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UNIVERSITY OF CALIFORNIA, SAN DIEGO
SAN DIEGO STATE UNIVERSITY

Cardiac progenitor cells support optimal heart function and repair

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Brandi Lynn Bailey

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2011

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2011

DEDICATION

Most of all, I would like to dedicate this dissertation to my husband, Mike, (you truly are my better half) and my parents, Bill and Michelle Bailey, (because work-a-holism runs in the family) and my sister, Revelle (my hero!), for continuous intravenous emotional support.

EPIGRAPH

"*Cynodon dactylon* produces significantly greater amounts of chlorophyll when located on the dorsal surface of the septic storage device."

Adapted from: *Erma Bombeck*

"The grass is always greener over the septic tank"

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LIST OF ABBREVIATIONS

α SMA	alpha smooth muscle actin
AWD	anterior wall dimension
BRDU	bromodeoxyuridine
BSA	bovine serum albumin
BZ	border zone
C57	C57/Bl6 mouse line
CPC	cardiac progenitor cell
ESD	end systolic diameter
EDD	end diastolic diameter
FBS	fetal bovine serum
GFP	green fluorescent protein
HSC	hematopoietic stem cell
IZ	infarct zone
NBF	neutral buffered formalin
NRCM	neonatal rat cardiomyocyte
NTG	non-transgenic
PBS	phosphate buffered saline
PWD	posterior wall dimension
Sca-1	stem cell antigen -1
ScaKI	sca-1 knock-in mouse
SEM	standard error of the mean

Topro	topro-3-iodide
TN	tris-NaCl buffer
Tmyo	tropomyosin
TNB	tris-NaCl blocking buffer
vWF	von Willebrand's factor

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Chapter I, in full, is a reproduction of the material as it appears in *Regenerative Medicine*, 2009. Cardiac stem cell genetic engineering using the α MHC promoter: Brandi Bailey, Alberto Izarra, Roberto Alvarez, Kimberlee M.

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- Immunohistochemistry, Immunocytochemistry, Fluorescence Confocal Microscopy
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ABSTRACT OF THE DISSERTATION

Cardiac progenitor cells support optimal heart function and repair

by

Brandi Lynn Bailey

University of California, San Diego 2011

San Diego State University 2011

Professor Mark A. Sussman, Chair

Heart disease remains a serious and intractable disease resulting in decreased productivity and increased mortality throughout the world. Cardiac Progenitor Cells (CPCs) have been shown to be a potential cellular therapeutic approach to blunt tissue damage and facilitate reparative and regenerative processes after myocardial infarction (MI).

Cardiac progenitor cells are important for maintenance of myocardial structure and function, but molecular mechanisms governing CPCs remain obscure and require elucidation to enhance regenerative therapeutic approaches. Normal CPCs engineered with fluorescent reporter protein constructs under control of the α MHC promoter show transgene protein expression, confirming activity of the promoter in CPCs. Cultured CPCs from both non-transgenic and cardiac-specific transgenic mice expressing survival kinases driven by the α -MHC promoter were analyzed to characterize transgene expression following treatments to promote differentiation in culture.

Green fluorescent protein under regulatory control of the endogenous Sca-1 promoter is present in vascular structures in mature mice. However, lack of Sca-1 does not result in a primary vascular defect. Instead, genetic deletion of Sca-1 results in early-onset cardiac contractile deficiency as determined by echocardiography and hemodynamics. ScaKI mice show compromised capacity to survive myocardial infarction relative to normal control subjects. Resident cardiac progenitor cells in ScaKI mice do not respond to pathological damage *in vivo*, consistent with observations of impaired growth and survival of ScaKI cardiac progenitor cells *in vitro*. Genetic deletion of Sca-1 causes primary cardiac defects in myocardial contractility and repair. These defects are likely due to impairment of resident cardiac progenitor cell proliferative capacity, possibly through altered Lef1 transcription factor expression. Thus, disruption of cardiac progenitor cells results in a heart that is slightly impaired in normal function with greatly reduced capacity to repair itself after injury.

CPCs have also been shown to respond to aging or general cardiomyopathy as damage. CPCs can be isolated and cultured from middle-aged hearts but they exhibit slightly impaired growth kinetics. The CPCs used for autologous cardiac progenitor cell therapy purposes would most likely benefit more from genetic engineering to increase proliferation and survival but they are still capable of supporting cardiac function in the context of aging and disease.

INTRODUCTION OF THE DISSERTATION

Cardiovascular Disease and Future Cell Therapies

Cardiovascular disease encompasses a range of specific symptoms and diagnoses including: high blood pressure, angina pectoris, myocardial infarction (MI) and congestive heart failure. Over 80 million Americans, or over 1 in 3 adults, have been diagnosed with cardiovascular disease in the United States¹. This percentage stands to increase both in the US and around the world as the population ages and adopts a more western, modern diet and sedentary lifestyle.

Current therapies for cardiovascular disease include: dietary and exercise behavioral intervention, pharmaceutical treatments and surgical interventions. Although there are many treatments which address the physiological symptoms of cardiovascular disease, no treatments are currently available which can replace aged or damaged myocardial tissue. Most incidences of cardiovascular disease, even with adequate medical intervention, will eventually result in a decline in cardiovascular function. The only treatment for end-stage heart failure is a heart transplant. The short supply of donor organs has limited heart transplant therapy to the most fortunate compatible recipients with the fewest complicating co-morbidities.

Recently, researchers in the cardiovascular field have uncovered a new potential therapy for cardiovascular disease and heart failure. The key to this therapy was the discovery of a small cardiac progenitor cell (CPC) within the adult heart of many mammals including: rodents², dogs³, and humans⁴. These cells bear a resemblance in their expression of stem cell markers c-kit and Sca-1

to hematopoietic stem cells (HSC) which are currently used clinically to repopulate the immune system after the treatment of certain cancers. Several studies to date have shown in multiple animal models that intramyocardial injection of CPCs to syngeneic animals has successfully mitigated the effects of a myocardial infarction in both mouse and rat models^{2, 5, 6}.

