using both pharmacological or genetic strategies can worsen mortality. Although a variety of different therapeutic strategies to improve cardiac function by minimizing fibrosis will be described below, it is important to that recognize ECM deposition after ischemic injury is a necessary process, and that the duration and timing of therapeutic treatments needs to be optimized to strike a balance between sufficient and excessive collagen production following interventions.

1.2 What is a cardiac fibroblast?

Cardiac fibroblasts (CFs) are defined as a population of mesenchymal cells residing in the interstitium of the heart that govern the dynamic equilibrium of its extracellular matrix (ECM). First described in the 1970s and 1980s in seminal studies by Zak and Nag (Nag, 1980; Zak, 1974), CFs were found to be the most abundant population of cells within the rodent myocardium (Banerjee et al., 2007). Although their role was initially described to be primarily involved in the regulation of the ECM, they have also been ascribed important roles in other electrical, chemical and biomechanical functions of the heart. Their principal role has often been thought to be the synthesis and deposition of fibrillar type I collagen and other ECM components, although these functions have typically not been used to identify CFs due to the historical difficulty in identifying such secretions. Instead, their definition has often been based
on their morphological and spatial characteristics, as well as a growing list of caveat-laden phenotypic markers that are not exclusive to CFs, such as vimentin, thymus cell antigen-1 (Thy-1), discoidin domain receptor-2 (DDR2) and fibroblast-specific protein-1 (FSP1/S1A004) (Krenning et al., 2010). CFs typically possess a flat shape with multiple spindle processes emerging from the cell body and, unlike all other cells in the heart, lack a basement membrane. Like many cell populations and fibroblasts from other tissues, the notion that CFs are a homogenous population of cells has largely been discredited as evidence of their heterogeneity has emerged. Still, a clear understanding of the various underlying cellular subsets that compose the classical definition of a CF has not been formed, and continues to be an area of intense study and therapeutic importance. A growing body of evidence supports the notion that the definition of a CF includes a population of cardiac-resident mesenchymal progenitors (described using International Society for Cellular Therapy [ISCT] terminology as cardiac “mesenchymal stromal cells” or cMSCs) and that this population plays a highly important role in physiological and pathological processes in the heart. The scope of this review will outline the current evidence regarding the identification of cMSCs and their therapeutic relevance in treating cardiac fibrosis.

1.2.1 Myofibroblast: questionably distinct

Dogma of cardiac pathology dictates that cardiac fibroblasts are activated to expand after myocardial ischemia and that this population subsequently differentiates into more mature “myofibroblasts” that are the causative cellular agent in the over-production of collagen and eventual manifestation of tissue fibrosis in the heart (Weber et al., 2013). This phenotypically transformed population is classically defined by the expression of alpha-smooth muscle actin
(αSMA) microfilaments and can be functionally identified by their hypersecretion of a variety of other protein markers including (but not limited to) periostin; collagen I and III; and fibronectin and its specialized ED-A isoform (Serini et al., 1998; Tomasek et al., 2002). In concert with inflammatory cells that infiltrate the myocardium, myofibroblasts are responsible for the coordination of a healing response that results in the eventual formation of scar tissue (Davis and Molkentin, 2014). The notion that myofibroblasts represent a distinct cell population that results from the differentiation of CFs has been questioned based on our lack of understanding of the cellular identity of CFs (Camelliti et al., 2005). Further, the classical experimental definition of myofibroblasts based on the presence of alpha-smooth muscle actin microfilaments does not necessitate it as differentiated progeny but may rather simply represent a distinctive phenotype in response to differing mechanical and environmental parameters (Camelliti et al., 2005).

1.3 Heterogeneity of mesenchymal cells in different tissues

Based on early cellular definitions, it was once thought that the bone marrow contained the primary reservoir of mesenchymal stromal cells in mammals (Phinney and Sensebe, 2013). This has largely changed in recent years as numerous cell populations possessing a high degree of similarity with BM-derived MSCs have been derived from numerous adult tissues, including adipose, heart, lung, kidney tissue, skeletal muscle and skin (Crisan et al., 2008; Pelekanos et al., 2012; Strioga et al., 2012). Identified both using numerous markers such as Sca-1, PDGFRα, Tie2 and collagen-1, and using isolation strategies such as their ability to efflux Hoechst dyes (Motohashi et al., 2008), it has been shown that these populations fit the classical definitions of a MSC and, further, were the mediators of fibrotic processes in numerous tissues. Stemming from
this involvement in pathological fibrosis, it has been demonstrated that pharmacological or genetic modulation of their behavior could lead to improved functional outcomes in situations of disease. In skeletal muscle, building on previous work describing the presence of populations of accessory cells that supported the regenerative capacity of satellite cells (Uezumi et al., 2006), our lab (Joe et al., 2010) and others (Uezumi et al., 2010) described the presence of a tissue-resident population of mesenchymal progenitors that possess both pro-regenerative and pro-fibrotic roles depending on the situation of disease (Joe et al., 2010). These initial studies have since been expanded upon to describe in detail how mesenchymal progenitors play important roles in regulating muscle regeneration following acute injury and, when this regeneration fails, are the cellular source of excess collagen and other ECM-proteins that become overly abundant in situations of tissue fibrosis (Uezumi et al., 2011). Similar roles for cell populations identified by Sca-1+ PDGFRα+ have also been found in the skin (Driskell et al., 2013; Schmidt and Horsley, 2013), where these tissue-resident fibroblasts/MSCs were the key mediator of fibrotic processes and resolution of injury.

1.4 Identification of cardiac mesenchymal stromal cells (cMSCs)

Since the first descriptions of stem cells in the adult mammalian heart (Beltrami et al., 2003; Oh et al., 2003), much effort has been invested in unraveling heterogeneity within this postulated cardiac cell population. A wide variety of identification methods have been used to identify and purify stem cells from the heart, including phenotypic identity (e.g. Sca-1 (Oh et al., 2003), ckit (Beltrami et al., 2003)), functional parameters (e.g ability to exclude Hoechst 33342 dyes (Hierlihy et al., 2002; Martin et al., 2004) and/or cell growth characteristics (e.g. ability to
generate spheres in culture (Messina et al., 2004)). These efforts have provided crucial insights into the underlying cellular biology of the heart, but also produced a highly fragmented field with numerous competing cellular definitions that likely contain considerable overlap (reviewed in Chong et al. 2014, and Garbern and Lee, 2013). A key parameter in assessing the therapeutic potential of these similar populations has been their capacity to generate cardiomyocytes both in vitro or, arguably more importantly, in vivo. Despite a wealth of preclinical data supporting the regenerative potential of cardiac stem cells and a number of clinical trials currently underway based on these data (Bolli et al., 2011; Chugh et al., 2012; Makkar et al., 2012; Malliaras et al., 2014), recent reports have cast doubt on the capacity of cardiac-resident cells to generate de novo myocardial tissue (van Berlo et al., 2014). Given the ascribed importance of a cardiogenic capacity for stem cells in the heart and resulting focus on this aspect of cardiac stem cell biology, it is of no surprise then that it was only recently demonstrated that the heart harbours a population of MSC-like progenitors with little to no capacity for generating cardiomyocytes.

Building on work in a variety of other organs, it was shown in 2011 (Chong et al., 2011) that the combined usage of the markers Sca-1 and PDGFRα allowed for isolation of a cell population which contained the entire “colony forming unit” (CFU) capacity, relying on a classical 1970 definition of a MSC as a CFU fibroblast (CFU-F). Utilizing this definition, Chong et al. (2011) identified a population of cardiac-resident MSC-like stem cells (termed cCFU-Fs) to which they ascribe the capacity to generate all cardiac lineages (cardiomyocyte by co-cultivation with neonatal rat cardiomyocytes, endothelial, and smooth muscle) based on in vitro and in vivo assays (transplantation into infarcted hearts). cCFU-Fs were shown to be highly enriched for the mesenchymal marker PDGFRα, distinct from rare ckit+ cells and not expressing the classical pericytes marker NG2. The role of this population, identified using Sca-1 and PDGFRα and
described as cardiac mesenchymal stromal cells (cMSCs) in this review, has since been much investigated in the context of development, health and disease. *In vitro* studies of cMSCs have revealed a transcriptome and immunophenotype highly congruent with that of better-characterized MSCs derived from bone marrow and kidneys. Despite maintaining unique properties based on their tissue of origin, all three populations were highly similar in most definitive properties of MSCs, including morphology, multipotency, expression of diagnostic MSC surface markers, and gene expression (Pelekanos et al., 2012). cMSCs have also been demonstrated to be highly similar to a more closely related population, the cardiac fibroblast. Building on previous studies describing the phenotypical similarity between MSCs and fibroblasts (Covas et al., 2008; Haniffa et al., 2009; Hematti, 2012), it has recently been shown that cardiac fibroblasts possess a high degree of overlap with cMSCs (Furtado et al., 2014a) and suggested that cMSCs likely represent a majority subset of this population. Taken together, these data provide a strong rationale that the classical definition of a cardiac fibroblast needs to be refined to recognize the numerous similarities with tissue-resident mesenchymal progenitors both in the heart and in other tissues. Normalizing the notion that CFs are largely composed of cMSCs represents a significant opportunity to the field as it would help in aligning research efforts towards a better understanding of how this population can be targeted therapeutically in situations of cardiac disease.

1.4.1 Developmental origins of cMSCs

With developmental origins first explored in the avian system, cardiac fibroblasts are known to be prominently derived from the epicardium (Gittenberger-de Groot et al., 2010; von Gise and
Pu, 2012), with an additional subset possibly arising from the endothelium (Ali et al., 2014; Moore-Morris et al., 2014b). Thus, given the high degree of overlap with cardiac fibroblasts (Furtado et al., 2014a), it is of no surprise that cMSCs were also found to have an epicardial origin and arise through post-embryonic EMT. Lineage tracing of the cells using a variety of Cre drivers demonstrated that cCFU-Fs were labeled by classical epicardial markers Wt1 and Gata5 as well as the mesodermal marker Mesp1, but not by cardiomyocyte markers Nkx2.5 (Chong et al., 2011). Transplantation of GFP+ bone marrow was used to rule out the contribution of circulating cells to cCFU-Fs both before and after injury. More detailed investigations into the importance of epicardial EMT in generating the mesenchymal component of the heart demonstrated that epicardial-specific deletion of PDGFRα led to a severe disruption of cardiac fibroblast development, while formation of coronary vascular smooth muscle was unperturbed (Smith et al., 2011). This developmental defect was later linked to function of the box helix-loop-helix (bHLH) transcription factor Tcf21 which, when deleted, led to similar effects on cardiac fibroblast formation (Acharya et al., 2012). More recently, the importance of cardiac fibroblasts to proper development of the myocardium was investigated by specifically deleting cardiogenic transcription factor T-box transcription factor 5 (Tbx-5) within the mesenchymal compartment using Cre driven by the matricellular protein periostin, one of the more robust markers of CFs. Building on previous work describing a regulatory role of cardiac fibroblasts in influencing the myocardial proliferation of cardiomyocytes during development (Ieda et al., 2009; Noseda and Schneider, 2009), the study – which also found that most fibroblasts express bona fide mesenchymal stem cell markers, further supporting the large degree of overlap between CFs and cMSCs – revealed the importance of cardiac transcription factor expression within the mesenchyme of the heart and found significant disruption to cardiac development.
when these factors were removed (Furtado et al., 2014a). Use of functional markers such as periostin, which has a known role in the maturation and regulation of the extracellular matrix (Snider et al., 2008), to identify and isolate CFs in combination with collagen-1, PDGFRα and Thy-1, have allowed the field to move away from less specific markers including FSP1 (Kong et al., 2013) and DDR2. Taken together, these studies support the hypothesis that within the classically defined population of a cardiac fibroblasts there exists a population of cells playing a crucial role in the development of the heart which also possesses a large number of markers and functions associated with MSCs from other tissues.

1.5 Role of cMSCs in cardiac injury and repair

As our understanding of the identity and development of cMSCs has grown, there has also been an increasing amount of evidence describing their role in injury and repair in the heart. The general importance of cardiac fibroblasts in the development of fibrosis in the heart has been long established, and as the definition of this population has been refined, a newfound clarity over the specific cellular processes involved in this pathological process has also been achieved. Earlier studies have demonstrated the role of epicardially derived cells in cardiac fibrosis (Zhou et al., 2010), and that excess collagen deposition in this pathological state is mediated by proliferation of non-myocyte cells (Teekakirikul et al., 2010), presumably fibroblasts. These outlined two important concepts that needed to be addressed: first, more detailed data delineating the functional role of subpopulations within the cardiac fibroblast definition and their differing roles in disease; and second, further clarification about the cellular origins of collagen-secreting cardiac fibroblasts in situations of injury, a line of research that has been plagued with
conclusions drawn from models with substantial shortcomings. Two recent murine studies (Ali et al., 2014; Moore-Morris et al., 2014a) utilizing a model of pressure overload by transaortic constriction (TAC) provided data to further both of these aims (Bursac, 2014; Moore-Morris et al., 2014b). Utilizing Thy1 (Ali et al., 2014) and Collagen-1 (Moore-Morris et al., 2014a) to identify CFs, both studies found that despite heterogeneity in the developmental origins of CFs, these differing populations demonstrated very similar patterns in proliferation after injury. Both studies also noted that further work in examining other functional parameters and means of subdividing the studied populations were warranted, as well as expanding such lines of inquiry into other models of cardiac injury. There are also potentially differences between the role that fibroblasts play in the development of cardiac fibrosis in acute versus chronic situations of disease. Ieronimakis et al. (2013) examined the differing roles in myodystrophic fibrosis and anatomical locations of PDGFRα+ cardiac fibroblasts expressing Sca-1+. The study denoted Sca-1+ cells as coronary adventitial cells, finding them to be highly involved in age-related cardiac fibrosis in mdx mice, while their Sca-1- counterparts were described as interstitial fibroblasts that were involved to a much lesser degree. These small rodent studies have been corroborated by a patient study that observed the presence of progenitor cells marked by PDGFRα (Sca-1 is not expressed in humans) in the diseased human myocardium as well (Chong et al., 2013). Based on strong evidence from studies in skeletal muscle, it has also been postulated that cMSCs may play a role in other cardiac pathologies, such as the characteristic fibrofatty infiltrate observed in arrhythmogenic cardiomyopathy (Paylor et al., 2013). Numerous scholars have forwarded the notion that our lack of in-depth knowledge of the cellular identity of cells involved in cardiac fibrosis has hampered efforts to develop effective therapeutics to target this pathological process. Indeed there are currently no clinically approved treatments to treat
cardiac fibrosis, and it is expected that as further studies emerge that are directed toward pharmacological or genetic manipulation of cMSCs, novel therapeutic strategies will emerge.

1.5.1 Possible sources of myofibroblasts in cardiac fibrosis – tissue-resident vs. circulating vs. EMT

Although there is general consensus about the developmental source of CFs, there continues to be debate about the cellular source of myofibroblasts in cardiac pathologies. Despite numerous thorough investigations utilizing more stringent cell markers and genetic fate mapping methodologies that refute the notion that cells other than tissue-resident progenitors contribute to this expanded cell population, many reviews of the field continue to state that various alternative sources – including epithelial-to-mesenchymal transition (EMT), endothelial-to-mesenchymal transition (EndMT) and/or circulating cells – are potential sources for cardiac myofibroblasts in situations of injury (Davis and Molkentin, 2014; Deb and Ubil, 2014; Lajiness and Conway, 2014). In light of this, it is important to take note of the wealth of high quality evidence demonstrating that this is not the case. Cardiac fibrosis is manifested as the result of tissue-resident populations expanding and differentiating into collagen-producing myofibroblasts, and it is important that this theoretical concept be normalized in the field of cardiac pathology. Although clinical studies have supported the notion that the majority of collagen-producing cells in the heart are derived from resident fibroblasts after injury (Pichler et al., 2012), it was not until recently that more stringent fate labelling studies have been used to verify this in a rodent model. Earlier studies supporting the notion that myofibroblasts are derived from intracardiac fibroblasts (Yano et al., 2005) were questioned by other data demonstrating that EMT/EndMT and
circulating cells were a significant contributing source (Chu et al., 2010; Falkenham et al., 2013; Rosin et al., 2012; Sopel et al., 2012; Zeisberg et al., 2007). Utilization of cell surface markers (e.g. Tie1 and FSP1 (Zeisberg et al., 2007)) that have been discredited as specific markers of mesenchymal fibroblasts (FSP1 (Kong et al., 2013)) or the endothelium (Tie1) is a longstanding problem in the field. However, more recent studies have improved on these methodological flaws by refining their analyses to use functional markers such as collagen-1 and periostin and found that although a subset of CFs arise from the endothelium during development (Ali et al., 2014), there is little to no contribution of either source to the expansion of cMSCs or to the generation of collagen-producing myofibroblasts in the adult (Chong et al., 2011; Furtado et al., 2014a; Ieronimakis et al., 2013; Moore-Morris et al., 2014a).

1.5.2 Interaction with inflammatory cells

As our understanding of heterogeneity within the mesenchymal subset of the heart has improved, so too has a greater appreciation for the presence of cardiac macrophages and their role in tissue repair in the organ. Although the role of inflammation and its cellular mediators, monocytes and macrophages, in the development of fibrosis have long been recognized (Cieslik et al., 2014), it has only been recently that the kinetics of this relationship have been described and the distinction between circulating and tissue-resident inflammatory cells has been reported. It has recently been demonstrated by two concurrent studies (Epelman et al., 2014; Heidt et al., 2014) that in physiological conditions, cardiac-resident macrophages are primarily derived from local proliferation rather than contributions of circulating monocytes. Further, it was found that after an acute ischemic event such as a myocardial infarction there is a large influx of bone-
marrow derived monocytes that give rise to macrophages. The differing roles of macrophages expanding locally versus infiltrating from circulation was also demonstrated, with the former primarily being involved in antigen sampling and efferocytosis, while the latter was shown to be the principal mediator of inflammation in the myocardium.

1.6 Therapeutic relevance and future prospects

Cardiac fibrosis is involved in almost all pathologies of the heart and leads to deleterious effects on function and increased mortality. With no clinically approved anti-fibrotic therapies currently available, applying our growing understanding of tissue fibrosis to the development of novel therapeutics is a research area of great interest. As our definition of a cardiac fibroblast and its progeny, the myofibroblast, becomes further detailed, so too will the availability of targeted experimental therapies designed to mitigate the excess deposition of ECM (Leask, 2010). With a focus on their role in targeting cMSCs, a variety of different therapeutic strategies to improve cardiac function by targeting the development of fibrosis after ischemic injury will be reviewed here.

1.6.1 Receptor tyrosine kinase inhibitors

With a large amount of evidence supporting a role for PDGF signaling in the progression of fibrosis in numerous tissues (Andrae et al., 2008; Bonner, 2004), the use of non-selective receptor tyrosine kinase inhibitors such as imatinib and nilotinib has been investigated in the context of cardiac fibrosis. Building on evidence supporting its anti-fibrotic effects in numerous
other tissues, imatinib has been demonstrated to reduce fibrosis in a variety of different models of cardiac injury, including uraemic cardiac disease (Baumann et al., 2010), chronic myocarditis (Leipner et al., 2008), and hypertension (Jang et al., 2014; Ma et al., 2012). Issues of timing and dosage are important to consider with this chemotherapeutic agent, however, as cardiotoxicity due to direct effects on cardiomyocytes has been reported (Herman et al., 2011; Kerkela et al., 2006), although the relevance of these rodent studies in a clinical context has been questioned (Breccia, 2011; Wolf et al., 2010). Nilotinib, a second generation RTK inhibitor that is structurally similar to imatinib, has not yet been investigated in the context of cardiac injury but has shown improved efficacy in ameliorating fibrosis over imatinib in a number of other tissues including liver (Shaker et al., 2011a; Shaker et al., 2011b; Shaker et al., 2011c), skin (Akhmetshina et al., 2008) and lung (Rhee et al., 2011). As an increased understanding of the role of cMSCs in the role of cardiac fibrosis emerges, the rationale of targeting this population using semi-selective inhibitors of proteins that are primarily expressed within them is strengthened. The development of more selective pharmacological inhibitors that target pathways involved in cMSCs during the manifestation of cardiac fibrosis thus represents an attractive future avenue for the development of novel therapeutics.

1.6.2 miRNAs

Increasingly, the importance of miRNAs, single stranded 22 nucleotide length molecules, in regulating both physiological and pathological processes is recognized in a wide variety of tissues. A number of studies have demonstrated that miRNAs – namely miR-21, miR-29, miR-30 and miR-133 (Bauersachs, 2010; Creemers and Pinto, 2011) – play an important role in the
development of fibrosis in the heart and, further, that their experimental manipulation can prevent the excess accumulation of collagen in the myocardium and improve organ function. Studies demonstrating the interaction of these miRs with key molecular mediators of fibrosis, including CTGF and TGF-β1, in different murine models of cardiac fibrosis have supported the notion that they represent potential therapeutic targets for the treatment of this pathological process. More recently, several other candidate miR targets have emerged that have been demonstrated to be involved in degenerative processes associated with aging (miR-34a, Boon et al., 2013) as well as cardiac regeneration (miR-590 and miR-199a, Eulalio et al., 2012). Although further work needs to be done to tie the causal influence of miR manipulation to the varying cell populations present in the myocardium – including cardiomyocytes, endothelial cells and cMSCs – as our understanding of the role of miRs in regulating pathological processes grows, so too does the notion that these molecules could be targeted therapeutically.

1.6.3 In vivo reprogramming and trans-differentiation

A final area of interest that has stemmed from insights provided by Dr. Shinya Yamanaka’s landmark description of methods to functionally reprogram somatic cells to an embryonic state has been growing evidence of the ability to reprogram adult cells in vivo (Takahashi and Yamanaka, 2006). Several recent studies have demonstrated that methods utilizing cardiac transcription factors, miRNA and/or small molecules can lead to trans-differentiation of cardiac fibroblasts into cardiomyocytes, raising the intriguing therapeutic possibility of conversion of scar tissue into functional myocardium (Jayawardena et al., 2012; Qian et al., 2012; Song et al., 2012; Wang et al., 2014). Although considerable work remains to be done on understanding if
such experimental strategies can be scaled to work in large mammals, this unique line of inquiry
represents a potentially paradigm shifting means to treat pathological conditions, including
cardiac fibrosis.

1.7 Conclusion

Efforts to develop new therapeutics to prevent and treat cardiac fibrosis are currently hampered
by a lack of thorough understanding of the heterogeneity represented by our classical definition
of a cardiac fibroblast. With numerous recent studies focusing on unraveling this complex
identity, it is important for the field to update standing dogma to reflect our newfound knowledge
regarding the differing cellular identities contained within the heart’s mesenchyme. Importantly,
it is now acknowledged that within much of the population of cells that has been defined as a
cardiac fibroblast is a tissue-resident population of mesenchymal progenitors (cMSCs) that give
rise to collagen-producing progeny following ischemic injury. Further, there currently exists
clear evidence that the cellular source of collagen-producing cells in the heart are tissue-resident
progenitors rather than previously hypothesized circulating or alternate lineage (EMT and
EndMT) sources. Continued efforts to demonstrate the role of these sources utilizing caveat-
laden methodologies that have been largely discredited due to their lack of stringency are a
disservice to the field and potentially a misuse of valuable research resources that could be
invested elsewhere. It can be expected that as our definition of cMSCs is further refined and a
greater understanding of the various subpopulation that this cellular subset contains is gained,
targeted therapies to mitigate the development of cardiac fibrosis following ischemic injury will
result. Given the importance of this pathological process in most cardiac diseases, it is vital to utilize these new research insights to the benefit of patients.

Building on the growing consensus over the presence of cMSCs within the heart, we hypothesize that:

1. Given the large conceptual degree of overlap with cardiac fibroblasts, cMSCs play an important role in the development and maintenance of cardiac fibrosis following acute injury,

2. Genetic abnormalities that lead to the development of cardiac fibrosis, such as chronic dystrophy processes present in patients with Duchenne’s Muscular Dystrophy, activate cMSCs to divide and secrete extracellular matrix proteins such as Type 1 collagen,

3. Developing an understanding of the phenotypic identity of cMSCs will allow for the development of targeted pharmacotherapeutics to modulate their role in the development of cardiac fibrosis, and potentially benefit cardiac function after injury.

Utilizing a series of transgenic murine studies involving a number of different models of cardiac disease, we designed an approach to test these hypotheses.
Chapter 2: Nilotinib attenuates the development of cardiac fibrosis by inhibiting the fibrogenic differentiation of cardiac-resident mesenchymal progenitors

2.1 Introduction

Cardiac fibrosis represents a key component in the majority of cardiac pathologies (Weber, 1997). The adult mammalian myocardium has limited regenerative capacity and the loss of functional cardiomyocytes due to ischemia results in their replacement by a collagen-rich scar (van Nieuwenhoven and Turner, 2013). While the scar itself is essential to retaining the heart’s ability to pump blood, fibrotic infiltrates often extend to the undamaged myocardial wall, with deleterious consequences to cardiac function. Long standing dogma has dictated that the principal cell population involved in the pathological deposition of collagen in the heart is the poorly defined cardiac fibroblast, and a growing body of evidence has implicated this vaguely defined population of mesenchymal cells in a variety of other patho- and physiological processes of the heart, underlying its importance (Souders et al., 2009). However, recent evidence suggests that our definition of a cardiac fibroblast actually encompasses several functionally distinct cellular subsets, including a range of cells spanning from mesenchymal precursors to differentiated collagen-producing myofibroblasts (Krenning et al., 2010).

We, as well as others, have identified a population of tissue-resident multipotent mesenchymal stromal cells (MSCs) in skeletal muscle that is identified by expression of PDGFRα and the stem
cell marker Sca-1 (Joe et al., 2010; Uezumi et al., 2010). Since initial reports describing their role in fibro-fatty degeneration, further evidence has been provided supporting MSCs’ involvement in fibrotic remodeling of skeletal muscle (Uezumi et al., 2014; Uezumi et al., 2011). Phenotypically identical MSCs are also found in numerous other tissues, including bone, kidney, skin (Festa et al., 2011), fat (Chun et al., 2013) and the heart (Chong et al., 2011), and these populations have been demonstrated to possess highly similar transcriptomes, albeit with some tissue-specific differences in gene expression patterns (Pelekanos et al., 2012). The presence of PDGFRα-expressing mesenchymal progenitors in the developing and diseased human heart (Chong et al., 2011; Chong et al., 2013) suggests their involvement in cardiac fibrosis and, by extension, the therapeutic potential of targeting these cells to modulate fibrotic degeneration following cardiac injury. Indeed, recent evidence in a murine model of muscular dystrophy implicated PDGFRα+ Sca-1+ progenitors as responsible for the development of perivascular fibrosis in dystrophic mice (Ieronimakis et al., 2013). Further, the presence of tissue-resident mesenchymal progenitors in the heart sheds new light on the potential source of characteristic fibrofatty infiltrate displayed in certain cardiac pathologies such as arrhythmogenic cardiomyopathy (Paylor et al., 2013).

With a well-established role for PDGF signalling in the development of fibrosis, its modulation with receptor tyrosine kinase (RTK) inhibitors represents an attractive therapeutic strategy (Distler and Distler, 2010). Imatinib mesylate (Gleevec) has been demonstrated to prevent fibrosis in a variety of preclinical animal models (Ito et al., 2013; Jang et al., 2014; Leipner et al., 2008; Ma et al., 2012), although sometimes not to the benefit of cardiac function (Baumann et al., 2010; Kerkela et al., 2006), a discrepancy that may highlight the importance of ECM
deposition at certain stages of the reparative processes following cardiac injury. However, an in-depth understanding of the cellular mechanisms mediating this anti-fibrotic effect is lacking. Nilotinib, a structurally similar RTK inhibitor with increased potency and selectivity for PDGFRα (Manley et al., 2010), has also been highlighted as a potential anti-fibrotic therapy (Rhee et al., 2011; Shaker et al., 2011c), although it has not yet been tested in a model of cardiac injury. The appeal of this inhibitor is enhanced by the fact that it also inhibits the tyrosine kinase c-abl, which is a transducer of Alk1 TGF-β1 receptor signalling (Liu et al., 2011; Rosenbloom and Jimenez, 2008). Thus, with evidence strongly implicating PDGF signalling and tissue-resident PDGFRα+ mesenchymal progenitors in the development of cardiac fibrosis, as well as TGF-β1 signalling in inducing their differentiation into myofibroblasts, targeting both pathways with nilotinib represents an attractive therapeutic strategy for the treatment of this pathological condition.

Utilizing two models of ischemic cardiac injury, daily injections of isoproterenol (Heather et al., 2009) and ligation of the LAD (Kolk et al., 2009), we demonstrate that cardiac-resident PDGFRα+ Sca-1+ mesenchymal progenitors (cMSCs) are strongly activated by damage and proliferate to eventually differentiate into PDGFRα+ Sca-1- collagen-producing cells. Through generation of parabiotic WT:Col1a1-3.6GFP pairs, we are able to rule out the contribution of circulating cells to collagen-producing cells in the LAD ligation model, firmly implicating local progenitors as a source of myofibroblasts. Finally, based on in vitro evidence demonstrating the ability of nilotinib to abrogate TGF-β1-induced upregulation of collagen in cMSCs, we show that
in vivo treatment following isoproterenol-induced cardiac injury leads to a reduction of fibrosis and following LAD ligation causes amelioration of cardiac dysfunction.

2.2 Methods

2.2.1 Animals

Mice were housed in an enclosed, pathogen-free facility, and experiments were performed in accordance with University of British Columbia Animal Care Committee regulations. Both male and female C57BL/6 mice (>8 weeks old) were used in all experiments unless otherwise specified. C57BL/6 and PDGFRα-H2B::EGFP mice were purchased from The Jackson Laboratory and were 8 to 12 weeks of age unless specified otherwise. Col1a1-3.6GFP mice were a gift from Dr. David W. Rowe (Center for Regenerative Medicine and Skeletal Development, University of Connecticut Health Center). Cardiac damage was induced by subcutaneous injection of 100 mg/kg of isoproterenol (Sigma) for five consecutive days. In vivo nilotinib (Novartis) treatment was achieved by daily intraperitoneal injections of 25 mg/kg at indicated times. To experimentally induce myocardial infarctions, mice were intubated, anesthetized and their left anterior descending artery ligated as previously described (Kolk et al., 2009). EdU (Life Technologies) was given for indicated time periods at 1mg/mouse injected intraperitoneally.

2.2.2 Histology and staining

Mice received an intraperitoneal injection of 0.5 mg/g Avertin, and were perfused transcardially with 20 ml pBS/ 2 mM EDTA, followed by 20 ml of 4% paraformaldehyde (PFA). The excised
muscles were fixed in 4% PFA overnight. The following day, the tissue was either prepared for paraffin embedding following the standard sequence of dehydration steps in ethanol, or transferred to 20% sucrose PBS and incubated overnight for subsequent optimal cutting temperature (OCT) formulation embedding and freezing. Standard methods were followed for cryosectioning. Tissue sections were either stained using Masson’s Trichrome Stain as previously described (Bostick et al., 2012), or processed for immunohistochemistry as follows. Tissue sections were permeabilized in 0.3% Triton X-100 (Sigma) in PBS, and blocked for 1 h at room temperature in PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100, 3% bovine serum albumin (BSA) and 0.1% NaN3. Cells were stained overnight at 4 °C using a primary antibody diluted in 10% NGS, 0.3% Triton X-100, 3% BSA and 0.1% NaN3. The primary antibodies used for immunofluorescence are listed in Table S1 of Supplementary Information. In all cases, the primary antibody was detected using secondary antibodies conjugated to Alexa 488, 555, 594, or 647 (Molecular Probes). Confocal microscopy was performed using a Leica SP5X White Light Laser Confocal Microscope equipped with a Koheras white light laser source whose emission covers the 470-670nm spectrum of wavelengths, a tunable prism-based spectral detector and a GaAsP hybrid detector. Figures were assembled using Illustrator CS3 (Adobe) and Photoshop CS3 (Adobe).

2.2.3 Flow cytometry

Mice were sacrificed using CO2 asphyxiation, their hearts excised and a single cell suspension created by digesting tissue for 30 minutes in Collagenase II followed by 1 hour in Collagenase:Dispase. Following washing, cell preparations were incubated with primary
antibodies against cell membrane markers for 30 min at 4 °C in supplemented PBS containing 2 mM EDTA and 2% FBS (FACS buffer) at ~3 × 10^7 cells per ml. The antibodies used in flow cytometry and the dilutions are listed in Table S2 of Supplementary Information. For *in vivo* proliferation assays, EdU (Invitrogen) was dissolved in PBS at 2 mg/ml solution and it was administered daily by intraperitoneal injection (40 mg/kg). For flow cytometric analysis, cells were stained for surface markers as previously described (Joe et al., 2010). The cells were then stained for EdU incorporation using the Click procedure as described in the manufacturer’s manual. Extracellular labelling followed EdU labelling. Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using FacsDIVA software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers. Sorting gates were strictly defined based on fluorescence minus one stains. Flow cytometry data analysis was performed using FlowJo 10.0.4 (Treestar) software.

### 2.2.4 *In vitro* culturing experiments

Cells were sorted from digested hearts using indicated gating into Dulbecco’s modified eagle medium (DMEM, Invitrogen), supplemented with 10% FBS and 10 ng/ml GMCSF1 (eBioscience). Media was changed every 3 days. For adipogenic differentiation, following 7 days of growth, media was changed to adipogenic differentiation media containing DMEM supplanted with 0.25µm dexamethosone, 0.5 mM Isobutylmethylxanthine, 1µg/ml insulin and 5µm troglitazone. Cells were cultured for an additional 14 days and adipogenesis assessed via Oil Red O staining. For osteogenic differentiation, following 7 days of growth, media was
changed to osteogenic differentiation media containing 10nm dexamethasone, 5mM β-glycerophosphate, 50 ug/ml ascorbic acid and 50 ng/ml BMP2 (R&D Systems). Cells were cultured for an additional 14 days and osteogenesis assessed via Alzarin Red staining. For \textit{in vitro} proliferation and differentiation studies, cells were grown for 3 days and treated with TGF-β1 (10ng/ml), PDGF-AA (10ng/ml) and/or nilotinib (1uM) for up to one week before being analyzed by immunochemistry or flow cytometry. EdU (1mg) was delivered for 2 hours before staining.

2.2.5 \textbf{Quantitative reverse transcription PCR}

RNA isolation was performed using RNeasy mini kits (Qiagen) and reverse transcription was performed using the Superscript Reverse Transcriptase (Applied Biosystems). Fibrogenic gene expression analysis was performed using Taqman Gene Expression Assays (Applied Biosystems), on a 7900HT Real Time PCR system (Applied Biosystems). Sequence information for the primers contained in the Taqman assays are not in the public domain, but ordering information is provided in Appendix A. Data were acquired and analyzed using SDS 2.0 and SDS RQ Manager software (Applied Biosystems).

2.2.6 \textbf{Echocardiography}

Cardiac function was evaluated by two-dimensional transthoracic echocardiography on isofluorane anesthesized mice using a VisualSonics Vevo2100 imaging system. All measurements were performed by a single experienced operator blinded to the mouse genotypes.
2.2.7 Parabiosis

Pairs of parabiotic mice were generated surgically as previously described (Ajami et al., 2007). Briefly, peripheral blood chimerism was induced by surgically joining syngenic mice in parabiosis. Surgically adjoined mice established rich anastomotic circulation leading to complete peripheral blood exchange and blood chimerism by day 10 as previous confirmed using GFP+/GFP- pairings where complete blood sharing (an overall average of 44% GFP+ cells in the GFP– partner) was verified 10d after surgery.