Adult Tissue Stem/Progenitor Cells and Stem Cell Markers

There are two common categories of stem cells described in the literature: embryonic stem cells (ESC) and adult stem cells. ESCs are derived from the inner cell mass of a blastocyst, which, for human derived cells, gives rise to ethical issues. They can become any type of cell in the organism but lack the potential to generate an entire living organism, thus, ESCs are described as pluripotent⁷. Adult stem/ progenitor cells are described as multipotent and will generally replace only a limited number of cell types within of their tissue of origin. Adult progenitor cells have been isolated and characterized from the mammalian: immune system^{8, 9}, brain^{10, 11}, skeletal muscle^{12, 13}, and, more recently, the cardiovascular system².

Cell surface markers common to progenitor cells from multiple organ systems and across multiple species are essential for the identification, isolation, culture and characterization of those cells. There are 3 common surface proteins commonly used to define a cell as an adult progenitor: c-kit (CD117), Stem Cell Antigen 1 (Sca-1, Ly-6A/E) and Multi-Drug Resistance Protein 1 (MDR-1, P-glycoprotein)¹⁴. c-kit is a receptor tyrosine kinase for which the ligand is Stem

Cell Factor¹⁵. Sca-1 is a phosphatidylinositol anchored surface protein that has effects on cell adhesion for which no intracellular signaling pathway has been described¹⁶. MDR-1 is an ATP binding transport protein responsible for the efflux of multiple substances including dyes and toxins from the cell¹⁷. These markers are not exclusive to stem cells and may be expressed on mature tissue types. Thus, a combination of the co-expression of more than one stem cell marker (c-kit, Sca1 or MDR-1) on a cell usually indicates adult progenitor cell status. For example, a HSC is usually defined and isolated as c-kit⁺, Sca-1⁺ and lineage marker negative (c-kit⁺, Sca⁺, lin⁻)¹⁸.

Cardiac Progenitor Cells

The paradigm of the heart as a post-mitotic organ has shifted in recent years to one that describes the cardiac tissue as containing a reserve of progenitor cells that can respond to injury or stress. It is important to understand the regular workings of these cells to maintain the myocardium throughout the lifespan of the organism.

Cardiac Progenitor Cells (CPCs), recently discovered in both humans and rodent experimental models, constitute an undifferentiated reserve population in the heart that will respond to insult or normal cell turnover. These CPCs can undergo replication and differentiation to replace myocardial, vascular, and endothelial cell types in the damaged heart². These relatively small CPCs may reside in a niche environment¹⁹ and characteristically express c-kit antigen and may also exhibit cardiac specific markers such as the transcription factors:

Gata4, Mef2c, or Nkx2.5². Endogenous CPCs also migrate into the infarct zone in response to MI, undergo differentiation, and express markers of cardiomyocytes, vascular smooth muscle, and endothelium along with c-kit²⁰.

The functional population of these cells declines with age as they cease to proliferate and become senescent²¹. Loss of a functional CPC population may be a primary cause of the aging associated loss of function of the heart in humans that is not associated with specific pathology²². Heart disease remains a serious and intractable disease requiring new treatments to be developed and the cellular etiology behind the decline in heart function more fully understood. Hence, the goal of the studies described herein is to investigate further the functional and signaling properties and explore the therapeutic potential of cardiac progenitor cells.

CHAPTER I:

Cardiac Progenitor Cell Engineering Using the α MHC Promoter

INTRODUCTION

The heart is an attractive candidate for regenerative tissue therapy due to the endogenous reservoir of stem cells that maintain the myocardium and replace myocytes over the lifetime of the organism²³. However, these endogenous stem cells do not have the capacity to repair the extensive damage caused by myocardial infarction (MI) or chronic cardiovascular disease, leading to deterioration of hemodynamic output and eventual cardiac insufficiency. Cardiac progenitor cells (CPC) are small, undifferentiated c-kit⁺ and/or Sca-1⁺ cells resident within the myocardium which may co-express cardiac specific markers such as Gata4, Nkx2.5 or Mef2c¹⁹. CPCs harvested from myocardial tissue and grown in culture improve cardiac function after injection following an MI in mouse^{4, 24}, rat^{2, 25} and large animal²⁶ experimental models. Adoptively transferred CPCs improve myocardial remodeling following injury by a combination of blunting cell death, recruitment of endogenous reparative mechanisms, and contribution of new cells to the damaged myocardium. Collectively, these salutary effects improve cardiac performance and ameliorate scar formation but usually the benefit is relatively modest and recovery of function still falls well below that of the healthy heart. One issue is the relatively short lived nature of the adoptively transferred cell population that results in limited engraftment and persistence after delivery^{27, 28}. Thus, molecular interventional strategies to enhance CPC proliferation, persistence, and engraftment upon delivery would be a valuable and important advance in

myocardial regenerative therapy. One such strategy involves the delivery of survival genes to the cells *ex vivo* prior to reintroduction into the myocardium, which improves and augments the beneficial effects of CPC therapy^{29, 30}. Appropriate regulatory elements to control these survival genes and their deployment in engineered stem cells remains an issue, since previous studies involve use of high level viral promoters with unrestricted tissue expression. A more desirable approach would involve the focused use of cardiac specific promoters to augment cardiogenic cell survival and commitment as part of an integrated stem cell engineering strategy.

Assessment of the cardioprotective effects mediated by multiple survival and angiogenic proteins has been facilitated through use of cardiac-specific transgenesis in genetically engineered mice. Dozens of transgenic mouse lines have been reported which overexpress a gene of interest specifically in cardiomyocytes by controlling transgene expression with a portion of the mouse α -myosin heavy chain (α MHC) promoter. Several mouse models have been used to confirm that pro-survival and anti-apoptotic genes protect the myocardium in α MHC driven transgenic mice³¹. Subramaniam *et al*³² developed and characterized the α MHC transgenic promoter system which is comprised of a 5.5kB region between the murine α - and β -myosin heavy chain genes. α MHC-driven transgene expression in the adult mouse was initially described as restricted to the myocardium and the large pulmonary veins of the lung³². The adjacent relationship between the α - and β -MHC gene structure is conserved

between human and mouse and the α MHC (MHC6) promoter regions have a 60% sequence identity using Smith-Waterman local alignment.