2.2.8 Statistics

All data are expressed as group mean values +/- SE. Statistical analysis was performed using Prism 5 for Mac OS (GraphPad Software, Inc.). Significant differences were determined by student t test between 2 groups with post hoc application of the Tukey test to adjust for multiple comparisons of > 2 groups. The level of significance was set at P < 0.05.
2.3 Results

2.3.1 Identification of cardiac-resident mesenchymal stromal cells using PDGFRα and Sca-1

Following enzymatic digestion of whole hearts isolated from 8-12 week old PDGFRα-H2B-EGFP mice, a population of CD45-:CD31-:PDGFRα+:Sca-1+ (0.7% +/- 0.2% of all events) cells was identified as previously described in both heart (Chong et al., 2011; Chong et al., 2013; Furtado et al., 2014a; Pelekanos et al., 2012) and skeletal muscle (Joe et al., 2010; Uezumi et al., 2010) (Figure 2.1a, Figure 2.3a). In line with previous reports, all Sca-1+ cells were either CD31+ (endothelial) or PDGFRα+ (mesenchymal, Figure 2.1a). Unlike skeletal muscle, however, where virtually all CD45-:CD31-:PDGFRα+ cells express Sca-1, we were able to identify an equally abundant population of Lin-:PDGFRα+:Sca-1- cells (0.6% +/- 0.2% of total sample, Figure 2.3a). Expression of ECM-associated genes was evident (Figure 2.1b) in both populations as previously described (Ieronimakis et al., 2013). Compared to sorted Lin-:PDGFRα- cells, both PDGFRα populations were found to be highly enriched for ECM-associated genes including TGF-β1, colla1, vimentin, fibronectin-1 and connective tissue growth factor (CTGF) while lacking myofibroblast marker alpha smooth muscle actin expression (αSMA) (Figure 2.1b). Sca-1+ cells expressed significantly more vimentin, fibronectin and CTGF as compared to Lin-:PDGFRα+:Sca-1- cells, but similar levels of collagen-1 and TGF-β1. In contrast, we were not able to detect any expression of collagen-1 in sorted CD45+ and CD31+ populations. Next, we visualized PDGFRα+ cells in the heart by immunofluorescence and found
them to be distributed throughout the ventricular myocardium with a higher density in the epicardium and surrounding αSMA+ vessels (Figure 2.1c). In contrast to previous reports (Ieronimakis et al., 2013) describing distinct anatomical locations for cardiac PDGFRα+ Sca-1+ (arrowheads, Figure 2.1c) and PDGFRα+ Sca-1- cells (arrows, Figure 2.1c), we found both populations to be residing in interstitial as well as perivascular spaces (Figure 2.1c). Similar to our qRT-PCR results (Figure 2.1b), PDGFRα+ cells did not express αSMA. Both Sca-1+ and Sca-1- cells were sorted and plated both in bulk cultures and clonally (Sca-1+ cells: 1 in 15 forming colonies) to assess their developmental potential (Pelekanos et al., 2012). We were able to observe both adipogenic and osteogenic differentiation of Sca-1+ bulk cultures using previously described conditions (Figure 2.2). However, we were unable to achieve reliable cell growth of Sca-1- cells in clonal conditions so they were excluded from further in vitro characterization. Based on their demonstrated multipotency and clonogenicity, and their close similarities to mesenchymal progenitors from other tissues, we will hereafter refer to cardiac PDGFRα+ Sca-1+ cells as cardiac mesenchymal stromal cells (cMSCs). Given the observation that PDGFRα+ Sca-1- cells are unable to grow clonally, which is suggestive of a more mature population, as well as their higher expression of ECM-associated genes, we will herein refer to cardiac Sca-1- PDGFRα+ cells with the more general terminology of cardiac fibroblasts (CFs).
2.3.2 Proliferation and upregulation of collagen-1 in cardiac PDGFRα+ cells during myocardial repair

To assess the roles of cardiac PDGFRα+ cells in myocardial injury, we utilized two well-characterized models of cardiac ischemic damage. In the first, isoproterenol-induced cardiac injury, mice were subjected to daily injections of isoproterenol (100mg/kg/day, 5 days; Figure 2.3b), a non-selective beta-agonist known to induce cardiac ischemia followed by subendocardial necrosis, fibrosis, oxidative damage and inflammatory cells infiltration similar to what is observed following a myocardial infarction (Heather et al., 2009). Isoproterenol treatment led to localized tissue damage and consequent regions of matrix deposition within the myocardium, identified by Masson Trichrome Staining (Figure 2.4a). We observed a significant increase in the relative quantity of PDGFRα+ cells in isoproterenol-damaged hearts of transgenic mice in which a nuclear-localizing EGFP cassette was inserted into the PDGFRα locus (Hamilton et al., 2003) (Figure 2.3c). This increase was suggestive of a proliferative response of these populations to cardiac injury (Figure 2.3c). Indeed, EdU labelling (1mg/day) in the final two days of isoproterenol treatment led to 12.4% +/- 5.4% of cMSCs and 7.0% +/- 3.2% of cardiac fibroblasts being labelled (Figure 2.3c, Figure 2.4 b-e). We also observed that proliferation of PDGFRα-EGFP+ cells was limited to the area of isoproterenol-induced collagen-1 deposits, in which these cells comprised over 50% of all EdU positive cells (Figure 2.4e). The remainder of EdU positive nuclei was found to belong primarily to CD68+ inflammatory cells infiltrating the site of injury (data not shown). To confirm cardiac PDGFRα+ cell involvement in myocardial repair in a more physiologically relevant model, we induced an experimental myocardial
infarction by ligation of the left anterior descending artery (LAD) in PDGFRα-EGFP+ mice. Mice were given daily injections of EdU (1mg/day) for two days post infarction (dpi), and proliferation was measured by flow cytometry in hearts sectioned into basal (undamaged), mid (infarct border region) and apical (infarct area) sections. Proliferation was found predominantly within the apical infarcted region of the hearts where 54.0% +/- 8.9% of PDGFRα+ cells incorporated EdU, with decreasing frequencies of positive cells observed in both mid and basal sections (Figure 2.5a). No significant difference was found in the extent of EdU incorporation between cMSCs and cardiac fibroblasts at this early time point utilizing the LAD ligation model. We isolated RNA from freshly sorted cMSCs and fibroblasts purified from all three cardiac regions of mice that had undergone LAD ligation surgeries and observed significant upregulation of col1a1 mRNA expression in both populations in all regions, with a predominance of expression within cells sorted from the apical infarcted region (Figure 2.5b). Again, expansion of PDGFRα+ cells co-localized with collagen-1 deposition. Further, the infarcted myocardium at 28 dpi was predominantly composed of PDGFRα expressing cells (57.2% +/- 16.8% freq. vs. Dapi+ cells) distributed through a collagen-rich scar tissue organized into a highly fibrillar pattern (Figure 2.6).

### 2.3.3 Loss of Sca-1 expression during pro-fibrotic differentiation of cardiac PDGFRα+ cells after damage.

To better understand the relationship between cMSCs and CFs during cardiac injury, a kinetic analysis of PDGFRα-EGFP+ cell expansion after isoproterenol treatment was performed.
Indicative of differing kinetics and reparative roles between the two populations, at day 6 after isoproterenol injury the increase in PDGFRα+ cells was limited to Sca-1+ cMSCs, while at day 13 we observed an equal increase in Sca-1- cardiac fibroblasts (Figure 2.7a). These findings were in contrast to the kinetics of PDGFRα+ cell proliferation observed in the LAD ligation model where both Sca-1+ and Sca-1- increased equally early after injury (Figure 2.5a), likely due to differences in the time point of observation (D3 days post infarction in the LAD model versus D6 following 5 days isoproterenol treatment) and differing degrees of severity of the damage. By thirty-six days following isoproterenol damage, both subsets returned to numbers comparable with those found in the undamaged heart. Similar kinetics were observed for infiltrating CD45+ positive inflammatory cells (Figure 2.8). qRT-PCR quantification of ECM gene expression in freshly sorted cMSCs and CFs at days 6, 13 and 36 after isoproterenol damage demonstrated upregulation of collagen 1a1, αSMA, and periostin (POSTN) in both populations (Figure 2.7b). However, the upregulation of these markers took place at earlier time points in cMSCs as compared their Sca-1- CF counterpart. Combined, the kinetics of expansion and gene expression in these populations supported the model that Sca-1+ cMSC cells are activated to proliferate and upregulate ECM-associated genes earlier than their Sca-1- counterparts. These observations suggest that cMSCs and CF may represent discrete components of a differentiation cascade, and that increases in the population of collagen-expressing Sca-1- CFs may, at least in part, be dependent on early expansion of their Sca-1+ cMSC counterpart.

To examine the different kinetics of expansion and potential lineage relationship between the two populations, we injected the nucleotide analogue EdU during the final two days of isoproterenol treatment. EdU is readily incorporated into proliferating cells, and can be detected
in their progeny for multiple rounds of division. Importantly, no incorporation was observed when EdU was injected following the discontinuation of isoproterenol treatment (Figure 2.7c), indicating that any positive cells observed past this time point could only have inherited the label because pre-labelled ancestry was proliferating in response to isoproterenol-induced damage. Immediately after the conclusion of isoproterenol treatment (D6), the predominance of labelled proliferating cells (62 +/- 3.4%) was found to be Sca-1+ cMSCs although a significant portion of Sca-1- fibroblasts was also labelled (39 +/- 1.9%). In contrast, at D13 most labelled cells (72 +/- 2.9%) were Sca-1- suggesting that following their initial expansion, a portion of cMSCs subsequently lose Sca-1 expression and are able to contribute to the Sca-1- population of cells (Figure 2.7c). Immunohistochemistry revealed that both Sca-1+ (arrows, Figure 2.9) and Sca-1- (arrowheads, Figure 2.9) EdU+ cells were preferentially located in areas of fibrotic collagen depositions (Figure 2.4b, Figure 2.9). To investigate whether this association was due to a role of these cells in collagen production, we performed LAD ligations on Collagen3.6:GFP transgenic mice in which GFP is present in cells expressing collagen-1. GFP expression was confirmed to co-localize with collagen-1 in the infarcted region via immunohistochemistry at 14 dpi and 28 dpi following LAD surgery (Figure 2.10). Flow cytometric analysis demonstrated that while GFP is found in the cMSCs at 3 dpi, at 14 dpi and 28 dpi its expression is shifted to CFs (Figure 2.11a). This temporal succession is compatible with a model in which collagen expression starts in Sca-1+ progenitors while mature collagen-expressing fibroblasts, appearing later within the fibrotic scar, have lost Sca-1 expression. Alternatively, contributions of non-cardiac cells to the Sca-1 negative, collagen-expressing fibroblasts could also explain our observations.
There is increasing consensus that almost all collagen-expressing cells in the myocardium arise exclusively from tissue-resident sources (Ali et al., 2014; Chong et al., 2011; Furtado et al., 2014a; Ieronimakis et al., 2013; Moore-Morris et al., 2014a; Pichler et al., 2012). However, to determine whether circulating cells can contribute to expansion of cMSCs and CFs, we generated surgical parabiotic pairs were generated with WT and Collagen3.6:GFP mice. Following confirmation of shared circulation, both mice were anesthetized and LAD surgery was performed on the WT parabiotic pair to induce an experimental MI (Figure 2.11b). At both day 14 and day 28 after LAD ligation, no GFP+ cells were detected in the infarcted myocardium of the WT pair using both flow cytometry (Figure 2.11c) and immunohistochemistry (not shown), providing strong evidence that collagen-producing cells that arise after myocardial infarction are not derived from a circulating precursor.

2.3.4 Nilotinib prevents proliferation and differentiation of cardiac MSCs in vitro

The involvement of PDGFRα signalling in fibrosis has been reported (Olson and Soriano, 2009). This, combined with our preliminary results demonstrating the role of cMSCs in the development of fibrosis, prompted us to investigate the potential of inhibition of PDGF signalling to modulate proliferation and differentiation of cMSCs as a means of reducing tissue fibrosis and improving cardiac function following injury. Nilotinib, a receptor-tyrosine kinase inhibitor with proposed anti-fibrotic effects (Rhee et al., 2011), was identified as a promising candidate based on its capacity to inhibit both PDGFRα signalling and the tyrosine kinases c-abl and p38, which are downstream of the pro-fibrotic factor TGF-β1 ALK1 receptor (Druker et al.,
1996; Kantarjian et al., 2006; Zerr et al., 2012). Indeed, when cMSCs were sorted from PDGFRα:EGFP mice and grown in vitro, nilotinib (1µM) was found to potently attenuate both basal and PDGF-AA (10ng/ml) induced proliferation of cells as assessed via EdU incorporation (Figure 2.12a, Figure 2.13). To investigate potential effects of nilotinib on fibrogenic differentiation, cMSCs were sorted from Collagen3.6:GFP mice and, following establishment in culture, treated for 1 week with the pro-fibrotic cytokine TGF-β1 (10ng/ml) and/or nilotinib (1µM). Flow cytometric analysis indicated that TGF-β1 treatment significantly (p<0.05) reduced the expression of Sca-1 and increased collagen:GFP expression in cultured cells (Figure 2.12b), supporting in vivo evidence pointing to the loss of Sca-1 expression during fibrogenic differentiation. Further, treatment with nilotinib was shown to attenuate TGF-β1 induced collagen expression in cultured cells (Figure 2.12b).

### 2.3.5 Nilotinib prevents proliferation and differentiation-induced loss of Sca-1 in cardiac MSCs and reduces cardiac fibrosis following ischemic injury.

Next, we performed an evaluation of the effects of nilotinib treatment on the response of PDGFRα+ cells to heart damage in vivo. Mice were treated with isoproterenol for 5 days following which they were given daily injections of nilotinib (25 mg/kg/day) for 7 days. Proliferating cells were labelled with EdU in the final two days of isoproterenol treatment. Nilotinib led to reduced collagen deposition (ISO – 5.8% +/- 2.9% vs ISO +NILO – 2.2 +/- 1.3% of total image area stained collagen positive, p<0.05) in the myocardium as assessed by immunohistochemistry (Figure 2.14) and Masson Trichrome Staining (Figure 15a), in keeping
with what was found following treatment with receptor tyrosine kinase inhibitors in numerous other models of fibrosis (Baumann et al., 2010; Distler and Distler, 2010; Ito et al., 2013; Jang et al., 2014; Leipner et al., 2008; Liu et al., 2011; Ma et al., 2012; Rhee et al., 2011; Rosenbloom and Jimenez, 2008; Shaker et al., 2011c). Flow cytometry of enzymatically digested injured hearts revealed a significant increase in the frequency of Sca-1+ cMSCs compared to Sca-1-fibroblasts at day 9 and day 13 after injury, supporting the hypothesis that nilotinib acts, at least in part, by blocking pro-fibrotic differentiation and loss of Sca-1 expression in PDGFRα+ cells (Figure 2.16a, compare to Fig 2.7c). In addition, nilotinib treatment significantly reduced proliferation of Sca-1+ cMSCs at day 6 after isoproterenol treatment, and of CFs at day 13 (Figure 2.16b). No change was observed in the total amount of CD45+ cells present in the treated animals, suggesting the observed effect arose from direct inhibition of signalling within cMSCs rather than through an inflammatory intermediate (Figure 2.15b). To investigate the effect of nilotinib on collagen expression in cMSCs and CFs in vivo after ischemic injury, Collagen3.6:GFP mice were given isoproterenol for 5 days followed by I.P. nilotinib (20mg/kg/day) for up to two weeks. At day 20 after injury, nilotinib-treated mice were shown to contain significantly (p<0.05) less collagen-GFP expressing cells, providing further evidence that the observed anti-fibrotic effects were due to direct inhibition of collagen production by both cMSCs and CFs (Figure 2.16b). Lacking a more specific inhibitor that specifically blocked either Sca-1 expression in cMSCs/CFs, proliferation of cMSCs/CFs, or collagen-production by these cell populations, we were not able to ascertain which of these effects was the primary cause of the reduced fibrosis that we observed.
2.3.6 Nilotinib improves cardiac function after LAD ligation

To assess if the observed anti-fibrotic effects of nilotinib were able to improve cardiac function following ischemic injury, echocardiography was performed on mice being given daily nilotinib treatment (20 mg/kg/day) at day 3, day 7 and day 14 following isoproterenol injury (100 mg/kg/day). No significant difference was found in ejection fraction (EF) or fractional shortening (FS) between isoproterenol, isoproterenol + nilotinib or untreated groups, making it impossible to evaluate the effects of this experimental treatment (data not shown). Moving to the more severe LAD ligation model, mice treated with nilotinib for 28dpi were found to have a significantly altered Sca-1+ to Sca-1- ratio in PDGFRα+ cells, similar to previous observations in vitro (Figure 2.12b) and in vivo in the isoproterenol model (Figure 2.16a), which demonstrated that nilotinib treatment led to a higher proportion of PDGFRα+ Sca-1+ cMSCs in relation to their Sca-1- CF counterparts (Figure 2.17a).

Assessment of cardiac function revealed that LAD ligation induced a progressive decline in LV ejection fraction at 7 and 28 dpi, which was associated with an increase in LV end diastolic volume (EDV) (Figure 2.17g). Stroke volume and cardiac output were not altered (Figure 2.17b, c). Additionally, LV chamber diameter as well as anterior and posterior wall thicknesses increased after LAD ligation, suggesting the development of cardiac dilation and hypertrophy, which are features of cardiac remodeling (Figure 2.17g, h). Interestingly, nilotinib treatment attenuated the decline in LV ejection fraction and completely prevented the increase in EDV and
LV chamber dilation induced by LAD ligation (Fig 2.17d,f,g,h). Anterior and posterior wall thicknesses were not significantly affected by nilotinib treatment (data not shown).

2.4 Discussion

Here we provide evidence that following acute myocardial injury, cardiac-resident mesenchymal progenitors (cMSC, identified phenotypically by Sca-1 and PDGFRα) proliferate, subsequently lose Sca-1 expression and contribute to the expansion of PDGFRα+ Sca-1- cardiac fibroblasts which upregulate collagen-I and are implicated in the development of fibrosis. Further, building on the well-known anti-fibrotic effects of several receptor tyrosine kinase inhibitors, we provide evidence that nilotinib is able to prevent the development of cardiac fibrosis through the inhibition of this proliferation and fibrogenic differentiation of cMSCs. We were able to demonstrate a benefit to ejection fraction in mice subjected to LAD ligation, highlighting the potential therapeutic benefit of such anti-fibrotic treatments.