Recent studies from our group have demonstrated that AKT (also known as Protein Kinase B) targeted to the nucleus protects the heart from ischemic injury without causing hypertrophy^{33, 34} as well as expands the CPC population *in vivo*³⁵ when expressed using the α MHC promoter. Another pro-survival kinase downstream of nuclear AKT known as Pim-1 is also highly protective in the myocardium in both infarction and pressure overload studies^{36, 37} when expressed using the α MHC promoter. Therefore, the capacity of the α MHC promoter to express cardioprotective genes within CPCs and cardiomyocyte could be advantageous in a genetic engineering approach to enhance the survival, persistence, proliferation, and engraftment of adoptively transferred CPS into the myocardium. This study characterizes α MHC promoter expression in cultured cardiac CPCs to assess transgene expression and cellular phenotype.

METHODS

Animal Models

All transgenic mice were derived in FVB strain (non-transgenic, NTG) mouse line. Animals are housed in approved facilities and all surgeries were performed under anesthesia according to Institutional Animal Care and Use Committee approved protocols. The α MHC^{GFP} mouse line expresses enhanced green fluorescent protein (GFP) under the control of the murine α MHC promoter, which is a 5.5Kb section of the endogenous mouse α MHC promoter commonly used for cardiomyocyte-specific transgenic expression³². The Pim-1 wild-type (Pim-wt) mouse line over-expresses the 33kDA human Pim-1 protein isoform as well as GFP driven by the α MHC promoter. The Akt-nuc mouse line expresses nuclear targeted AKT/PKB that is myc-tagged on the C-terminus driven by the α MHC promoter. Neonatal rat cardiomyocytes (NRCMs) were harvested from 2-3 day old Sprague-Dawley rats as previously described³⁸ and cultured in M199 media supplemented with 10% FBS.

Cardiac Progenitor Cell Isolation and Culture

CPCs were isolated and cultured as previously described². Briefly, two mice per preparation are anesthetized using ketamine-xylazine solution and the heart was cannulated through the aortic arch and perfused at 37°C in oxygenated basic buffer (J-MEM, 0.7 g/L hepes, 1.25g/L taurine, 20units/L insulin, Pen/Strep/Glutamine, Amphotericin, Gentamicin, pH 7.3) on a Radnotti

apparatus. The heart was then digested for 12 minutes at 37°C in 320units/mL of collagenase II in oxygenated basic buffer. Afterwards, the heart was then minced in basic buffer +0.5% BSA and the cardiomyocytes pelleted for 1 min at 100g and discarded. Remaining cells in the supernatant were passed through a 25µM filter and pelleted. The cell pellet was resuspended and incubated with anti-c-kit (CD117) Miltenyi beads in PBS + 0.5% BSA; washed and then isolated on a magnetic column to extract c-kit⁺ CPCs according to manufacturer's instructions. CPCs are cultured according to standard tissue culture protocols in CPC media (DMEM/F12, 10% Embryonic Stem Cell-Grade FBS, PSG, Insulin-Transferrin-Selenium, 1000u/mL LIF, 40ng/mL EGF, 20ng/mL bFGF).

Bone Marrow Isolation and Culture

Bone marrow cells (BMC) were flushed from both tibias and femurs of each mouse using PBS + 1%FBS buffer. BMCs were separated from red blood cells by density centrifugation using Histopaque reagent according to manufacturer's instructions. The BMCs were either assayed by FACS analysis fresh or cultured in serum-free StemSpan media supplemented with 1% PSG, 100ng/ml h-SCF, 100ng/ml Flt-3, 20ng/ml TPO, 20ng/ml IL-3, 20ng/ml IL-6.

Trypan Blue Assay

Cell cultures were counted after passage using 50% trypan blue solution by hemocytometer determination. For trypan blue assay (Figure 1.3E), 20,000 cells per well were seeded in a 6 well plate and counted at two or four days post plating.

FACS Analysis and Treatments

CPCs were seeded into 6 wells plates in CPC media for at least 24 hours before treatment. Treated cells received 400 μ M hydrogen peroxide (H₂O₂) for 4 hours. After peroxide incubation, treated cells and untreated controls were labeled with BD Pharmingen Annexin V-PE Apoptosis Detection Kit (559763) according to manufacturer instructions. Briefly, cells were suspended at 100,000 cells per 100 μ L in Annexin V Binding Buffer. Each set of treated and untreated cells was incubated for 20 minutes at room temperature in the dark with 0.75 μ L Annexin V and 2 μ L 7-AAD per 100,000 cells. 300 μ L of Binding Buffer was added post incubation and the cells were analyzed by flow cytometry with a BD FACSaria instrument. Unstained and single stain controls were used to establish baseline fluorescence levels for the samples.

RNA Isolation and PCR

CPCs were cultured according to standard procedure in CPC media and then harvested, counted and pelleted. One to two million cells from each CPC cell line were used to extract RNA using a Qiagen RNeasy RNA Isolation kit according to manufacturer's protocols. cDNA was synthesized using 2 μ g of RNA from each CPC line, random hexamer primers and the Applied Bioscience High Capacity Reverse Transcription kit according to the manufacturer's instructions. cDNA was amplified using primers specific for each transgene as a well as GAPDH as a control.

Immunocytochemistry

CPCs were plated on Permanox chamber slides for at least 24 hours. NRCMs were cultured on laminin-coated glass slides. After incubation, the slides were then washed once with PBS and fixed in 4% Paraformaldehyde (PFA) for at least 20 minutes at 4°C. After fixation, the slides were washed twice with PBS, permeablized with PBS+0.2% Triton-X 100; washed again and blocked with blocking buffer (PBS + 10% Horse Serum) for 30 minutes. Primary antibodies were diluted in blocking buffer and incubated on the slides overnight at 4°C and then washed 3 times in PBS. Fluorescently conjugated secondary antibodies were diluted in blocking buffer, incubated at room temperature for 2 hours and then washed 3 times in PBS. A table of antibodies and dilution ratios is available in Supplemental Table 2. Topro-3-Iodide (Topro) was included in the final wash to fluorescently label the DNA/nucleus. The coverslip was mounted with Vectashield to preserve fluorescent signal. All slides were imaged using a Leica TCS SP2 Confocal Microscope.

Immunohistochemistry

The hearts were fixed by retroperfusion of 10% neutral buffered formalin and paraffin embedded as previously described²⁰. Paraffin sections of mouse hearts cut at 4µm were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed in 10mmol/L citrate pH 6.0 using 1100-W microwave oven for 3 minutes at high power and 12 minutes at 50% power. The slides were allowed to cool to room temperature,

washed in 3 times in TN buffer (NaCl 150 mmol/L, Tris 100mmol/L, pH 7.5) and quenched with 3% hydrogen peroxide in TN buffer for 20 minutes to block endogenous peroxidase activity. Slides were then washed in TN buffer and blocked for 1 hour in TNB (1X TN buffer containing 0.5% Blocking Buffer, proprietary formula from Perkin ElmerTM kit). Primary antibodies were diluted in TNB and incubated overnight at 4°C. Slides were then washed 3 times in TN and incubated 2 hours at room temperature in the dark with species-specific secondary antibodies conjugated to a fluorophore in TNB. Nuclei were stained for 20 minutes with Topro. Detection of some antigens required signal amplification using horseradish peroxidase (HRP) conjugated antibodies or streptavidin followed by reaction with a fluorescent tyramide substrate. Coverslips were mounted with Vectashield and imaged by confocal microscopy.

Treatments to promote differentiation

For the purposes of these studies, the term “differentiation” refers to the cellular phenotypic responses to culture conditions involving inductive stimuli to promote lineage commitment. Induction of cardiomyogenic differentiation for imaging was performed by culturing CPCs on a layer of fixed NRCMs. Briefly, NRCMs were cultured for 3 days on laminin-coated glass chamber slides. Conditioned media was removed and stored for later use at 4°C. The NRCMs were washed twice with PBS and fixed in 4% PFA for exactly 20 minutes then washed 3 more times with PBS. The PFA was neutralized by a 30 minute incubation of 0.1M Glycine followed a PBS wash and an overnight incubation with 10% FBS media. CPCs

were passaged according to standard procedure, pelleted, resuspended in NRCM conditioned M199 +10% FBS media and added to the layer of fixed NRCMs for 7 days. Fresh media M199 +10% FBS media was supplemented to the slides on Day 4. On day 7 the slides containing CPCs differentiated on NRCMs were fixed and stained for immunocytochemistry. Non-specific control differentiation treatment involved CPC culture on Permanox slides for at least 24 hours in CPC media and then replacing the media with Differentiation Media (α -MEM, 10%FBS, PSG, 10nM dexamethasone, Sodium Bicarbonate pH 7.3). The CPCs were incubated with the Differentiation Media for 7 days and then fixed and stained for immunocytochemistry.

Cardiomyogenic Differentiation for qPCR Analysis

NTG CPCs were transduced with a lentiviral vector encoding GFP and subsequently co-cultured with pre-plated NRCMs in M199 + 10% FBS for 7 days. Co-cultured cells were trypsinized and cells with high level GFP expression were separated from NRCMs by FACS. RNA was isolated from the GFP+ fraction using Trizol/chloroform extraction. cDNA was obtained with the Applied Bioscience High Capacity cDNA Reverse Transcription Kit. Taqman qPCR assays (Applied Biosystems) were performed using an MJ Research Opticon instrument for the following genes: troponin T (Mm00441922_m1), tropomyosin (Mm00600378_m1) and α -smooth muscle actin (Mm01546133_m1). β -actin served as an internal control. Data were analyzed

using the Opticon instrument software with calculated Ct values and the $\Delta\Delta C_t$ method for determining relative RNA/cDNA quantities.

Plasmid Constructs and Transfection

Plasmids were constructed using a pEGFP-C1 backbone. The CMV-td-Tomato-Flag construct expresses the Tomato fluorescent protein fused to a C-terminal Flag-tag under the control of the cytomegalovirus (CMV) promoter. The α MHC-GFP construct expresses EGFP under the same 5.5 kb α MHC promoter used to create transgenic mice. The α MHC-YFP-HA construct expresses Yellow Fluorescent Protein (YFP) fused to a C-terminal HA-tag under the α MHC promoter. Transfections were accomplished using 0.25-0.5 μ G of purified plasmid per slide chamber in 1mL of CPC media using the Eugene 6 transfection reagent according to manufacturer's instructions.

RT-PCR Primer sets and sequences

All primers were designed using Vector NTI 10 from Invitrogen. Transgene specific primers were designed for the sequence of DNA that was used to create the respective transgenic animals. Primer sequences, annealing temperatures and expected product lengths are available in Supplemental Table 1.

RESULTS

CPCs express fluorescent markers and tags when transfected with plasmid constructs under the control of the α MHC promoter

NTG CPCs were transfected with plasmids encoding fluorescent/tagged proteins driven by either the α MHC or CMV promoter to assess transgene expression. CPCs were transfected with a plasmid encoding the Red/Orange-fluorescing td-Tomato, with a C-terminal Flag-tag under control of the constitutively active CMV viral promoter. CPCs expressing td-Tomato protein were readily evident within 24-48 hours post-transfection. Immunocytochemistry colocalized td-Tomato positive CPCs with both c-kit and Flag-tag markers (Figure 1.1A). Transfection of CPCs with a constructs encoding fluorescent reporters under control of the α MHC promoter also resulted in expression for GFP (Figure 1.1B) or YFP (Figure 1.1C). Transgene expression regulated by the α MHC promoter is specific to CPCs relative to either c-kit⁺ bone marrow stem cells (Figure 1.2) or HEK-293 cells (data not shown) where fluorescent reporter protein expression was not observed.