The definition and cellular identity of the cardiac fibroblast remains a highly contentious issue and unresolved problem in our understanding of cardiac development and pathology (Krenning et al., 2010). Our evidence supports previous reports (Chong et al., 2011) demonstrating that within cardiac PDGFRα+ mesenchymal cells there is a population of Sca-1+ progenitors (previously referred to as cardiac-resident colony forming units – fibroblast, or cCFU-Fs) that possess a highly similar functional and phenotypic identity to classically defined mesenchymal stromal cells (MSCs) (Pelekanos et al., 2012). We did not seek to confirm whether this population contains a bona fide stem cell population as reported by Chong et al. (2011) and
instead confirmed the presence of cells that, at least in part, adhered to the basic definition of a “mesenchymal stromal cell” (Dominici et al., 2006) and sought to investigate the role of these cells in reparative processes following acute cardiac damage. Utilizing two models of myocardial injury, our data demonstrate that cMSCs expand in situations of cardiac injury and subsequently differentiate, losing Sca-1, into collagen-producing progeny. Although there remains considerable ambiguity in the literature about the definition of PDGFRα+ cells in the heart, utilizing EdU labelling and the flow cytometric analysis of collagen-1:gfp expressing cells after injury, we provide the first evidence of a relationship between Sca-1+;PDGFRα+ and Sca-1-;PDGFRα+ cells in the development of cardiac fibrosis. Regardless of the terminology used to define this population – including cCFU-Fs (Chong et al., 2011), coronary adventitial cells (Ieronimakis et al., 2013), or cardiac fibroblasts (Acharya et al., 2012; Moore-Morris et al., 2014a) – the combined use of Sca-1 and PDGFRα allows the delineation of a multipotent mesenchymal progenitor population in the heart (Figure 2.1, Figure 2.3) that responds early to ischemic injury (Figure 2.3, Figure 2.7) and subsequently loses Sca-1 in response to TGF-β1 induced signalling in a fibrotic environment (Figure 2.12). Our findings also support previous evidence (Chong et al., 2011) distinguishing cardiac MSCs from classically defined “pericytes” (identified by PDGFRB, NG2 and αSMA), as we were unable to observe any αSMA expression in this population via either IHC or PCR (Figure 2.1b,c). This builds on previous developmental studies outlining a model in which PDGFRα+ progenitors contribute to the generation of fibrogenic cells and PDGFRB+ progenitors to coronary vascular smooth muscle cells (cVSMC) (Smith et al., 2011), following reports by Olson and Soriano in disrupting signalling in both of these RTKs (Olson and Soriano, 2009, 2011).
TGF-β1 has long been known as a pro-fibrotic cytokine in a wide variety of tissues (Border and Noble, 1994), and its role in mediating the progression of cardiac fibrosis is widely accepted. Our data supports previous reports indicating pro-fibrotic effects of TGF-β1 on non-myocytes in vivo (Teekakirikul et al., 2010) and provides newfound insight into the cellular mechanisms by which this occurs. Although we were unable to observe a significantly differential pattern in αSMA expression between cMSCs and CFs at D13, our PCR data demonstrating upregulation of both collagen-1 (p<0.05) and periostin (not significant), both markers of differentiated fibroblasts (Oka et al., 2007; Snider et al., 2008), in the Sca-1- subset after ischemic injury indicates that they likely represent a mature fibroblast population. Although our in vitro data demonstrates a clear role of both PDGF-AA and TGF-β1 in mediating this progression, it is likely that other pro-fibrotic stimuli including connective tissue growth factor and fibronectin also play a role, which remains to be elucidated (Leask and Abraham, 2004).

In addition to debate on the cellular identity of cardiac fibroblasts, there has not yet been consensus reached on the cellular source of their differentiated progeny, αSMA+ myofibroblasts. PDGFRA signalling has been shown to be vital for the formation of interstitial cardiac fibroblasts via endothelial-to-mesenchymal transition of multipotent epicardial progenitors during development (Acharya et al., 2012; Smith et al., 2011), but debate continues about where collagen-producing fibroblasts arise from after injury. Our data disputes any significant direct contribution of circulating cells to collagen production in the fibrotic heart, and supports numerous previous reports in both the heart (Chong et al., 2011; Chong et al., 2013; Moore-Morris et al., 2014a), and other tissues (Barisic-Dujmovic et al., 2010). In agreement with Chong et al. (2011) and Ieronimakis et al. (2013), we found no contribution of circulating cells to
collagen-producing cells in the heart using a parabiotic model in which a WT animal was given a LAD ligation while sharing circulation with a Col1a1-3.6GFP+ partner (Chong et al., 2011; Ieronimakis et al., 2013). This matches clinical evidence demonstrating that collagen production occurs from tissue-resident cells in sex-mismatched heart transplant recipients (Pichler et al., 2012). We were unable to assess the relative contribution of endo- or epithelial-to-mesenchymal transformation in generating collagen-producing myofibroblasts, but other recent studies employing strict lineage tracing in other tissues have cast significant doubt on the importance of this developmental process in the creation of adult collagen-producing fibroblasts after injury (Rock et al., 2011; Scholten et al., 2010).

Our data demonstrate the anti-fibrotic effects of nilotinib treatment and are consistent with a growing body of evidence in numerous different situations of tissue injury in which nilotinib treatment leads to improved organ function {Rhee, 2011 #133;Shaker, 2011 #132;Shaker, 2011 #130}. Identification of the cellular target of receptor tyrosine kinase inhibition represents a significant step forward in developing this therapeutic avenue and further effort needs to be invested in delineating the effects on different signalling molecules, including c-abl, PDGFRs and downstream TGF-β1 signalling molecules such as SMADs. Although we were able to observe direct effects of Sca-1+ PDGFRα+ cells in vitro and were able to demonstrate no difference in total inflammatory infiltrate after injury, further study of the relative effect of nilotinib treatment on the multiple cell types involved in the development of fibrosis, including infiltrating inflammatory cells, is warranted.
In conclusion, use of Sca-1 and PDGFRα to investigate changes in the cellular composition of the damaged myocardium provides novel insights into the role of tissue-resident mesenchymal populations in situations of cardiac injury. Differing kinetics of expansion and gene expression support a model in which PDGFRα+ Sca-1+ progenitors respond early to ischemic injury, and subsequently either die by apoptosis or lose Sca-1 expression while differentiating into more mature Sca-1- cardiac fibroblasts. In support of this model, we were able to demonstrate that the RTK inhibitor nilotinib is able to reduce fibrosis in the myocardium and improve cardiac function through a combination of blocking proliferation and differentiation of cMSCs, as well as directly inhibiting the expression of *coll1a1*. This evidence supports the potential use of nilotinib as an anti-fibrotic therapy to prevent or reduce fibrosis in situations of cardiac injury, a much-needed line of therapy given the clinical prevalence of this pathological process.
Figure 2.1 - Characterization of cardiac MSCs in the murine heart
A. All cardiac Sca-1+ cells are either CD31+ or PDGFRα+. Nucleated cells were selected from enzymatically digested murine hearts based on their FSC/SSC profile as well as staining negative for Propidium Iodide and in the mid-region for Hoechst. Gating on Sca-1+ cells demonstrated that nearly all cells were either CD31+ or PDGFRα+, with no co-expression of the two markers.
B. ECM gene expression of cardiac MSCs. RNA was isolated from freshly sorted Sca1−PDGFRα−, Sca1−PDGFRα+ and Sca1+PDGRα+ cells from undamaged hearts and expression of TGFβ-1, Collagen-1, Vimentin, Fibronectin-1 and CTGF determined by quantitative real-time PCR. Expression is normalized to GAPDH and presented relative to the PDGFRα− Sca1− * - p < 0.05 vs. PDGFRα-Sca1- expression, n=3, student t test.
C. Cardiac MSCs do not express αSMA in the undamaged myocardium. 10µm sections of cardiac tissue from PDGFRα-EGFP mice were stained with anti-αSMA and anti-Sca-1 antibodies and visualized with PDGFRα-EGFP and EdU.
Figure 2.2 - Adipogenic and osteogenic differentiation of cardiac MSCs.
PDGFRα+Sca1+ cells were sorted in 96-well plates and grown for 1 week in growth media followed by 1 week in either adipogenic or osteogenic differentiation media. Differentiation was assessed by visualizing successful Oil Red O (left) and Alzarin Red (right) staining.
**Figure 2.3 - Isoproterenol-induced injury causes expansion of cardiac MSCs**

A. Gating strategy used to identify and isolate Live Lin- PDGFRα+ Sca1+ cardiac cells. Nucleated cells were selected from enzymatically digested murine hearts based on their FSC/SSC profile as well as staining negative for Propidium Iodide and in the mid-region for Hoechst. Endothelial and hematopoietic cells were then excluded using CD31 and CD45 antibodies and the remaining cell population fractionated on Sca-1 and PDGFRα expression.

B. Isoproterenol and EdU treatment schematic.

C. Isoproterenol causes proliferation of cardiac MSCs. Mice were given EdU (1mg/day) for the final two days of isoproterenol treatment (5 days). On day 0 after treatment, hearts were excised and digested enzymatically and EdU detected. Frequency of PDGFRα+ cells (left) was shown to increase from 10 +/- 0.9% to 12.5 +/- 0.72% and the % of GFP+ that were EdU+ increased from 0.52 +/- 0.09% to 9.82 +/- 3.2%. * - p<0.05 vs. non-damage, n=5, student t test.
Figure 2.4 - Isoproterenol-induced injury causes expansion of cardiac MSCs and upregulation of ECM genes

A-E. Isoproterenol-induced cardiac injury. Adult mice were treated with isoproterenol (100mg/kg/day) for 5 days and hearts excised, and perfused with 4% PFA. Masson’s Trichrome Stain was performed on 10µm sections and fibrosis visualized. 10µm sections of cardiac tissue were stained with an anti-collagen antibody and visualized with PDGFRα-EGFP and EdU. The quantity of EdU+ cells that were also GFP+ was calculated to be 52.2 +/- 4.8% in isoproterenol-treated animals. n=3, 5 sections examined per heart, * - p<0.05 vs. non-damage, student t test.
**Figure 2.5 - LAD artery ligation induces proliferation and upregulation of collagen in cardiac MSCs**

A – LAD ligation leads to proliferation of cMSCs. EdU (1mg/day) was given to mice for 2 days prior to LAD ligation and hearts excised at 3dpi. Following enzymatic digestion, EdU incorporation within the basal (non-infarct), mid (border zone) and apical (infarct region) regions of the myocardium was assessed by flow cytometry.

B. Localized upregulation of collagen-1 mRNA in cardiac MSCs in the infarct region. RNA was extracted from freshly sorted Sca1-PDGFRα- cells, cMSCs and CFs from basal (non-infarct), mid (border region) and apical (infarct region) areas of the heart. Expression of collagen-1 determined by qRT-PCR. *-p<0.05 vs. Non-dmg group, n=3, student t test.
Figure 2.6 - LAD artery ligation induces proliferation and upregulation of collagen in cardiac MSCs

Expanded cMSCs numbers after LAD ligation persist within collagen-rich scar at 1 month. EdU (1mg/day) was given to mice for 2 days prior to LAD ligation, and hearts of mice were excised at 3 days, 7 days and 28 days following surgery. 10µm sections of cardiac tissue were stained with an anti-collagen (red) and anti-CD68 antibodies (light blue) and visualized with PDGFRα-EGFP (green), EdU (purple) and Hoechst (blue) on a Leica spectral scanning confocal microscope.
Figure 2.7 - Isoproterenol treatment causes expansion in cardiac MSCs which subsequently lose Sca-1 expression
A. Kinetics of isoproterenol-induced cardiac MSC expansion. Hearts of mice treated with isoproterenol were sacrificed at day 6, day 13, and day 36 after treatment and enzymatically digested. Relative quantity of cMSCs and CFs was determined. * - p<0.05 vs. indicated grouping, n=4-7, student t test
B. Isoproterenol causes upregulation of ECM-associated genes in cardiac MSCs. RNA was isolated from freshly sorted cMSCs and CFs after damage and gene expression normalized to GAPDH and represented as fold change relative to non-damaged samples. * - p<0.05 vs indicated grouping, n=4-7, student t test
C. EdU labelling reveals loss of Sca-1+ in proliferating PDGFRα+ cells. Mice were given a 2 day pulse of EdU (1mg/day) at the end of 5 days of isoproterenol (100mg/kg/day) treatment and flow cytometry used to track labelled proliferating cells at day 1, 3 and 7 after injury. * - p<0.05 vs indicated grouping, n=3, student t test
Figure 2.8 - Isoproterenol-induced cardiac injury leads to infiltration of circulating cells in a time-dependent manner.
Hearts of mice treated with isoproterenol were sacrificed at day 6, day 13, and day 36 after treatment and enzymatically digested. Relative quantity of CD45+ cells was determined.
Figure 2.9 - Both Sca-1+ and Sca-1- PDGFRα cells colocalize with collagen after isoproterenol-induced injury.

Mice were treated with isoproterenol for five days and hearts excised at D6 following initiation of treatment. 10µm sections of cardiac tissue were stained with an anti-collagen (red) and anti-Sca-1 antibodies (purple) and visualized with PDGFRα-EGFP (green) and Hoechst (blue) on a Leica spectral scanning confocal microscope.
Figure 2.10 - LAD ligation in Col1a1-3.6GFP transgenic mice causes temporal progression of GFP expression from Sca-1+ to Sca-1- cells
LAD artery ligation causes expansion of Col3.6:GFP+ cells within the infarct zone. Hearts of Col3.6:GFP mice were excised at 14 days and 28 days post-ligation. 10µm sections were stained with Collagen-1 (red) and CD68 antibodies (light blue) and visualized with GFP (green) and Hoechst (blue) on a spectral scanning confocal microscope.
Figure 2.11 - LAD ligation in Col1a1-3.6GFP transgenic mice causes temporal progression of GFP expression from Sca-1+ to Sca-1- cells

A. Kinetics of Col-GFP+ cell expansion following experimental MI. Hearts of Col-GFP+ mice were enzymatically digested after 3 days, 14 days and 28 days following LAD artery ligation and kinetics of GFP expression determined.

B. Schematic of parabiotic pairings. WT (C57B/6) and Col3.6:GFP mice were surgical paired and shared circulation verified by flow cytometry after 4 weeks.

C. Following LAD ligation, there is a lack of Col-GFP+ cells within myocardium of WT parabiotic pair. LAD ligation surgery was performed on the heart of the WT partner in parabiotic pair with a Col3.6:GFP. After 3 days, hearts were excised and digested enzymatically and presence of GFP+ assessed by flow cytometry.
Figure 2.12 - In vitro modulation of proliferation and differentiation of cardiac MSCs using nilotinib
A. Nilotinib prevents PDGF-AA induced proliferation of cardiac MSCs. Cardiac MSCs were sorted from PDGFRα-EGFP mice and cultured in a 96-well plate. Following 3 days of growth, PDGF-AA and/or nilotinib (1mg/ml) was added for 48 hours. EdU was added 2 hours before the cells were fixed and EdU incorporation assessed. *-p<0.05 vs. Ctl, #-p<0.05 vs. PDGF, n=8, student t test

B. TGFβ1 and nilotinib have opposing effects on Sca-1 and Col3.6:GFP expression in cultured cardiac MSCs. Cardiac MSCs were sorted from Col3.6:GFP mice and cultured in a 96-well plate. Following 1 week of growth in nilotinib (1µM) and/or TGFβ1 (10ng/ml), cells were analyzed for Sca-1 and GFP expression by flow cytometry. *-p<0.05 vs. CTL, %-p<0.05 vs. TGFβ1, n=8, student t test
Figure 2.13 - Nilotinib prevent PDGF-AA induced proliferation of cardiac MSCs in vitro.
Cardiac MSCs were sorted from PDGFRα-EGFP mice and cultured in a 96-well plate. Following 3 days of growth, PDGF-AA and/or nilotinib (1mg/ml) was added for 48 hours. EdU was added 2 hours before the cells were fixed and EdU incorporation assessed.

Figure 2.14 - In vivo modulation of proliferation and differentiation of cardiac MSCs using nilotinib.
Nilotinib reduces collagen deposition following isoproterenol damage. Mice were treated with isoproterenol (100mg/kg/d) for 5 days and nilotinib (25mg/kg/day) for 7 days. Hearts were excised and collagen visualized by immunohistochemistry. 10µm sections were stained with anti-collagen antibody and the amount of collagen (collagen pixel area / total pixel area) quantified. *-p<0.05 vs. ISO (n=3, 5 sections, student t test)
Figure 2.15 - Nilotinib treatment prevent isoproterenol-induced cardiac injury

a. Nilotinib prevents the development of isoproterenol-induced cardiac fibrosis. Adult mice were treated with isoproterenol (100mg/kg/day) for 5 days with or without nilotinib (25mg/kg/day) and hearts excised, and perfused with 4% PFA. Masson’s Trichrome Stain was performed on 10µm sections and fibrosis visualized.

b. Nilotinib does not prevent the infiltration of CD45+ circulating cells. At D13 following treatment with isoproterenol (100mg/kg/day) and with or without nilotinib (25mg/kg/day), hearts were excised and enzymatically digested. Relative quantity of CD45+ cells was determined.
Figure 2.16 - *In vivo* modulation of proliferation of cMSCs using nilotinib.

A. Nilotinib treatment prevents the loss of Sca-1 and inhibit proliferation in PDGFRα+ following isoproterenol damage. Mice were treated with isoproterenol for 5-days followed by nilotinib (25mg/kg/day) for up to 7 days. At indicated time points, hearts were excised and digested enzymatically and analyzed by flow cytometry. * - p<0.05 vs. indicated grouping, n=4-7, student t test

C. Nilotinib reduces collagen expression in PDGFRα+ cells after isoproterenol damage. Mice were treated with isoproterenol for 5 days followed by nilotinib for up to 14 days. Col3.6:GFP expression was analyzed by flow cytometry at D20 following treatment. * - p<0.05 vs. indicated grouping, n=5, student t test
Figure 2.17 - Nilotinib prevents LAD-induced decline in cardiac function

A-H. LAD artery ligation impairs cardiac function. Successful surgery was confirmed in live mice using VEVO2100 echocardiography at 7 days after surgical ligation. Impaired strain (shown) and strain rate (not shown) was observed in both the posterior and anterior apical regions. The relative decline in regional function was quantified (* - p<0.05 vs. indicated grouping, n=3, vs. average at baseline, student t test).
Figure 2.18 – Schematic of healthy myocardium.
Figure 2.19 – Schematic depicting the proposed model of progression from Sca-1+ cMSC to Sca-1- CF following acute cardiac injury.
Chapter 3: Chronic imatinib treatment reduces cardiac fibrosis and improves function in aged *mdx* mice

3.1 Introduction

Duchenne muscular dystrophy (DMD) is a degenerative muscle disorder caused by a deficiency of dystrophin, a large protein that links the intracellular cytoskeleton to the extracellular matrix. DMD primarily affects skeletal and cardiac muscle and there are currently no effective therapies for this disease, which is present in an estimated 1 in 3500 male births (Emery, 1991). As improved noninvasive respiratory support has extended the lifespan of patients with DMD, mortality due to cardiomyopathies has increased, and is now estimated to account for up to 20% of DMD deaths (Shirokova and Niggli, 2013). To develop new therapies to treat this debilitating disease, much preclinical effort has been invested in preventing the progression of DMD cardiomyopathy in *mdx* mice, a murine model of DMD lacking dystrophin that develops skeletal and cardiac dystrophy that largely mimics human disease processes (Quinlan et al., 2004; Van Erp et al., 2010).