CPCs isolated from mice created by α MHC-specific transgenesis show reporter gene expression as well as the pro-proliferative and anti-apoptotic effects of these genes

CPCs were previously reported to be expanded due to α MHC-driven Akt-nuc expression *in vivo*.³⁵ Consistent with this finding, immunohistochemical staining

of myocardial sections obtained from a transgenic mouse expressing GFP under control of the α MHC promoter ($_{MHC}GFP$) heart section show colocalization of c-kit and GFP (Figure 1.3A). GFP was also expressed in mature myocytes as shown by colocalization of GFP with desmin staining (Figure 1.3A). Next, CPCs were isolated from $_{MHC}GFP$ as well as two additional cardiac-specific transgenic lines expressing survival kinases driven by the α MHC promoter: $_{MHC}Akt-nuc$ and $_{MHC}Pim-wt$ ^{33, 39}. The Akt-nuc construct possesses three C-terminal nuclear localization sequence (NLS) repeats followed by an C-terminal myc-tag that allows for specific primers designed for the NLS/Myc-tag sequence (“Akt-myc”, Figure 1.3B). GFP mRNA expression was detected in cultured CPCs derived from $_{MHC}GFP$ and $_{MHC}Pim-wt$ myocardium by RT-PCR (Figure 1.3B) together with GAPDH expression a quality control. Protective effects of both Pim-1 and Akt-nuc overexpression are evident after hydrogen peroxide treatment for four hours. NTG CPCs show significant induction of Annexin V/ 7-AAD staining (10 fold) indicative of apoptosis, whereas $_{MHC}Pim-wt$ (2-fold) and $_{MHC}Akt-nuc$ (5-fold) CPCs show a lesser induction (Figure 1.3C and 1.3D). $_{MHC}Pim-wt$ and $_{MHC}Akt-nuc$ CPCs exhibit faster proliferation rates during *in vitro* culture by trypan blue exclusion assay (Figure 1.3E).

CPCs express transgenes controlled by the α MHC promoter in the appropriate subcellular localization in culture by confocal microscopy

CPCs derived from transgenic mouse lines express fluorescent reporter constructs as confirmed by confocal microscopy. CPCs obtained from NTG,

$_{MHC}Akt-nuc$ and $_{MHC}Pim-wt$ mouse lines show immunoreactivity for appropriate expressed exogenous proteins. A representative scan shows increased Pim staining in the cytoplasm of the $_{MHC}Pim-wt$ CPCs (Figure 1.4A and 1.4B). Similarly, CPCs from $_{MHC}Akt-nuc$ mice show nuclear localization of the exogenous protein by immunolabeling for the myc-tag relative to the NTG sample (Figure 1.4C and 1.4D). Bone marrow cells isolated from $_{MHC}Pim-wt$ and $_{MHC}GFP$ transgenic mouse lines do not express GFP by FACS analysis (Figure 1.5).

Cardiomyogenic differentiation of CPCs results in persistent alphaMHC driven transgene expression

Cardiomyogenic induction of lineage commitment for CPCs can be promoted by co-culture with Neonatal Rat Cardiomyocytes (NRCMs)⁴⁰. Monolayers of NRCM used in co-culture studies are fixed prior to addition of CPCs to eliminate potentially confounding results caused by cell fusion. Conditioned media from the NRCM cultures is added to provide for cell-cell as well as soluble differentiation signals. The fixed layer of NRCMs lose Topro-dependent nuclear staining after 7 days of incubation (Figure 1.6), allowing for visualization of nuclei present only in CPCs. Cultured CPCs derived from NTG, $_{MHC}Akt-nuc$ and $_{MHC}Pim-wt$ myocardial tissue were subjected to co-culture with fixed NRCMs for 7 days and then immunolabeled for nuclei (Topro) and and alpha-sarcomeric-actinin (α -Sarc-Actinin) to identify myocytes as well as either Pim and GFP ($_{MHC}Pim-wt$) or myc-tag ($_{MHC}Akt-nuc$). CPCs derived from $_{MHC}Pim-wt$ mice display increased levels of Pim (Figure 1.7A) and GFP (Figure 1.7C) immunoreactivity

relative to NTG controls (Figure 1.7B and 1.7D). CPCs derived from _{MHC}Akt-nuc mice show nuclear-localized myc-tag (Figure 1.7E), whereas the myc-tag is absent in the NTG control group (Figure 1.7F). Further characterization of CPC differentiation was performed using NTG CPCs lentivirally transduced to express high-levels of GFP and co-cultured with NRCMs. Cocultured CPCs were isolated after 7 days based on GFP expression and mRNA levels of cardiac specific and smooth muscle-specific genes were assessed by RT-qPCR. CPCs plated in these co-culture studies express approximately 16-fold more troponin T, 33-fold more tropomyosin and 90-fold more α -smooth muscle actin mRNA levels relative to control samples. (Figure 1.7G).

Non-cardiogenic differentiation leads to loss of transgene expression in cultured CPCs

Dexamethasone steroid treatment of CPCs has been reported to promote cardiomyocyte-like, endothelial cell² or smooth muscle². Treatment of cultured CPCs derived from _{MHC}Pim-wt mice with 10nM dexamethasone results in loss of immunoreactivity for both c-kit and Pim protein (Figures 1.8A and 1.8B). Identical treatment of cultured CPCs derived from _{MHC}Akt-nuc mice (Figure 1.8C) results in loss of nuclear myc-tag immunoreactivity after 7 days of exposure to dexamethasone (Figure 1.8D).

DISCUSSION

Since heart disease remains a major cause of morbidity and mortality, the treatment of this intractable disease by regeneration of damaged tissues or weakened cells remains a novel and exciting therapeutic approach. Initial steps toward implementation of regenerative therapy have already been accomplished: 1) reliable isolation and culture conditions exist for CPCs^{2, 4, 19}, 2) animal studies have indicated that injection of syngeneic or autologous CPCs regenerate myocardium, decrease infarct size and improve function relative to controls^{2, 32, 41, 42} and 3) some stem cell types are adept at avoiding allogeneic rejection even without immunosuppressive drugs⁴¹. Studies following CPC injection in several models have failed to demonstrate any evidence of oncogenic transformation^{26, 27, 29, 30}. However, the persistence, long term engraftment, and replicative capacity of adoptively transferred CPCs remain weak aspects of their use in the clinical setting. Increased efficacy of CPCs for clinical treatment of heart failure may rest upon genetic modification using signaling molecules with demonstrated propensity for enhancing survival and proliferation without inhibiting cardiogenic commitment or leading to oncogenic transformation. These questions can be studied using CPCs in vitro with well known signaling molecules to optimize gene expression, characterize the phenotypic properties of genetically engineered cells, and assess safety issues prior to use in experimental animal models.

The focal point of this report is the demonstration of transgene expression in CPCs driven by the α MHC promoter in cell lines obtained from both NTG as

well as three genetically engineered transgenic lines. Although α MHC promoter expression is generally considered to primarily occur in cardiomyocytes, the expression observed in CPCs may represent initial expression levels occurring at a very early stage of cardiogenic lineage commitment. Transgenic mRNA expression is maintained during *ex-vivo* culture and expansion of CPCs (Figure 1.3) and appropriate protein localization was confirmed by immunocytochemistry (Figure 1.5). These data indicate that cultured CPCs maintain these essential *in vivo* phenotypic characteristics even through stressful isolation procedures and subsequent artificial *in vitro* culture conditions.