Although advances have been made towards restoring functional dystrophin expression using experimental strategies such as gene therapy (Bostick et al., 2011; Yue et al., 2004) or exon skipping (Alter et al., 2006), the majority of existing preclinical research has focused on treating DMD cardiomyopathies by preventing the pathological complications of the disease, most notably the development of cardiac fibrosis. Numerous studies have demonstrated that a variety of different drugs – including sildenafil (Adamo et al., 2010; Khairallah et al., 2008), losartan
(Bish et al., 2011; Spurney et al., 2011b), pirfenidone (Van Erp et al., 2006) and deacetylase inhibitors (Minetti et al., 2006) – have the ability to prevent the development of cardiac fibrosis and improve cardiac function in *mdx* mice.

Numerous studies have shown the ability of imatinib, a tyrosine kinase inhibitor with known anti-fibrotic effects, to inhibit the development of muscle fibrosis in *mdx* mice (Bizario et al., 2009; Huang et al., 2009). However, to date the efficacy of this compound in preventing the development of DMD cardiomyopathy has not been demonstrated. The therapeutic relevance of imatinib is supported by evidence demonstrating its effects on tissue-resident Sca-1+ PDGFRα+ mesenchymal progenitors that are known to mediate fibrotic processes in both skeletal and cardiac muscle of *mdx* mice. Herein we demonstrate that chronic treatment with imatinib via drinking water reduces the development of cardiac fibrosis in *mdx* mice and prevents age-related decline of their cardiac function.

### 3.2 Methods

#### 3.2.1 Mice

Mice were housed in an enclosed, pathogen-free facility, and experiments were performed in accordance with University of British Columbia Animal Care Committee regulations. Male C57BL/6 mice (>8 weeks old) were used in all experiments unless otherwise specified. C57BL/6, *mdx* and PDGFRα-H2B::EGFP mice were purchased from The Jackson Laboratory and were 8 to 12 weeks of age unless specified otherwise. *In vivo* imatinib (LC Labs) treatment
delivered in the drinking water by dissolving 165 mg/250mL of drinking water (0.66g/L) and water was available ad libitum for the duration of the study (15 months).

### 3.2.2 Flow cytometry

Mice were sacrificed using CO₂ asphyxiation, their hearts excised and a single cell suspension created by digesting tissue for 30 minutes in Collagen II followed by 1 hour in Collagenase:Dispase. Following washing, cell preparations were incubated with primary antibodies against cell membrane markers for 30 min at 4 °C in supplemented PBS containing 2 mM EDTA and 2% FBS (FACS buffer) at ~3 × 10⁷ cells per ml. The antibodies used in flow cytometry and the dilutions are listed on Table S1 (Appendix A). Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using FacsDIVA software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers. Sorting gates were strictly defined based on fluorescence minus one stains. Flow cytometry data analysis was performed using FlowJo 10.0.4 (Treestar) software.

### 3.2.3 TGF-β1 ELISA

Whole hearts were homogenized in 500 µl of complete RIPA lysis buffer using a tissue smasher and the homogenates were centrifuged at 10,000 rpm for 2 min. The supernatants were aliquoted and kept at -80 °C. TGF-β1 content was measured using the TGFb Platinum ELISA kit (eBioscience), following the manufacturer’s instructions.
3.2.4 Immunohistochemistry

Mice received an intraperitoneal injection of 0.5 mg/g Avertin, and were perfused transcardially with 20 ml pBS/ 2 mM EDTA, followed by 20 ml of 4% paraformaldehyde (PFA). The excised muscles were fixed in 4% PFA overnight. The following day, the tissue was either prepared for paraffin embedding following the standard sequence of dehydration steps in ethanol, or transferred to 20% sucrose PBS and incubated overnight for subsequent OCT embedding and freezing. Standard methods were followed for cryosectioning. Tissue sections were permeabilized in 0.3% Triton X-100 (Sigma) in PBS, and blocked for 1 h at room temperature in PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100, 3% bovine serum albumin (BSA) and 0.1% NaN3. Cells were stained overnight at 4 °C using a primary antibody diluted in 10% NGS, 0.3% Triton X-100, 3% BSA and 0.1% NaN3. The primary antibodies used for immunofluorescence are listed on Table S1 of Supplementary Information. In all cases, the primary antibody was detected using secondary antibodies conjugated to Alexa 488, 555, 594, or 647 (Molecular Probes). Confocal microscopy was performed using a Leica SP5X White Light Laser Confocal Microscope equipped with a Koheras white light laser source whose emission covers the 470-670nm spectrum of wavelengths, and a tunable prism-based spectral detector and a GaAsP hybrid detector. Figures were assembled using Illustrator CS3 (Adobe) and Photoshop CS3 (Adobe).
3.2.5 **Echocardiography**

Cardiac function was evaluated by two-dimensional transthoracic echocardiography on conscious mice using a VisualSonics Vevo2100 imaging system. All measurements were performed by a single experienced operator blinded to the mouse genotypes.

3.2.6 **Statistics**

All data are expressed as group mean values +/- SE. Statistical analysis was performed using Prism 5 for Mac OS (GraphPad Software, Inc.). Significant differences were determined by student t test between 2 groups with post hoc application of the Tukey test to adjust for multiple comparisons of > 2 groups. The level of significance was set at P < 0.05.

3.3 **Results**

3.3.1 **Study design and mortality**

Male C57BL/6 (n=12) and mdx mice (n=32) were weaned at 3 weeks and randomized to either standard drinking water (wt: n=6, mdx: n=16) or drinking water supplemented with imatinib mesylate (0.64g/L) for fifteen months (Figure 3.1a). Water was provided ad libidum. By the study endpoint, 56.25% of untreated mdx mice (9/16) and 75% of treated mdx were alive (12/16,
p = 0.25, Figure 3.1b). All wild-type mice from both treated and untreated groups were alive at the study endpoint (100%).

### 3.3.2 Chronic imatinib treatment reduces fibrosis in diaphragm and heart

Although acute administration (daily treatment for 6 weeks) of imatinib mesylate in *mdx* mice has been previously demonstrated to ameliorate the dystrophic phenotype (Bizario et al., 2009; Huang et al., 2009; Ito et al., 2013), delivery of this therapeutic compound in a chronic setting of dystrophy has never been studied. Chronic treatment of *mdx* mice with imatinib via drinking water led to attenuated *mdx* pathologies within the diaphragm where a reduction in the collagen content (assessed via Masson’s Trichrome Staining, Figure 3.2a) and increased diaphragm width was observed (untreated – 245 +/- 7µm, treated – 514 +/- 34µm, p<0.05, n=5, Figure 3.2c).

Further, by breeding *mdx* mice with animals in which a GFP cassette has been inserted into the PDGFRα locus (PDGFRα:EGFP), we were able to observe that imatinib treatment led to a reduced density of cells (untreated 1.47 +/- 0.2 cell/mm² image, treated – 0.84 +/- 0.12 cell/mm² image, p<0.05, n=5, Figure 3.2b,d) labelled by this mesenchymal marker.

Following confirmation of previous results about the effect of imatinib on skeletal muscle fibrosis in *mdx* mice, we focused our efforts on assessing its anti-fibrotic effects in the heart.

Hearts of *mdx* mice treated with imatinib were found to have significantly less collagen deposition within the myocardium (3.8 +/- 0.6% *mdx* +imatinib vs 5.7 +/- 1.8% collagen+ *mdx* untreated, p<0.05, n=3), highlighting the efficacy of this delivery method in preventing the age-dependent development of fibrosis (Figure 3.3).
3.3.3 Anti-fibrotic effects of imatinib treatment are not due to intermediary effect on infiltrating inflammatory cells

We next investigated whether the observed reduction of cardiac fibrosis was due to indirect effects of imatinib through inhibition of signaling within infiltrating inflammatory M1 macrophages, which have a well-known role in the progression of dystrophic processes. Although a significant increase in the amount of Ly6c+ M1 macrophages was observed in the hearts of 15-month mdx mice as compared to young mdx mice, treatment with imatinib was found to have no effect on this increase (Figure 3.4), indicating that anti-fibrotic effects were likely due to direct inhibition of tissue-resident PDGFRα+ cells. Another important component of the development of fibrosis in the heart of mdx mice is a proposed role of endothelial secretion of the pro-fibrotic cytokine TGF-β1 (Ieronimakis et al., 2013). We observed no change in the total amount of TGF-β1 between treated and untreated 15-month-old mdx mice, although there was significantly more TGF-β1 as compared to young WT mice (Figure 3.5). These observations support the hypothesis that the observed anti-fibrotic effects were due to direct inhibition of pro-fibrotic signaling within mesenchymal cells in the heart.

3.3.4 Imatinib reduces the amount of PDGFRα+ Sca-1+ cMSCs in the hearts of aged mdx mice

Building on previous work by others and us that has demonstrated that tissue-resident PDGFRα+ Sca-1+ mesenchymal progenitors play an important role in the development of pathological fibrosis in both skeletal muscle and the heart, we next sought to determine the effect of chronic
imatinib treatment on this population in the hearts of mdx mice. Quantity (cells/mg of tissue) of PDGFRα+ Sca-1+ cardiac mesenchymal stromal cells (cMSCs) but not PDGFRα+ Sca-1- cardiac fibroblasts (CFs) was found to be significantly increased in the hearts of 15-month mdx mice, with a far greater prevalence of cMSCs compared to their Sca-1- CF counterparts in aged mdx mice than in aged WT mice (Figure 3.6). Treatment with imatinib led to significant reductions in the total quantity of Sca-1+ cMSCs in the myocardium of aged mdx mice, but no significant difference in WT mice (Figure 3.6). Building on previous evidence demonstrating reduced fibrosis in the hearts of treated mdx mice, these data supported the model that imatinib treatment prevented the expansion of cMSCs leading to reduced ECM deposition in aged animals. With reduced amounts of fibrogenic cells present and decreased levels of fibrosis, we next sought to assess whether chronic imatinib treatment would benefit cardiac function at this time point.

3.3.5 Imatinib improves cardiac function in aged MDX mice

To assess whether the prevention of excess myocardial ECM deposition by continuous treatment with imatinib led to amelioration of cardiac dysfunction in aged mdx mice, echocardiography was performed at baseline (8 weeks) and after 6, 12 and 15 months of continuous treatment. The ratio of early diastolic velocity (Ea) to peak velocity with atrial contraction (Aa, Ea/Aa ratio), which is a sensitive measure of left ventricular diastolic function, was calculated. Although a progressive decline in function was observed up until the study endpoint at 15 months, it was only at the final time point that a significant different (p<0.05) in Ea/Aa ratio between untreated mdx and WT mice was detected (Figure 3.7). At this time point, however, we were also able to
observe a significant improvement in the Ea/Aa ratio of *mdx* mice that had been treated with imatinib, highlighting the potential functional benefits of reduced tissue fibrosis in this pathological state.

### 3.4 Discussion

Currently there are no effective treatments for DMD. It is expected that further understanding of the underlying cellular processes which direct its degenerative pathologies could lead to new targeted therapies. Building on previous work implicating the role of tissue-resident mesenchymal progenitors in the development of skeletal muscle and cardiac fibrosis (Ieronimakis et al., 2013; Ito et al., 2013; Trensz et al., 2010; Uezumi et al., 2011), we have demonstrated for the first time that oral administration of imatinib via drinking water is an effective way to elicit its anti-fibrotic effects and prevent the development of chronic cardiomyopathy in aged *mdx* mice. Matching previous studies that looked at earlier time points and used different delivery methods (Bizario et al., 2009; Huang et al., 2009), this treatment methodology was able to both reduce fibrosis in the diaphragm and the heart, and lead to a significant reduction in the total amount of PDGFRα+ Sca-1+ cMSCs in the myocardium. Finally, to assess the therapeutic relevance of these cellular and histological effects, we demonstrated that chronic treatment with imatinib was able to preserve left ventricular diastolic function in aged *mdx* mice.

Similar to previous studies that have characterized the time-dependent effects of the deletion of the dystrophin gene on cardiac function, we observed that *mdx* mice displayed impaired cardiac
function and diffuse myocardial fibrosis at 15 months of age. Unlike others, we did not observe a significant impairment of cardiac function at 12 months of age, perhaps due to inter-operator differences in echocardiography methodologies or gender influences as we only used male mice and gender differences in mdx mice has been reported (Bostick et al., 2010). It could be expected that use of more refined echocardiography methodologies such as speckle based tracking, which has been shown to be more sensitive to early changes in cardiac functional parameters, would improve our ability to detect beneficial effects at earlier time points (Spurney et al., 2011a). Despite this, the demonstration that long-term imatinib treatment is able to significantly ameliorate impaired cardiac function in mdx mice provides validation of an oral delivery method that has been demonstrated to work efficiently with a number of other compounds (Adamo et al., 2010; Spurney et al., 2011b; Van Erp et al., 2006) but, until now, not with imatinib in the context of muscular dystrophy. We were also able to observe beneficial effects on the degree of tissue fibrosis present in the diaphragms of mice at 15 months of age, which differs from a similar study utilizing losartan treatment for 2 years (Spurney et al., 2011b), supporting the hypothesis of increased efficacy of anti-fibrotic effects with imatinib at the selected dose.

There continues to be great preclinical progress made in developing therapies to target the underlying cause of muscular dystrophy, such as use of gene therapy to restore expression of dystrophin (Alter et al., 2006; Bostick et al., 2011; Yue et al., 2004), but evidence supporting the use of existing and approved pharmacological therapies has the potential result in benefit to a great number of patients in the nearer term. Although there have been previous reports of the cardiotoxicity of imatinib (Hu et al., 2012; Kerkela et al., 2006), subsequent studies have revealed the importance of dosage of the drug in preclinical studies and have revealed difficulties
in translating murine studies into human clinical outcomes (Breccia, 2011; Wolf et al., 2010).

Given that imatinib has been shown to prevent fibrosis in a wide variety of different disorders in both rodents (Daniels et al., 2004; Distler et al., 2007; Kuo et al., 2012; Ma et al., 2012) and humans (Gordon and Spiera, 2011), there is a strong rationale at this point to evaluate the effectiveness of this compound in preventing the development of cardiomyopathies in DMD patients.
Figure 3.1. Schematic and mortality of chronic imatinib treatment in mdx mice
A. Experimental methodology. 8 week mdx mice were randomized to either treated (imatinib) or untreated groups. Imatinib was delivered via drinking water for up to 15 months.
B. Mortality of mdx mice during imatinib study. Survival of four experimental groups (n=6-16) WT (CTL), WT (Imatinib), mdx (CTL) and mdx (Imatinib) was followed for duration of study. No significant difference in mortality was detected.
Figure 3.2. Continuous imatinib treatment leads to reduced skeletal muscle fibrosis in mdx mice

A,C Imatinib prevents the development of mdx age-dependent skeletal muscle fibrosis. Adult mdx mice were treated with imatinib (50mg/kg/day) 15-months in the drinking water. Mice were perfused with 4% PFA and diaphragms excised. Masson’s Trichrome Stain was performed on 10µm sections and fibrosis visualized. * - p<0.05 vs. indicated grouping, n=5, student t test

B,D. Imatinib reduces the accumulation of PDGFRα+ cells in the diaphragm of aged mdx mice. Adult PDGFRα:EGFP:mdx mice were treated with imatinib (50mg/kg/day) 15-months in the drinking water. At experimental endpoint, mice were perfused with 4% PFA and diaphragms excised. GFP+ cells and diaphragm width were assessed in 10µm sections. * - p<0.05 vs. indicated grouping, n=5, student t test.
Figure 3.3 - Imatinib prevents the development of mdx age-dependent cardiac fibrosis.
Adult mdx mice were treated with imatinib (50mg/kg/day) 15-months in the drinking water. At experimental endpoint, mice were perfused with 4% PFA and hearts excised. Masson’s Trichrome Stain was performed on 10µm sections and fibrosis visualized. Collagen was quantified using ImageJ. * - p<0.05 vs. indicated grouping, n=3, student t test
Figure 3.4 - Chronic imatinib treatment does not affect macrophage polarity in aged *mdx* mice.

Hearts of mice from the following experimental groups: WT Young (8 week), MDX Young (8 week), MDX old (15 month) and MDX old + imatinib (15 month) were excised and enzymatically digested. Relative percentage of Ly6c+ cells was quantified within CD45+ cells. * - p<0.05 vs. indicated grouping, n=4, student t test
Figure 3.5 Chronic imatinib treatment down not affect mature TGF-β1 levels in the hearts of aged mdx mice.
Hearts of mice from the following experimental groups: WT Young (8 week), MDX old (15 month) and MDX old + imatinib (15 month) were excised and enzymatically digested. Concentration of mature TGF-B1 was quantified using Platinum ELISA kit (eBioscience) according to manufacturers’ instructions.
Figure 3.6 - Chronic imatinib treatment reduces the quantity of cMSCs and CFs within the aged mdx myocardium.
Hearts of mice from the following experimental groups: WT Young (8 week), MDX old (15 month), MDX old + imatinib (15 month), WT Old (15 month) and WT Old + imatinib (15 month) were excised and enzymatically digested. Quantity of cMSCs (PDGFRα+ Sca-1+) and CFs (PDGFRα+ Sca-1-) were divided by the total pre-digestion wet weight of hearts to calculate concentration (cell / mg tissue) for each group. * - p<0.05 vs. indicated grouping, n=3-5, student t test
Figure 3.7 - Chronic imatinib treatment prevents age-related decline in cardiac function in *mdx* mice.
Cardiac function of WT and MDX mice (untreated = regular drinking water, treated = drinking water + imatinib) was assessed at 8 weeks (baseline), 6 months, 12 months and 15 months. Ea/Aa ratio was calculated by tissue doppler echocardiography. * - p<0.05 vs. indicated grouping, n=8-16, student t test.
Treatment of mdx mice with imatinib delivered via drinking water for 15 months was able to prevent age-related increases in Sca-1+ cMSCs as well as the development of cardiac fibrosis. It also lead to improved heart function at 15 months of age as evidenced by increased Ea/Aa ratio.

Figure 3.8 – Schematic of experimental findings in Chapter 3
Chapter 4: Tissue-resident PDGFRα+ Sca-1+ mesenchymal progenitors are the cellular source of fibrofatty infiltration in arrhythmogenic cardiomyopathy

4.1 Introduction

Arrhythmogenic cardiomyopathy (AC) is a heterogeneous disease of the heart associated with an increased risk of both ventricular arrhythmias and sudden cardiac death. Although the exact pathogenesis of this disease is unknown, mutations in genes coding for the five major proteins of the desmosome – namely, plakoglobin, desmoplakin, plakophilin-2, desmoglein (Yang et al., 2006) and desmocollin-2 (Syrris et al., 2006) – have been strongly implicated. The penetrance of these desmosomal mutations in AC patients has been estimated to be between 40 and 90% by different studies (Cox et al., 2011; Kapplinger et al., 2011; Protonotarios et al., 2011), making identification of at-risk individuals by clinical genotyping difficult. As such, there is currently no single test to diagnose AC, although improved quantitative functional parameters associated with the disease as well as identification of pathogenic mutations in first-degree relatives have improved both the sensitivity and specificity of current diagnostic criteria (Marcus et al., 2010). Current therapeutic and management paradigms rely on symptomatic impact, including anti-arrhythmic drugs and lifestyle modifications, and it is hoped that probing the link between desmosomal gene defects and the progression of AC will lead to more effective disease-targeted therapies (Basso et al., 2012).
The name arrhythmogenic cardiomyopathy has gradually evolved since the initial classification of this disease in 1982, when it was called “right ventricular dysplasia” (Marcus et al., 1982) as it was thought to be caused by a developmental defect of the heart before birth. It was soon determined that the symptoms and signs were in fact a progressive cardiac disease, and thus dysplasia was replaced with “cardiomyopathy” (Thiene et al., 1988). Clinically, since the main symptoms that appear during the progression of the disease are right ventricular arrhythmias, the term arrhythmogenic right ventricular cardiomyopathy (ARVC) became the most commonly used name. With a growing body of evidence demonstrating left ventricular involvement in this disease (Nucifora et al., 2012; Sen-Chowdhry et al., 2008), the current terminology of arrhythmogenic cardiomyopathy (AC) was adopted in 2010 (Basso et al., 2012). Several disease patterns are encompassed by this definition, including right dominant, left dominant and biventricular AC (Basso et al., 2012; Marcus et al., 2010).

4.1.1 Pathophysiological mechanisms of arrhythmogenic cardiomyopathy

While several etiopathogenic theories for the development of AC have been proposed (Ananthasubramaniam and Khaja, 1998; Dokuparti et al., 2005), including dysontogenic (dysplasia) (James et al., 1996), apoptotic (Mallat et al., 1996; Runge et al., 2000) and transdifferentiative (d'Amati et al., 2000) processes, it is most widely accepted today that degenerative and dystrophic mechanisms underlie its progression (Basso et al., 2012). This latter model, proposed well before the discovery of associated desmosomal mutations, draws from histopathological similarities between AC and skeletal-muscular dystrophies, which are both characterized by progressive muscle damage with associated replacement with fibrofatty
connective tissue. Experimental data have demonstrated that cardiomyocyte death, either by apoptosis or necrosis, is the primary initiating trigger that eventually is followed by fibrofatty replacement of functional myocardium (Li et al., 2011; Pilichou et al., 2009). The molecular pathways that underlie the progressive loss of cardiomyocytes in AC continue to be investigated (reviewed in (Shirokova and Niggli, 2012), with the ultimate goal of developing targeted preventives or therapies. It should be noted that while the presence of fibrofatty tissue is pathognomonic for this disease, Burke et al. (Burke et al., 1998) suggest that the fibrofatty infiltration is most likely secondary to associated desmosomal gene mutations. Progressive loss of cardiomyocytes due to mutated desmosomal gene products may activate reparative processes and lead to progressive accumulation of diffuse fibrofatty tissue, with concomitant alterations in cardiac electrophysiological and contractile function. Although it is widely accepted that a key pathological link between desmosomal mutations and the often fatal ventricular arrhythmias of this disease is the development of the characteristic fibrofatty infiltrate, the cellular source of the fibroblasts and adipocytes that compose this connective tissue currently remains unclear. Two recent studies in murine models of the disease have implicated cardiac progenitor cell (CPC) populations as the most likely candidate, but noted that further work was necessary to clearly identify the cells involved (Lombardi et al., 2011; Lombardi et al., 2009).

4.1.2 The hypothesis

We propose that reparative processes in the heart triggered by myocardial dystrophy lead to the differentiation of tissue-resident cardiac Sca-1+ PDGFRα+ mesenchymal stromal progenitors (cMSCs) into both fibroblasts and adipocytes, resulting in the characteristic fibrofatty lesion
observed in AC. Here we present preliminary evidence supporting this hypothesis and demonstrating that cMSCs can give rise to adipocytes in the myocardium following deletion of a transcription factor that regulates quiescence in mesenchymal cell populations.

Recent genetic analyses of AC patients (Basso et al., 2009) have identified the causative role of desmosomal mutations in its progression and have aided in the clinical diagnosis of the disease, but it has been the development of numerous in vivo transgenic murine models of AC (Pilichou et al., 2011) that have greatly furthered our fundamental understanding of the disease.

Utilizing lineage tracing and genetic fate mapping in a murine model of AC, Lombardi et al. showed recently that second heart field CPCs are a source of adipocytes (Lombardi et al., 2009) in a murine model of AC. The second heart field is a developmental definition that identifies the progenitors that give rise to the last region of the myocardium added to the outflow tract. Through use of a series of conditionally expressed reporter strains, which concomitantly delete the desmosomal protein desmoplakin in cardiac myocyte lineages and permanently activate yellow fluorescent protein expression in the deleted cells, this group elegantly demonstrated a contribution of second heart field CPCs to adipogenesis. Further, they provided strong evidence implicating perturbations in Wnt/Tcf7l2 signaling as a molecular mechanism underlying this progression. Although these experiments have provided compelling data to support the molecular mechanisms governing the development of fibrofatty scar tissue in AC, other than demonstrating the involvement of Isl-1+ second heart field progenitors, the lineage tracing strategies were unable to provide substantive evidence as to the identity of cells involved in fibrofatty scar development. Further, they were unable to conclude that the second heart field CPCs are the sole
cellular source since the Cre-drivers used (Nkx2.5, Mef2C, α-MyHC) are unable to distinguish the involvement of pericytes, fibroblasts or circulating cells.

It was demonstrated previously that CPCs identified using the marker Sca-1 can give rise to adipocytes *in vitro* (Matsuura et al., 2004), yet evidence implicating Sca-1+ CPCs as the cellular source of adipocytes in models of AC *in vivo* is still lacking. Lombardi et al. (Lombardi et al., 2011) have identified a role for Sca-1+ CPCs in the enhanced adipogenesis of AC mice harbouring mutations in the desmosomal protein plakoglobin. They did not, however, perform the strict lineage tracing experiments required to quantitatively assess the relative contribution of CPCs to the adipogenesis in their models. The long standing dogma of AC being primarily a right ventricular disease was supported by demonstration of the involvement of second heart field progenitors, but this proposed model of AC fails to accommodate recent reports of similar pathological processes in the left ventricle (d'Amati et al., 2000; Michalodimitrakis et al., 2002). Indeed, data demonstrating that fibrofatty scarring and functional deterioration occur in both ventricles in AC argues that the progenitor cell subset responsible for generation of fibroblasts and adipocytes in this disease is distributed throughout the heart. If such is true, a population of subepicardial progenitors may be a good candidate (Gittenberger-de Groot et al., 2010). Such a model is strongly supported by recent studies demonstrating that both the murine (Chong et al., 2011) and human (Chong et al., 2013) hearts harbour a population of pro-epicardially-derived tissue-resident mesenchymal progenitors, which express Sca-1 and PDGFRα. These studies demonstrated the broad trans-germ layer differentiative capacity of this cardiac-resident progenitor population (Chong et al., 2011) and characterized their presence in both fetal and diseased human myocardium (Chong et al., 2013), but did not thoroughly investigate their role in
regeneration and repair of the heart. The notion that the heart harbours a population of Sca-1+ PDGFRα+ mesenchymal progenitors able to generate both fibroblasts and adipocytes aligns with the hypothesis that this population is the most significant cellular source of fibrofatty infiltration in AC.

Although fibrofatty replacement of the myocardium is the hallmark feature of AC, the consistent observation of small lymphocytic foci in the disease (Campian et al., 2010; Campuzano et al., 2012; Jordan et al., 2010) further supports the involvement of cardiac-resident Sca-1+ PDGFRα+ mesenchymal progenitors in its progression, as these progenitors have been recently shown to contribute *in vivo* to lymph node stroma (Benezech et al., 2012) and follicular dendritic cells (Krautler et al., 2012). Thus, the milieu of chronic myocardial inflammation may trigger their differentiation not just into adipocytes and myofibroblasts, but also possibly into cells capable of attracting and supporting lymphocytes.

Although studies in murine models of AC employing the strict lineage tracing methods required to determine the cellular source of adipocytes have yet to be performed, studies in skeletal muscle (SM) further support our hypothesis in implicating Sca-1+ PDGFRα+ tissue-resident mesenchymal progenitors. Recent studies (Joe et al., 2010; Uezumi et al., 2010) accomplish the prospective isolation and purification of a population of SM resident Sca-1+ PDGFRα+ mesenchymal progenitors, which were further shown to be the sole SM-derived population with fibro-adipogenic potential. Additional evidence from *mdx* mice, a murine model of Duchenne muscular dystrophy (Uezumi et al., 2011), strongly supports the hypothesis that tissue-resident Sca-1+ PDGFRα+ cells are the principal cell population involved in the generation of fibrofatty
scars in situations of chronic muscle damage. Interestingly, a robust regenerative capacity of SM was demonstrated by these studies, and others (Mathew et al., 2011; Murphy et al., 2011), to be at least partly due to paracrine roles of these tissue-resident mesenchymal progenitors. In light of this evidence, the therapeutic potential of modulating proliferation and differentiation of cardiac Sca-1+ PDGFRα+ progenitor cells in situations of acute or chronic damage is certainly intriguing. With a large body of clinical evidence demonstrating the beneficial effects of transplanting bone marrow derived mesenchymal stromal cells (MSCs) into patients afflicted with a variety of cardiovascular disorders (Clifford et al., 2012), and numerous data highlighting the similarities between MSCs derived from different tissues (Pelekanos et al., 2012), the identification of a tissue-resident counterpart presents an attractive candidate for pharmacological modulation. Such manipulation could lead to therapeutic benefits, similar to those observed with exogenous delivery of similar cells but devoid of the adversities stemming from \textit{ex vivo} manipulations, including (but not limited to) transplantation, immunogenicity issues and challenges involved in the use of GMP facilities for clinical-grade cell preparations.

\textbf{4.1.3 Further research required to test the hypothesis}

In order to unequivocally demonstrate the contribution of cardiac-resident Sca-1+ PDGFRα+ mesenchymal progenitors to fibrofatty scarring in AC, a number of questions must be addressed.

First, a more thorough characterization of these progenitor cells must be performed to determine both their functional role in health and disease, as well as determine the homogeneity of this population. It is very possible that within this phenotypic identity, several functionally different
cellular subsets are present, and the unravelling of these hierarchies could provide significant insight into cellular processes in the regenerating or degenerating myocardium. Dularoy et al. (Dulauroy et al., 2012) identified a fibrogenic subpopulation within PDGFRα+ Sca-1+ progenitor in the SM using ADAM12+, highlighting heterogeneity, and supporting the need to further distinguish between functional subsets of this mesenchymal population. It will also be highly important to determine whether the desmosome plays a direct role in PDGFRα+ Sca-1+ progenitor cell function, or whether the arrhythmogenic reparative disorder observed in AC is to be ascribed solely to continued loss of cardiomyocytes due to desmosomal gene defects.

Second, further lineage tracing experiments using several inducible Cre-drivers such as PDGFRα-CreER in combination with either previously described (Pilichou et al., 2011) or novel models of AC will allow identification of the role of this population in AC.

Finally, the limitations of the murine Sca-1 (Ly6A/E) as a marker of tissue-resident progenitors should be addressed since there is currently no known human homolog for this gene. Such is the case despite the hypothesis that a broad range of functions mediated by this marker are probably assumed by other Ly6 proteins in humans (Holmes and Stanford, 2007).

4.1.4 Consequences of the hypothesis

With no current treatment available for AC, it is highly attractive to consider that manipulation of molecular mechanisms underlying the development of the cardiac fibrofatty infiltration could mitigate both functional deterioration as well as survival of AC patients. Clinical trials in patients
suffering from Duchenne muscular dystrophy utilizing anti-fibrotic strategies may provide valuable insight into the beneficial effects of preventing development of fibrofatty scars in the functional myocardium (Lehmann-Horn et al., 2012). Additionally, with several recent reports describing in vivo reprogramming of cardiac fibroblasts into functional cardiomyocytes (Jayawardena et al., 2012; Qian et al., 2012; Song et al., 2012), it could be postulated that targeting of existing fibrofatty scars could be therapeutically beneficial. With a growing recognition of functional and theoretical overlap between fibroblasts and mesenchymal stem cells (Haniffa et al., 2009; Hematti, 2012) there is a strong possibility that the beneficial effects seen in recent cardiac studies are due to reprogramming of these tissue-resident progenitor cells. Recent technical advances involving patient-specific induced pluripotent stem cells (iPSCs) (Kim et al., 2013), as well as further development of aforementioned reprogramming strategies, may enable novel targeted approaches to specific cell populations with enhanced transdifferentiative capacities, and could provide a basis for next-generation therapy of AC.

In summary, recent evidence that the murine heart harbours a population of Sca-1+ PDGFRα+ mesenchymal progenitors has provided new directions for defining the cellular source of the fibrofatty infiltrate that is characteristic of AC. A growing understanding of the role of tissue-resident mesenchymal progenitors in numerous other tissues, most notably skeletal muscle, offers strong support for our hypothesis. Potential avenues for novel targeted therapeutics may emerge to benefit patients with AC.
4.2 Methods

4.2.1 Mice and surgery

Mice were housed in an enclosed, pathogen-free facility, and experiments were performed in accordance with University of British Columbia Animal Care Committee regulations. Both male and female C57BL/6 mice (>8 weeks old) were used in all experiments unless otherwise specified. Hic-1-CreERT2:R26R-LSL-TdTomato and Hic-1-floxed:UBCCreERT2 mice were provided by Dr. Michael Underhill (Biomedical Research Centre, University of British Columbia). To experimentally induce myocardial infarctions, mice were intubated, anesthetized and their left anterior descending artery ligated. EdU (Life Technologies) was given for indicated time periods at 1mg/mouse injected intraperitoneally. To induce Cre drivers, tamoxifen (2mg/day, i.p.) was delivered for 5 days (Hic1-CreERT2) or 3 days (UBCCreERT2).

4.2.2 Immunohistochemistry

Mice received an intraperitoneal injection of 0.5 mg/g Avertin, and were perfused transcardially with 20 ml PBS/2 mM EDTA, followed by 20 ml of 4% paraformaldehyde (PFA). The excised muscles were fixed in 4% PFA overnight. The following day, the tissue was either prepared for paraffin embedding following the standard sequence of dehydration steps in ethanol, or transferred to 20% sucrose PBS and incubated overnight for subsequent OCT embedding and freezing. Standard methods were followed for cryosectioning. Tissue sections were
permeabilized in 0.3% Triton X-100 (Sigma) in PBS, and blocked for 1 h at room temperature in PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100, 3% bovine serum albumin (BSA) and 0.1% NaN3. Cells were stained overnight at 4 °C using a primary antibody diluted in 10% NGS, 0.3% Triton X-100, 3% BSA and 0.1% NaN3. The primary antibodies used for immunofluorescence are listed in Table S1 of Appendix A. In all cases, the primary antibody was detected using secondary antibodies conjugated to Alexa 488, 555, 594, or 647 (Molecular Probes). Confocal microscopy was performed using a Leica SP5X White Light Laser Confocal Microscope equipped with a Koheras white light laser source whose emission covers the 470-670nm spectrum of wavelengths, and a tunable prism-based spectral detector and a GaAsP hybrid detector. Figures were assembled using Illustrator CS3 (Adobe) and Photoshop CS3 (Adobe).

4.3 Results

4.3.1 Hic-1 labels proliferating cMSCs following LAD ligation

Hic-1 (Hypermethylated In Cancer 1) is a tumor suppressor gene that encodes a transcriptional repressor with multiple targets and well known roles in several broad biological roles, including control of cell growth, survival, migration and motility (Rood and Leprince, 2013). Although it is most well known for its role in cancer initiation and progression, it has also recently been shown to specifically label mesenchymal cells in numerous tissues, and has been demonstrated to show a high degree of overlap with PDGFRα expression in the heart (data unpublished, Michael Underhill). Utilizing a transgenic murine strain harboring Hic-1-CreERT2:R26R-LSL-TdTomato
transgenes, we were able to induce permanent labelling of a population of cells through the myocardium with tamoxifen (Figure 4.1) that mimicked previous observations of physiological distribution of PDGFRα:EGFP+ cells in the heart (Figure 4.1). Experimental induction of a myocardial infarction led to massive proliferation of Hic-1-Tomato+ cells within the ischemic region in a manner that was highly similar to the proliferative response observed in PDGFRα+ cells in Chapter 2 (Chapter 2: Figure 2.3). Given the observed labeling overlap and high degree of similarity to cMSC distribution within the myocardium, we next sought to genetically manipulate this population by deleting the Hic-1 locus.