CPCs exhibiting cardiogenic commitment should retain α MHC-driven gene expression whereas those moving toward non-myocyte lineages stop expressing α MHC-driven genes. Indeed, CPCs in differentiating co-culture with fixed NRCMs persist in α MHC driven transgene expression (Figure 1.6) whereas those subjected to dexamethasone treatment lose expression mediated by the α MHC promoter (Figure 1.8). Cardiogenic-associated transgene expression could be valuable in a therapeutic setting to augment persistence and proliferation of only those CPCs destined for myocardial fates. Cells committing to adipocyte or fibroblast types would lose the growth and survival-promoting effects mediated by the transgene, leaving the cell without a persistent advantage. Thus, use of cardiac-specific promoters that are activated early in the CPC lineage program would provide a distinct advantage for the expansion of that progenitor cell population as well as their engraftment and retention in the myocardium, presumably leading to increased reparative and regenerative processes.

The feasibility of this *ex vivo* engineering strategy depends upon the capacity to modify isolated CPCs under *in vitro* culture conditions to express transgenes driven by the α MHC promoter. Clearly, CPCs express fluorescent proteins driven off the α MHC promoter after simple plasmid transfection (Figure 1.1). There may be advantages to the use of plasmid-based strategies to circumvent issues of long term gene expression and potential for oncogenic transformation. If desired, greater efficiency and permanent transgene expression could be obtained by lentiviral or retroviral transduction instead of plasmid transfection and we are assessing the utility of those genetic engineering approaches with ongoing studies.

The genetic modifications most likely to provide salutary effects in CPCs are those associated with increased cellular proliferation, survival, and engraftment in a regulated fashion that would not result in loss of capabilities for differentiation as seen in oncogenic cell lines. Fortunately, as far as a target tissue, the myocardium is notoriously resistant to oncogenic transformation even when tumorigenic genes are expressed at high levels in cardiomyocytes by transgenesis. The inherent inert quality of the adult heart as a predominantly post-mitotic organ may prove a saving feature for cells delivered for regenerative therapy, as this characteristic would reduce the potential for oncogenic genes to promote uncontrolled cell growth in the cardiomyocyte context. As previous studies have already pointed toward a number of intriguing cardioprotective genes such as Akt-nuc, Pim-wt, IGF-1 and hGF, future studies will need to

determine the feasibility of creating genetically engineered CPCs that possess enhanced survival and proliferation for efficacious treatment of heart disease.

CPC-mediated regenerative cell therapy is a promising new treatment for heart disease, but the outcome of treatment could be markedly improved by increased survival and proliferation of cardiogenic cells. Genetic modification of CPCs with cardiac-specific promoters analogous to the murine α MHC promoter that is expressed early in cardiogenic lineage commitment could provide an important advance in our efforts to implement regenerative medicine with increased benefit with lowered safety risks.

SUMMARY POINTS

- CPCs express α MHC-driven transgenes *in vivo* and *in vitro*, demonstrating both early commitment to cardiomyocyte lineage *in vivo* and persistence of pre-cardiac cell phenotype *in vitro*.
- Cardiac-specific expression of survival and proliferative signaling will be advantageous for selective repopulation of myocardial tissue.
- NTG CPCs express exogenously created gene constructs under control of the α MHC promoter when transfected into the cultured cells.
- CPCs exposed to cardiogenic differentiation signals continue to express α MHC-driven transgenes.
- Future CPC therapies will potentially benefit from engineering the donor cells with cardioprotective genes such as Akt-nuc, Pim-1 or other pro-survival proteins.
- CPC engineering could be accomplished safely and effectively by putting the cardioprotective genes under the control of the α MHC promoter.

FIGURES

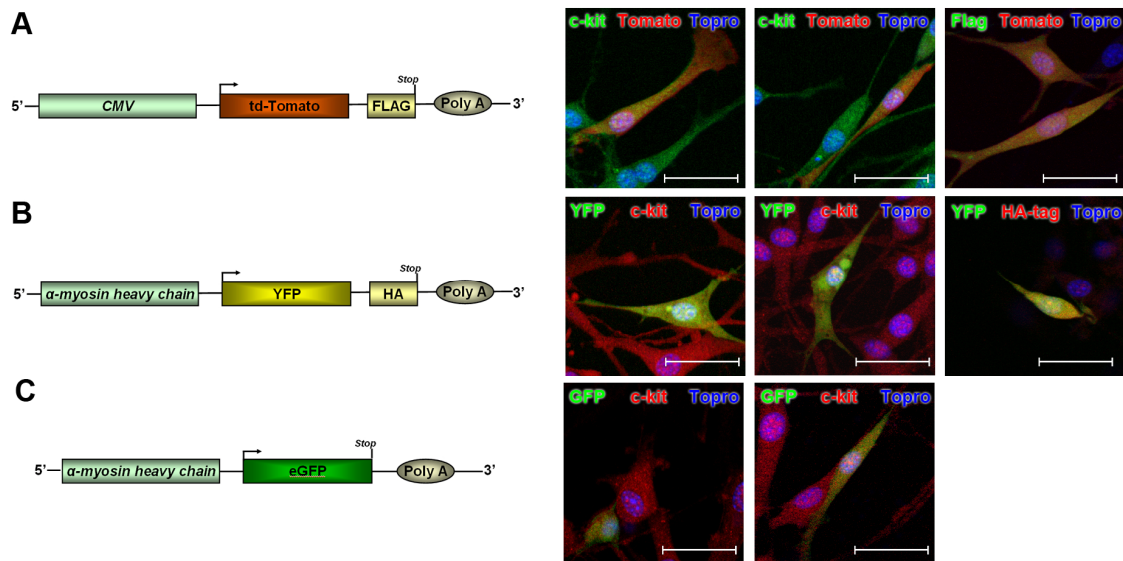


Figure 1. 1: CPCs express transfected fluorescent tags under control of the alphaMHC promoter.