4.3.2 Genetic deletion of Hic-1 leads to hyperproliferation of cMSCs and de novo generation of adipocytes within the myocardium

Previous work in Dr. Michael Underhill’s group has demonstrated that genetic deletion of floxed Hic-1 using tamoxifen-inducible Cre in Hic-1-floxed:UBCCreERT2 mice led to a hyperproliferative response in mesenchymal cell populations, leading to their eventual accumulation within the interstitial space of a variety of organs. In the heart, deletion of Hic-1 led to the progressive accumulation of labelled cells in the interstitial space (data unpublished, Michael Underhill). Three months following tamoxifen treatment to induce deletion, the emergence of perillipin+ adipocytes was observed in the myocardium of Hic-1-floxed:UBCCreERT2 mice, and these cells were predominantly located in the proximity of the endocardial surface (Figure 4.2). Based on the overlap between Hic-1 and PDGFRα labelled cells (Figure 4.1), this experimental evidence supports the hypothesis that PDGFRα+ Sca-1+ cMSCs are the cellular source of the fibrofatty infiltrate in AC, as hyperproliferation is
postulated to lead to accumulation and eventual differentiation into adipocytes in this model (Figure 4.3).

4.4 Discussion

Arrhythmogenic cardiomyopathy (AC) is a disease of the heart involving myocardial dystrophy leading to fibrofatty scarring of the myocardium, and is associated with an increased risk of both ventricular arrhythmias and sudden cardiac death. It often affects the right ventricle but may also involve the left. Although there has been significant progress in understanding the role of underlying desmosomal genetic defects in AC, there is still a lack of data regarding the cellular processes involved in its progression. The development of cardiac fibrofatty scarring is known to be a principal pathological process associated with ventricular arrhythmias, and it is vital that we elucidate the role of various cell populations involved in the disease if targeted therapeutics are to be developed. The known role of mesenchymal progenitor cells in the reparative process of both the heart and skeletal muscle has provided inspiration for the identification of the cellular basis of fibrofatty infiltration in AC. Here we provide preliminary data in support of the hypothesis that reparative processes triggered by myocardial degeneration lead to the differentiation of tissue-resident cardiac mesenchymal stromal cells (cMSCs) into adipocytes and fibroblasts, which compose the fibrofatty lesions characteristic of AC.

In the absence of a robust model of AC, we utilized an available transgenic strain, Hic-1-floxed:UBCCreERT2 mice, in which tamoxifen induced Cre expression to delete Hic-1, a transcription factor that has been shown to regulate quiescence specifically in mesenchymal
cells. Although these conditions do not entirely mimic the chronic dystrophic processes that lead to the formation of fibrofatty infiltrate in AC, the hyperproliferative effects of this deletion do lead to the emergence of adipocytic infiltration within the myocardium. Given that this deletion is specific to the mesenchymal subset of the heart, which includes PDGFRα+ Sca-1+ cMSCs and PDGFRα+ Sca-1- CFs, the atypical emergence of adipogenic cells within the myocardium is indicative of the in vivo PDGFRα+ cells to differentiate into this cell type. This data supports our hypothesis of cMSCs being the cellular source of fibrofatty infiltrate in AC, although further experimentation using a specific deletion of desmosomal proteins in conjunction with lineage tracing of cMSCs using PDGFRα or Sca-1 Cre- drivers remains the definitive experimental proof of this hypothesis.
Figure 4.1. LAD ligation in Hic-1-CreERT2:R26R-LSL-TdTomato transgenic mice leads to widespread labelling within the ischemic myocardium. Following tamoxifen induction to activate Tomato expression under the Hic-1 locus, transgenic Hic-1-CreERT2:R26R-LSL-TdTomato mice underwent LAD ligation surgery. 7 days post infarction, hearts were excised and tomato expression viewed in 10µm section utilizing a Leica white light confocal microscope.
Figure 4.2. Hic-1 deletion leads to the emergence of adipocytes within the myocardium. 5 months following deletion of Hic-1 expression in Hic-1+ cells using a tamoxifen-inducible Cre-driver combined with a floxed Hic-1 locus, hearts were excised and perfused with 4% PFA. 10µm sections were stained with perillipin (red) antibodies and visualized with Dapi (blue) on a spectral scanning confocal microscope.
Figure 4.3. Proposed model depicting the role of cMSCs in the development of fibrofatty infiltrate in arrhythmogenic cardiomyopathy
Chapter 5: Discussion and conclusion

5.1 General conclusions

Cardiac fibrosis is a pathological condition that is manifested in most cardiac diseases and, despite its deleterious effects on the function of the heart as well as its contributing role to patient mortality, there are currently no effective therapies for its treatment. Longstanding dogma in the field of cardiovascular research has dictated that cardiac fibrosis results from the excessive deposition of ECM components, including collagen, by a population of cells known as cardiac fibroblasts and their differentiated progeny, myofibroblasts. Progress in the development of new therapeutics to target this pathological condition has been severely hampered by a lack of understanding regarding the cellular identity of cardiac fibroblasts, as well as the mechanisms involved in their expansion and differentiation after injury. The goal of this thesis is to provide much-needed insight into the role of tissue-resident mesenchymal progenitors, identified as cardiac PDGFRα+ Sca-1+ mesenchymal stromal cells (cMSCs), in the development of cardiac fibrosis.

In Chapter 2, we presented evidence that cardiac mesenchymal stromal cells (cMSCs) possess a number of classical characteristics of MSCs in other tissues and are highly enriched for a number of ECM related genes, highlighting their involvement in excess deposition of extracellular matrix. cMSCs were demonstrated to be activated to proliferate after acute ischemic injury and subsequently differentiate, losing Sca-1, into collagen-producing cardiac fibroblasts (CFs). Further, we demonstrated that treatment with the
PDGFRα/c-abl/p38 inhibitor nilotinib was able to block both the proliferation and differentiation of cMSCs, leading to preserved cardiac function after ligation of the left anterior descending artery (LAD).

In Chapter 3, we built on the model of cMSCs’ involvement in cardiac fibrosis by investigating their role in the development of cardiomyopathy in mdx mice, a murine model of Duchenne Muscular Dystrophy (DMD). Continuous treatment with imatinib mesylate, an anti-fibrotic compound, was able to partially prevent the development of cardiac fibrosis in mdx mice at 15 months of age. Flow cytometry and immunohistochemistry were used to demonstrate that the quantity and concentration of cMSCs was reduced in both the diaphragms and hearts of the mice, indicating that treatment with the inhibitor was able to reduce the accumulation of ECM-associated PDGFRα+ cells over the life of the animals. This inhibitor had not been studied in mdx mice using the oral delivery model we used to achieve long-term administration. Our data demonstrate a beneficial effect to cardiac function at the 15 month study endpoint, suggesting that these inhibitors should be investigated in human clinical trials.

Finally in Chapter 4, based on observations made in skeletal muscle in the context of highly similar degenerative processes, we hypothesized that the characteristic fibrofatty infiltrate observed in arrhythmogenic cardiomyopathy likely arises from the differentiation of cMSCs due to chronic injury. In the absence of an available murine model of AC based on the desmosomal mutations common in this disease, we were able to gain insight in another murine model that develops adipocyte infiltration within the
myocardium. Genetic deletion of Hic-1, which was shown to be expressed in mesenchymal cells in the myocardium both before and after a LAD ligation, led to proliferation of the labeled subset and eventual accumulation of adipocytes within the endocardium. These observations provide much needed insight into the cellular processes by which a fibrofatty infiltrate arises in AC and highlights potential pathways by which this pathological process could be mitigated.

Taken in its entirety, the data and interpretation included herein demonstrate that:

1. Cardiac-resident PDGFRα+ Sca-1+ cells (cMSCs) represent a population of mesenchymal progenitors that are activated to proliferate and differentiate into collagen secreting PDGFRα+ Sca-1- cardiac fibroblasts (CFs) after cardiac injury.

2. Both nilotinib and imatinib, kinase inhibitors that inhibit the activity of PDGFRα as well as that of c-abl and p38 downstream of TGFβ, exert anti-fibrotic effects through the blockade of proliferation, differentiation and accumulation of cMSCs and their more differentiated progeny.

3. Preliminary evidence supports the hypothesis that cMSCs are a cellular source of fibrofatty infiltrate in the heart, and likely involved in the clinical manifestation of this pathology that is observed in arrhythmogenic cardiomyopathy (AC).
5.2 The role of PDGFRα+ Sca-1+ cMSCs in the fibrotic response after acute and chronic myocardial injury

Greater clarity has emerged regarding the diversity contained within the mesenchymal component of the heart in recent years. Studies demonstrating the existence of cMSCs in both rodents (Chong et al., 2011; Chong et al., 2013) and humans (Chong et al., 2013), the importance of this population in development (Acharya et al., 2012; Furtado et al., 2014b; Smith et al., 2011) and disease (Ieronimakis et al., 2013), as well as evidence demonstrating their similarities to other mesenchymal populations within both the heart (Furtado et al., 2014b) and other tissue (Pelekanos et al., 2012), have all contributed to this newfound understanding. Still, details are lacking regarding the role of cMSCs in the response to ischemic injury in the heart and, more specifically, the resulting development of cardiac fibrosis. The data included herein provide first insights into mechanistic details of the involvement of tissue-resident PDGFRα+ Sca-1+ mesenchymal progenitors in reparative processes in four different models of cardiac injury, namely isoproterenol treatment, LAD ligation, chronic cardiomyopathy present in muscular dystrophy, and the development of a cardiac fatty infiltrate.

Following an ischemic injury, the stepwise progression from inflammation towards an eventual mature fibrotic scar has been documented and investigated extensively (Shinde and Frangogiannis, 2014). Although the role of cardiac fibroblasts in this process is well-
known, lacking a clear understanding of the phenotypic identity of this population has severely limited our ability to translate our growing understanding of this role into new therapeutics. Standard medical therapies for acute myocardial infarction include a number of pharmacologics that deal with several of the complications of loss of viable myocardium such as decreased contractility, increased blood pressure, myocardial remodeling and eventual heart failure. Unfortunately, the ability to manipulate the pathophysiological processes that underlie the development of these complications has remained a therapeutic prospect. Data contained herein that demonstrate the role of cMSCs in generating mature CFs following ischemic injury provides a novel therapeutic pathway in which the development of cardiac fibrosis could be manipulated to the benefit of cardiac function. Indeed, in both an experimental MI model (LAD ligation) and chronic dystrophic model (mdx mice), we were able to observe improved cardiac function following treatment with TKIs with previous demonstrated anti-fibrotic properties, imatinib and nilotinib. Providing insight into the cellular processes following cardiac injury is thus clearly a priority in the field and it is the hopes of this author that these insights will be further expanded in a number of research directions proposed below.

The results found in Chapter 2 support results found in three recent research reports documenting the developmental origins of cardiac fibroblasts and their roles in reparative responses of the heart (Ali et al., 2014; Furtado et al., 2014b; Moore-Morris et al., 2014a), and expand them in a novel direction. Similar to what has been reported in other tissues (Barisic-Dujmovic et al., 2010) and echoing a growing consensus in the field, we were unable to observe any significant contribution of circulating cells to the generation of
collagen-producing fibroblasts following an ischemic injury in parabiotic mice. However, it is important to note a difference in the transgenic Colla1-3.6GFP mouse strain used in this thesis (Kalajzic et al., 2002) and Collagen-GFP transgenics used in the above noted reports. While we were only able to detect a very low level of GFP expression in steady state conditions, other reports observed widespread GFP expression driven by collagen 1a1 through the myocardium (Acharya et al., 2012) due to a different section of the colla1 promoter being used in the development of the transgenic, which was selected for the highest level of GFP expression (Lin et al., 2008). While our model lacked baseline sensitivity and exhibited differing kinetics of GFP as compared to collagen 1a1 expression by immunohistochemistry (Figure 2.4), this may have allowed us to better detect differences due to experimental treatments because of a lesser risk of saturation of GFP expression (i.e., all cells expressing GFP). Further, unpublished RNAseq data from our lab demonstrating that the GFP+ cells express a coordinated fibrogenic transcriptional program support the notion that this system is only activated when cells differentiate towards the fibrogenic lineage. This allows the utilized Collagen-GFP to mark this coordinated response, and provide a tool to study its modulation. Thus, although stain differences may result in difficulties in comparing effects of treatments between papers, the utilized strain is effective in assessing the effect of in vivo nilotinib treatment on collagen expression at a cellular level.

Although we were able to observe similar effects on the development of cardiac fibrosis and preservation of function with both therapeutics (imatinib and nilotinib), there were differences. Both in vivo and in vitro evidence in Chapter 2 supports the notion that
cMSCs expand after injury and subsequently generate mature CFs, and that this progression can be blocked using pharmacological treatment with nilotinib. Data obtained in aged mdx mice is somewhat at odds with this model, as in fibrotic hearts obtained from 15-month mice there was a drastic shift towards the prevalence of cMSCs rather than CFs, and treatment with imatinib reduced overall numbers rather than skewing the ratio (as observed in both isoproterenol and LAD ligation damage models in Chapter 2). These differences could be in part attributed to the cause of myocardial damage that differed in the acute and chronic models utilized. It could be postulated that mild chronic damage models present a less inflammatory environment that supports expansion of cMSCs but not their differentiation into mature CFs as observed in a scar resulting from acute ischemic injury. Alternatively, the chronic conditions present in mdx mice could lead to differentiation of cMSCs into a more mature collagen-expressing phenotype without the loss of Sca-1 expression as seen in Chapter 2. Further experimentation would be necessary to unravel these differences and represents an important future research direction stemming from this work.

5.3 Future directions and research perspectives

The data contained herein provides much needed insight into the roles of PDGFRα+ cells in reparative processes of the heart and, further, raises several important questions that need be resolved before therapeutic benefit can be achieved. To the issue of heterogeneity within broadly defined cell populations, although we were able to provide evidence of differing roles and kinetics of PDGFRα+ Sca-1+ (cMSCs) and PDGFRα+ Sca-1- (CFs)
cells in the heart, it is highly likely that both of these groups can be further divided into functional sub-populations. With access to high resolution techniques such as single cell PCR (Liu et al., 2014) and mass cytometry (Tanner et al., 2013) becoming increasingly available, it is expected that the improved capacity for understanding cell behavior at an individual cell level, rather than a phenotypic population level, will allow for further unraveling of the aforementioned groups. Indeed, recent studies have revealed considerable heterogeneity within cardiac fibroblast populations (Furtado et al., 2014b), which often demonstrate highly overlapping functional roles utilizing relatively basic criteria to determine 1) identity and 2) function. Insights from other tissues such as skeletal muscle have provided evidence that there do exist distinct subsets (e.g. ADAM12+) within fibroblast populations that are largely responsible for the deposition of excess collagen in fibrotic states and, further, that these populations can be targeted for therapeutic benefit (Dulauroy et al., 2012). As further understanding of the differing cellular identities and their distinct roles in various physiological and pathological processes emerges, we can expect that further refined pharmacological strategies can be devised to exploit this important insight.

Crucial to these expected therapeutic strategies, additional tools must be developed that are able to specifically target cellular subpopulations and the molecular pathways they use in pathological states. Although we were able to demonstrate the functional benefit of TK inhibitors imatinib and nilotinib (Chapter 2, Chapter 3), these compounds are known to inhibit a wide variety of different pathways and, despite PDGFRα being included in this list, it is impossible to conclude based on our data that the benefits observed are due to
inhibition of this receptor. Additionally, all drug-based therapies present unique issues with dosing-based toxicity and, given reports of cardiotoxicity of some TKIs like imatinib (Kerkela et al., 2006), it is important that the clinical potency of these compounds be integrated into research efforts to evaluate their potential benefit to human patients. Use of more specific transgenic tools that allow for knocking out of specific components of pathways associated with fibrosis in cMSCs and CFs (e.g. PDGFs, SMADs, Wnt) in concert with studies that utilize genetic ablation strategies (e.g. through use of iDTR) to remove certain cell populations at crucial points of the reparative process will be of great value to this effort. Further, research efforts to develop new small molecule inhibitors and derivatives of existing pharmacologics that better target pathways and molecules highlighted in this thesis could potentially provide great improvements on the described effects of imatinib and nilotinib herein.

Focusing largely on the in vivo behavior of cMSCs and CFs following cardiac injury, another important area of research stemming from the data contained here is a more thorough and detailed study of cMSCs in vitro and the molecular signals that control their fate decisions. A better understanding of how crucial processes such as proliferation and differentiation are regulated within cardiac PDGFRα+ Sca-1+ progenitors would further refine efforts to develop new targeted pharmacological inhibitors of this process, as described above. Building on a large body of data describing the developmental origins of mesenchymal populations in the heart, recent studies demonstrating that epicardial progenitors can be generated from embryonic stem cells (Witty et al., 2014) provide evidence that such knowledge can be used to develop methodologies to create large
sources of cMSCs in vitro. The potential therapeutic potential of such methods is
discussed below.