A) Diagram of CMV-Td-Tomato-Flag construct (left). Cells were transfected with a Tomato-Flag construct under the viral CMV promoter as a control and stained with c-kit or Flag-tag (green) and Topro (blue), td-Tomato protein fluorescence (red) (right). B) Diagram of α MHC-YFP-HA construct (left). Cells were transfected with a YFP-HA construct under the α MHC promoter and stained with c-kit or HA (red), Topro (blue) and YFP fluorescence (green) (right). C) Diagram of α MHC-GFP construct (left). Cells were transfected with a GFP construct under control of the α MHC promoter and stained with c-kit (red), Topro (blue) and GFP protein fluorescence (green) (right). Scale bar is 40 μ m.

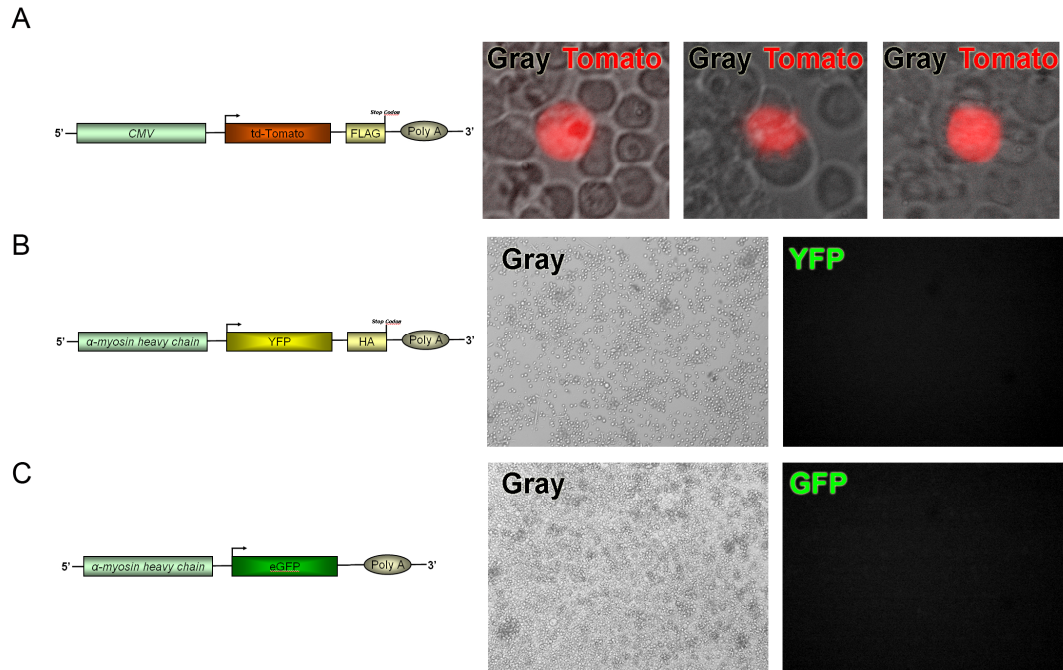
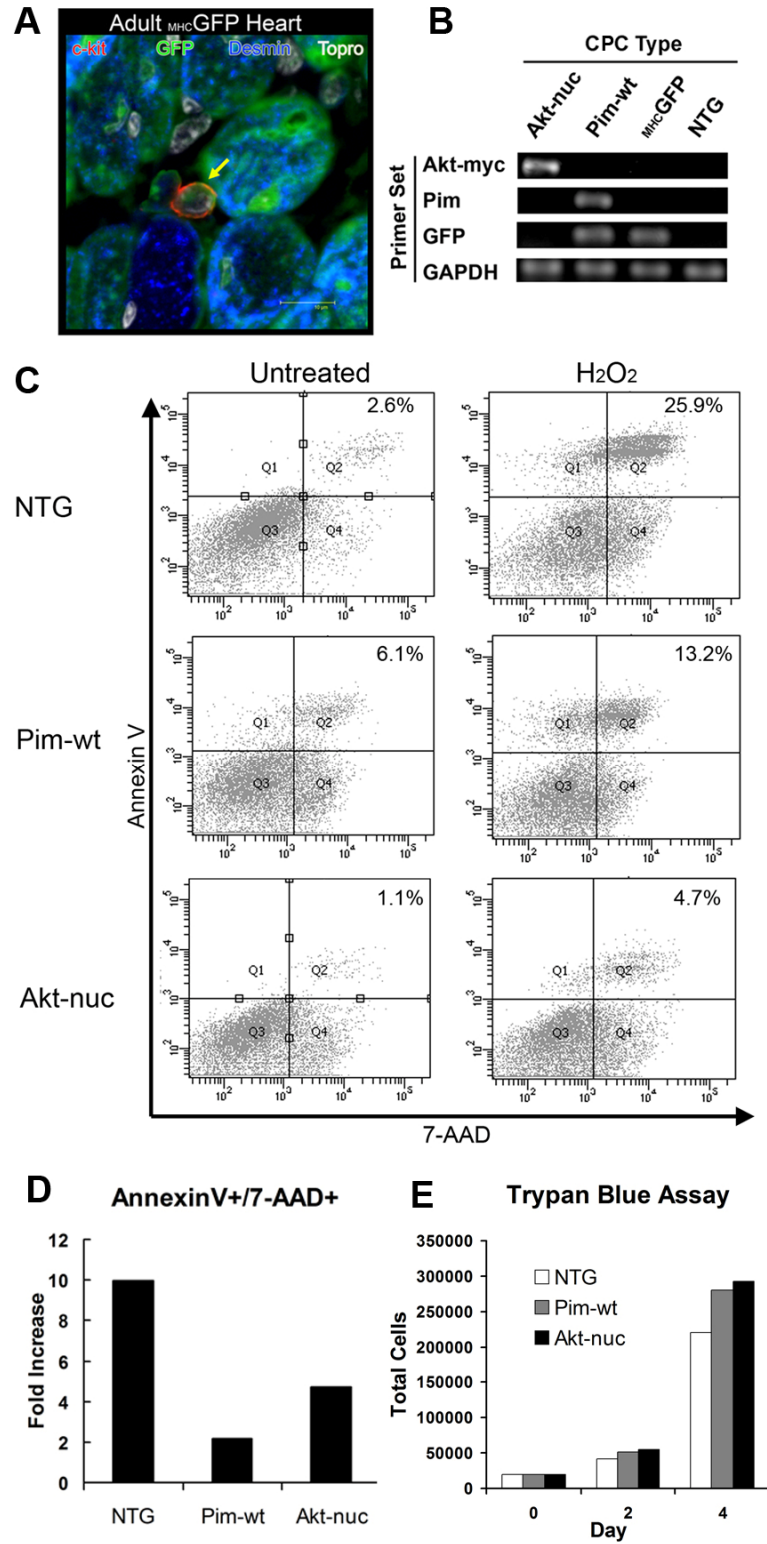


Figure 1. 2: The α MHC promoter is not active in primary cultured bone marrow stem cells.