5.4 Clinical implications

Utilizing two different clinically approved RTK chemotherapeutics, imatinib and
nilotinib, we were able to demonstrate a beneficial effect on cardiac function following
both acute and chronic injury. The clinical importance of such findings is very clear,
especially given the prevalence of cardiac fibrosis in most cardiac pathologies, and also
given that there are currently no clinically approved drugs for use as anti-fibrotic therapies
(Li et al., 2014). Although there has been some worry over TKIs such as imatinib due to
reported cardiotoxicity, most recent evidence has cast doubt on the validity of these earlier
studies due to the reported dosages used and clinical cohorts examined (Breccia, 2011;
Wolf et al., 2010). Outside of direct toxicity on cardiomyocytes, usage of anti-fibrotic
therapies must be done within the boundaries of how much ECM deposition is necessary
after injury, as several reports have demonstrated that complete abrogation of collagen
secretion can cause increases in mortality after induction of an experimental MI (Garcia et
al., 2007; Shimazaki et al., 2008). Taking heed of these concerns, it is the conclusion of
this thesis that there is therapeutic potential to modulate the excess deposition of ECM
after cardiac injury using pharmacological compounds which target signal transducers that
are present and active on tissue-resident mesenchymal progenitors after injury. The limits
of this modulation are not considered in this set of experiments and certainly warrant
further investigation.
The prospect of using cellular therapies to treat cardiovascular disease is an area of research that has been subject to considerable growth in recent years (Boudoulas and Hatzopoulos, 2009). Early data which reported the potential for bone marrow derived stem cell populations to differentiate into cardiomyocytes generated a large degree of excitement and led to a large number of research groups working on the area as well as the initiation of several clinical trials ((Bolli et al., 2011; Clifford et al., 2012). Although the notion that circulating cells can contribute to de novo formation of functional myocardial tissue to any significant level has largely been discredited (Balsam et al., 2004; Murry et al., 2004; Nygren et al., 2004), research into the use of bone marrow derived progenitors to treat cardiovascular disease continues and has been shown to be both safe and provide modest therapeutic benefit over existing therapies (Clifford et al., 2012). The mechanisms for such effects are generally considered to be due to a wide variety of paracrine roles that circulating cells play in aiding regeneration and modulating repair in damaged tissue, with much of these roles being attributed to bone-marrow derived mesenchymal stromal cells (BM-MSC). Given the highly transient presence of transplanted BM-MSCs within the heart (Mirotsou et al., 2011), it is thus of considerable interest that a population of tissue-resident MSCs have been described in the heart (cMSCs) as much of the observed beneficial effects derived in the transplantation of BM-MSCs is a result of their paracrine effects on cMSCs. Although the potential of isolating and expanding cMSCs to sufficient numbers to transplant back into the damaged myocardium has not yet been studied, based on the well-documented beneficial effects of BM-MSC transplantation after ischemic injury (Clifford et al., 2012), it is very possible that the pro-fibrotic environment that is present after an ischemic event would not direct their differentiation into collagen-
producing fibroblasts similar to their non-transplanted analogue residing in the tissue. This line of inquiry leads to an important and outstanding research question: what conditions ultimately dictate the ability of MSCs isolated from various tissues to play either positive or negative roles in regards to their effects on cardiac function following injury? With a growing body of evidence demonstrating that MSCs isolated from a number of sources – including BM, adipose tissue and dental pulp – can have beneficial effects on heart function after injury (Przybyt and Harmsen, 2013), it is important that we understand how these effects are achieved and the differing roles played by transplanted MSCs in injured tissue.

Another area of considerable interest is the transplantation of cardiac-derived stem cells or other stem cell populations to achieve true regeneration of the myocardium after injury. Although the field has been clouded by numerous definitions and continuing disagreement over the exact mechanisms of the modest cell turnover that has been demonstrated in the heart (the rate of which is also disagreed upon), there remains belief that this area of research contains the possibility to regenerate damaged myocardial tissue, a therapeutic prospect that cannot be achieved with existing therapies. With several clinical trials running utilizing differing cell sources and/or preparations, it has been highlighted that increased understanding of the interactions of different cardiac cell populations both before transplantation (\textit{in vitro}) and afterwards (\textit{in vivo}) must be achieved for the true potential of these therapies to be realized (Garbern and Lee, 2013). The recent demonstration that ES cells can be differentiated into epicardial progenitors is thus of considerable interest to the field (Witty et al., 2014), as it is expected that modeling \textit{in vivo}
conditions and generating physiologically relevant *in vitro* tissue systems which contain the numerous cell populations present in the adult myocardium will further improve the outcome of cell therapy strategies.

The elucidation of differing cellular roles within heterogeneous populations is, and will continue to be, an area of intense scientific scrutiny in the next five to ten years. It is expected that as our understanding grows of how individual cells interact, make fate decisions, and contribute to physiological and pathological processes in various tissues, this insight can be harnessed to greatly improve our ability to promote health and treat disease. The data contained within this thesis help shed light on the heterogeneity contained within the mesenchymal stromal subset of the heart and, further, demonstrate how a mechanistic understanding of reparative processes can be exploited to the benefit of cardiac function following injury. Moving past the importance of developing a greater understanding of such processes, it is also vital to be cognizant of the challenges in having this information properly accommodated in subsequent efforts to translate scientific knowledge into clinically available therapies, as well as to set policy agendas to facilitate this process. Although it is somewhat outside of the scope of the biological focus of this thesis, some broader societal considerations of how public perceptions of scientific progress can shape medical progress are included in Appendix 2, framed in the context of neurological diseases.
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Appendices

Appendix A  Supplementary tables and figures

Table A.1. List of antibodies used in immunofluorescence

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<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Rabbit anti-collagen 1</td>
<td>-</td>
<td>1/1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>(specific to Type 1</td>
<td>-</td>
<td>1/500</td>
<td>Serotec</td>
</tr>
<tr>
<td>collagen)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat anti-CD68</td>
<td>ED1</td>
<td>1/500</td>
<td>Serotec</td>
</tr>
<tr>
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<td>D7</td>
<td>1/500</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>Rabbit anti-αSMA</td>
<td>-</td>
<td>1/1000</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>Rat anti-perilipin</td>
<td>-</td>
<td>1/500</td>
<td>Sigma</td>
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<tr>
<td>Chicken anti-GFP</td>
<td>-</td>
<td>1/500</td>
<td>Abcam</td>
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Table A.2. List of antibodies used in flow cytometry

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<th>Conjugate</th>
<th>Dilution</th>
<th>Source</th>
</tr>
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<td>MEC13.3</td>
<td>APC</td>
<td>1/500</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Rat anti-CD45</td>
<td>30-F11</td>
<td>APC</td>
<td>1/500</td>
<td>BD Biosciences</td>
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<tr>
<td>Rat anti-Ly6a</td>
<td>D7</td>
<td>PeCy7</td>
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<td>eBiosciences</td>
</tr>
<tr>
<td>Rat anti-CD140a</td>
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<td>APC</td>
<td>1/1000</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>Rat anti-Ly6c</td>
<td>Hk1.4</td>
<td>PerCp</td>
<td>1/500</td>
<td>eBiosciences</td>
</tr>
</tbody>
</table>
Appendix B  Collision or convergence? Beliefs and politics in neuroscience discovery, ethics, and intervention

Democratizing decision-making

In the aim of democratizing the process by which health policy is formed, scholars and policy makers have begun to converge on the importance of including the voice of the public in agenda-setting and prioritization (Stirling, 2012). Strategies to create constructive public deliberation have focused on facilitating informed, effective and legitimate participation, and taken heed of the ways in which the process of developing evidence-based policy can go awry. Understanding the capacity of stakeholder capture in influencing public policy, a process in which groups exert undue influence over public participation given specific expertise and vested interests, is crucial to efforts to navigate the complex process through which public opinion influences policy. None could be more important than those that relate to emerging health policy domains for neurologic disease, which pose serious risk to both physical well-being and personal identity (Staton et al., 2012).

Many scholars have identified public trust in science as a vital component of the policy influence of stakeholder and patient groups, and have expounded on the policy implications at both the individual and societal level when trust is breached (Engdahl and Lidskog, 2012). For example, in 1999, the death of gene therapy patient Jesse Gelsinger led to a loss of both public and government trust with long-lasting implications on the
field. In 2007, the revelation that Dr. Hwang Woo-suk fabricated data from stem cell and human cloning research followed a similar trajectory for that field. The rapid pace of progress and emergence of new medical bio- and neuro-technologies continually test the boundaries of trust as a lack of public understanding of scientific progress can easily be exploited or misdirected.

In a comparative review of five cases chosen for their enduring or contemporary nature, and involving stem cells and neurodegenerative disease, blood flow intervention for multiple sclerosis, vaccines and autism, vegetative states and the right to life, and the Gulf War Syndrome (Box 1), we highlight this phenomenon in detail.

**Core challenges**

Neurological disease represents one of the most significant global sources of disability and its prevalence continues to grow as populations become older worldwide. Disorders associated with pathologies of the nervous system are often unique in their capacity to provoke strong emotional responses from patient groups on the numerous issues related to social perception and treatment of them. The tendency for public opinion to influence government action on health policy decisions related to neurological disease is a common and rising phenomenon. The cases we highlight here variously illustrate how calculations of health risk and consent, considerations of health privacy, and decision-making alongside the involvement of caregivers, create a perfect storm for public disenchantment with the scientific progress and the consequences of under or over government
intervention (Table 1). Separately and together, the cases also raise questions about values, autonomy, justice and trust in biomedicine against a backdrop of other clear successes, and they illustrate the harms of communication failure or shortfall.

**Values, autonomy, justice and trust:** Respect for autonomy and justice represent primary values underlying the five cases. Achieving the best interest of a patient, as in the case of Terry Schiavo, has long been a fundamental principle for health care regulators and practitioners. Similarly, promoting autonomy and freedom of choice to accept or decline vaccination or to pursue the remediation of multiple sclerosis following the theory of chronic cerebrospinal vascular insufficiency (CCSVI) is another. Upholding the right of access to health resources, as highlighted in the case of soldiers returning from the Gulf War, closes the health care ethics loop. In areas of medical uncertainty, however, balance in decision-making is especially difficult to strike. The loss of hope and threat to identity associated with incurable neurological conditions further inflame public opinion. On this tightrope are also differences in stakeholder tolerance for risk, with patients and their advocacy groups often placing a far greater importance on rapid access to treatments rather than their demonstrated safety or efficacy (Chafe et al., 2011). The examples of CCSVI and Stamina Foundation further illustrate high degrees of frustration when regulatory processes (Pullman et al., 2013) are privileged over individual will. Similarly, the continued public rejection of vaccination represents the rejection of the notion that health policy should trump autonomy. And, certainly, few better cases than that of Gulf War Syndrome (GWS) illustrate how access to care can be compromised when the line
between objective medical measures are challenged by suspicion, uncertainty, and misunderstanding.

How does trust factor into this equation? Unmet expectations are the greatest challenge to this complex phenomenon and, in biomedicine, hype and premature promises of benefit are the underlying culprits (Master and Resnik, 2013). Fruitless suppositions lead to impressions of deception, inaction and indecision that all conspire to compromise trust. Instances in which regulatory officials bow to public pressure in the face of scientific advice serve to satisfy public demand and provide near-term political gains, but they undermine the process by which evidence-based health policy is informed. If public engagement and education are not sufficiently addressed, then calls for caution and restraint in the progress of medical therapies will go unheard.

*Communication of uncertainty:* The myriad challenges surrounding public understanding of scientific progress are ever-evolving with the dynamic content and pace of biomedicine, and significant advances in models of neuroscience communication, knowledge translation and public education (Bubela et al., 2009) have been made in an effort to keep apace. Nonetheless, a dated Information Deficit Model that describes how medical controversies stem from limited public understanding is still held as the prevailing view by scientists and policy makers. Science communication and education initiatives designed to fill a void in public understanding have been shown to fall short (Einsiedel), and are instead being increasingly replaced with a more interactive public engagement approach that emphasizes the facilitation of dialogue in which the plurality of viewpoints
are heard and can inform research priorities and science policy (Bubela et al., 2009). Success has been achieved in some fields, but there remains a strong need to normalize these new engagement models across scientific domains. It has been stated that the scientific community’s ‘license to practice’ can no longer be assumed and, rather, must emerge through processes that include public engagement and dialogue. Following this, determination of scientific quality can only be achieved through the representation of the plurality of social perspectives where publics are a part of a broader community that enact ‘extended peer review’ (Funtowicz, 1994). Whether even the most harmonized efforts could have mitigated the public response to any of the cases we featured here given the presence and extra layers of political and financial conflict, however, is an open question.

*The other side of the coin:* Public advocacy and stakeholder influence do not by definition negatively affect the development of health policy for conditions that affect the central nervous system. Amid those that have been unconstructive are others where patient advocacy successfully guided policy decisions to the benefit of public health. Access to experimental HIV/AIDS treatments in the 1980s, and the influence of Mothers Against Drunk Driving (MADD) on drug and alcohol policy are among a few. In Canada, both public and academic support contributed to preventing the closure of Insite, North America’s first supervised drug injection facility that has served over 8000 people since 2003. Despite evidence supporting its cost effectiveness and benefit to fatal overdose rate in the area, the facility was at risk for closure by an unsupportive federal government. Sustained advocacy from both academic and patient communities drove continued media coverage and public support of the facility, enfeebling government efforts to withdraw
financial backing and legal privilege for operation. These particular cases illustrate how well formed relationships between stakeholders from the public and patient communities, academics, and health professionals can be aligned toward the common goal of beneficence and reduction of harm.

**Conclusion**

Court action involving the Stamina Foundation is ongoing today. Advocates of CCSVI for MS are holding on to a last, but unraveling thread of hope. The anti-vaccination campaign retains a small yet vocal stronghold that continues to be heard. Terry Schiavo is dead, but the lessons learned from her suffering and those around her live on. War fighters are deployed to restore peace abroad, and many return to anything but a peaceful health setting in which to revive normal healthy lives.

Where do we go from here? The neuroscience community has a duty to explicitly recognize and address the powerful emotive capacity of neurologic disease to galvanize stakeholders. The community must take heed of the importance of collective understanding surrounding experimental therapies as they emerge, and inspire action that promotes understanding. Targeted communication strategies using the tools of knowledge translation (Bubela et al., 2009), social media, and deliberative decision-making will lead to better framed messages that co-originate with patient groups affected by neurologic disorders, advance accurate reporting on regulatory principles for clinical trials, and
mitigate controversies that are both sensitive and fundamental at an individual and societal level.

**BOX 1**

**Stamina Foundation – Stem cell therapy now**: Unproven, self-described stem cell therapy for central nervous system diseases is offered by private non-medical organization in Italy. The provider is shut down due to non-compliance with clinical good manufacturing practice (GMP) regulation. Supporters mount a legal challenge on grounds of compassionate therapy. Popular tabloid television show features distressed parents and ill children, and catapults Stamina to the forefront of public discourse with outspoken celebrities alongside. The case draws sharp criticism from scientific communities nationally and worldwide (Bianco et al., 2013) with a marginal correcting effect.

**Multiple Sclerosis – The call for correcting cerebrovascular insufficiency**: Italian radiologist proposes controversial explanation and intervention for multiple sclerosis (MS) based on blood flow that brings unprecedented disease-liberating hope to sufferers worldwide. The study has significant methodological limitations, is met with skepticism from academic communities, yet still gains high profile exposure. Patient advocacy groups rally behind trials of new procedure and pressure health officials to act and provide resources with unprecedented response. The race to demonstrate treatment efficacy is on and the momentum fuels both media hunger for promising news and public confusion.
MMR – The debate that does not abate: British physician publishes papers suggesting link between measles, mumps and rubella (MMR) vaccine and autism. It goes largely unnoticed until it is featured in a television press conference. Emboldened by famous supporters, fears about vaccines spread rapidly throughout UK and North America, and childhood vaccination rates drop. Decades of research fail to support causal link. Nonetheless, measles that was once largely eradicated in the developed world reemerges. The widespread and dangerous misconception about vaccines endures as one of most damaging medical fallacies of the century.

Persistent Vegetative State (PVS) – Right to die meets the political right: A chilling debate fueled by politics and emotion on an individual’s right-to-die is brought to the foreground by the case of 26-year old Terri Schiavo, a patient in PVS following hypoxic-ischemic brain damage. In the absence of hope of recovery, her surrogate decision maker wishes to withdraw life support. Other family members vehemently object based on video images of responsiveness that is interpreted to be voluntary. The unscientific evidence weighs heavily into the discussion about life sustaining interventions that comes to involve both legal and political stakeholders.

Gulf War Syndrome (GWS) – Biological and social constructions of illness: Reports emerge about unique multi-symptom illness involving chronic headaches, deficits of memory and concentration, persistent fatigue, mood disturbances, and widespread pain in soldiers returning from the Iraq war. Termed Gulf War Syndrome (GWS), the condition appears to be distinct from the class of post-traumatic stress disorders. Academic debate
ensues over whether GWS is a single unique pathological entity with a neurologic origin, or a social construction fueled by rising levels of stigma and frustration with war fighters struggling to reintegrate into society (Durodie, 2006). The latter notion is promulgated by the press.
Table B.1 Comparative analysis

<table>
<thead>
<tr>
<th>Key theme</th>
<th>Stamina Foundation</th>
<th>Chronic Cerebrospinal Venous Insufficiency (CCSVI)</th>
<th>MMR Vaccine in Autism</th>
<th>Case of Terry Schiavo</th>
<th>Gulf War Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time range</td>
<td>2013 to present</td>
<td>2009 to present</td>
<td>1998 to present</td>
<td>1990 to present</td>
<td>1991 to present</td>
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<tr>
<td>Time to policy impact</td>
<td>3 months</td>
<td>24 months</td>
<td>24-36 months</td>
<td>N/A (used as legal precedent)</td>
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<tr>
<td>Diseases and intervention</td>
<td>Numerous: Parkinson’s, Alzheimer’s, Muscle-wasting disorders, progressive congenital neurological deficits</td>
<td>Multiple Sclerosis (MS)</td>
<td>Measles/Mumps/Rubella Vaccination, Enterocolitis, Autism</td>
<td>Vegetative state</td>
<td>CNS syndromes</td>
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<td>Terminal condition</td>
<td>Yes</td>
<td>No</td>
<td>No (Autism)</td>
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<td>Initial sample size</td>
<td>32 (No study)</td>
<td>65 (Zamboni et al., 2009)</td>
<td>12 (Wakefield et al., 1998)</td>
<td>1</td>
<td>At least 167,500</td>
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<td>Analysis of media coverage</td>
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<td>Funding</td>
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<td>Government-funded research programs and institutes</td>
<td>N/A</td>
<td>Government-funded patient support programs and research</td>
</tr>
<tr>
<td>Regional focus</td>
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<td>Canada</td>
<td>UK</td>
<td>USA</td>
<td>USA</td>
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<td>Stakeholder capture</td>
<td>Stamina Foundation</td>
<td>Italian Health Ministry, Political right</td>
<td>Academics</td>
<td>Inventor Media, MS patients, Government</td>
<td>Research sponsors Professional health associations Media Government</td>
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<tr>
<td>Key ethical and regulatory issues</td>
<td>Stamina Foundation</td>
<td>Chronic Cerebrospinal Venous Insufficiency (CCSVI)</td>
<td>MMR Vaccine in Autism</td>
<td>Case of Terry Schiavo</td>
<td>Gulf War Syndrome</td>
</tr>
<tr>
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</tr>
<tr>
<td>Informed consent</td>
<td>Waiver of regulatory process Right-to-hope</td>
<td>Efficacy</td>
<td>Safety</td>
<td>Right-to-life Right-to-die</td>
<td>Medical legitimacy</td>
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<td>Health risk</td>
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<tr>
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<td>Yes</td>
<td>No</td>
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<td>Justice and rights</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Caregiver involvement</td>
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<td>Yes</td>
<td>Yes</td>
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