Non-transgenic bone marrow stem cells (BMSCs) will not express fluorescent markers and tags when transfected with plasmid constructs under the control of the α MHC promoter. NTG BMSCs were cultured in growth media; transfected with plasmid constructs; allowed to express for 48 hours. A) Cells were transfected with a Tomato-Flag construct under the viral CMV promoter as a control. Td-Tomato protein fluorescence in red and phase contrast pictures are in gray-scale. B) Cells were transfected with YFP-HA construct under the control of the α MHC promoter. No fluorescence was observed (green) in a wide field of cells (gray-scale). C) Cells were transfected with GFP construct under the control of the α MHC promoter. No fluorescence was observed (green) in a wide field of cells (gray-scale).

Figure 1. 3: CPCs from transgenic mice express alphaMHC driven transgenes in vivo and in culture.

A) Immunolabeling of adult MHC^{GFP} mouse heart section shows colocalization of c-kit (red) and GFP (green) staining. Desmin (blue) also colocalizes with GFP in mature myocytes. Scale bar is $10\mu\text{M}$. B) CPCs isolated from MHC^{GFP} , $\text{MHC}^{\text{Akt-nuc}}$ and $\text{MHC}^{\text{Pim-wt}}$ were cultured in growth media and harvested for RNA isolation and cDNA synthesis. cDNA was amplified using primers specific for the transgene inserted into each CPC line. C) FACS analysis of NTG, $\text{MHC}^{\text{Pim-wt}}$ and $\text{MHC}^{\text{Akt-nuc}}$ CPCs either untreated (left) or H_2O_2 treated (right) stained for AnnexinV and 7-AAD apoptosis markers (representative of $n=2$ independent experiments). D) Fold-increase quantitation of AnnexinV+/7-AAD+ CPCs after H_2O_2 treatment in C. E) Trypan blue exclusion hemocytometer counts of NTG, $\text{MHC}^{\text{Pim-wt}}$ and $\text{MHC}^{\text{Akt-nuc}}$ CPCs seeded at equal amounts on Day 0 and counted 2 or 4 days later (representative of $n=3$ independent experiments).



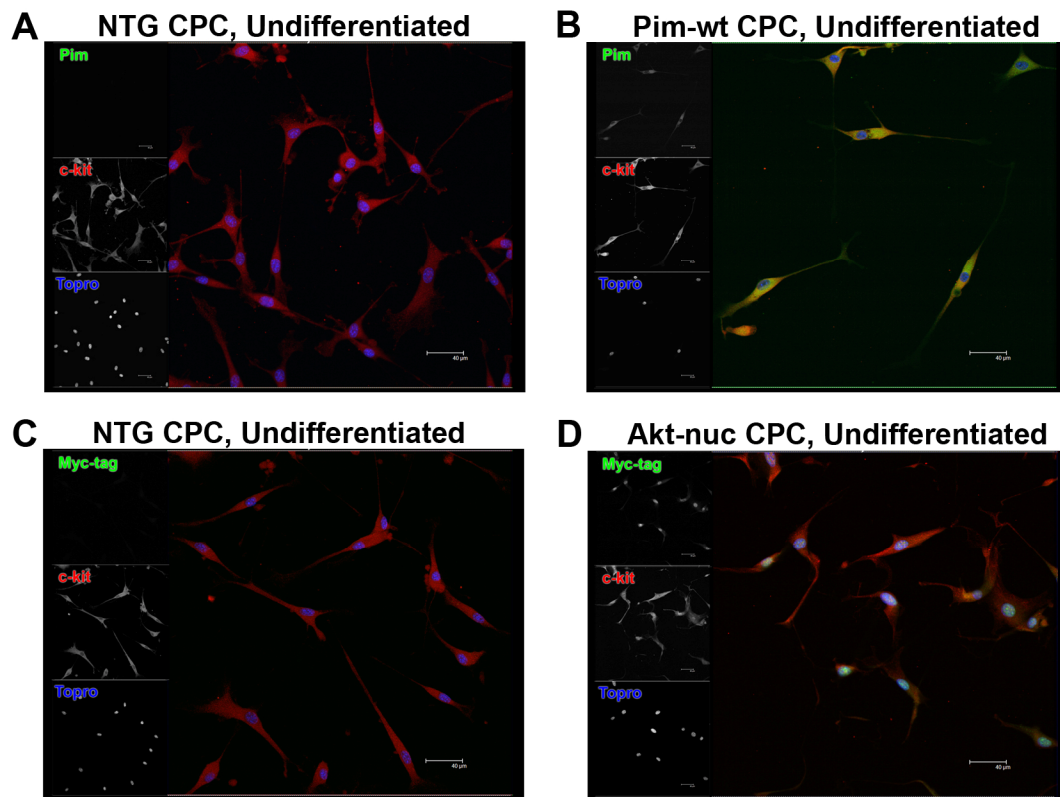


Figure 1. 4: CPCs express transgenes controlled by the α MHC promoter in the appropriate subcellular localization in culture by confocal microscopy.

A) NTG and B) $_{MHC}$ Pim-wt CPCs cultured in growth media, fixed, and immunolabeled with antibodies to Pim (green), c-kit (red) and Topro (blue). $_{MHC}$ Pim-wt CPCs show increased Pim expression localized to the cytoplasm. C) NTG and D) $_{MHC}$ Akt-nuc CPCs cultured in growth media, fixed and immunolabeled with Myc-tag (green), c-kit (red) and Topro (blue). $_{MHC}$ Akt-nuc CPCs show nuclear-localized myc-tag expression. Scale bar is 40 μ m.

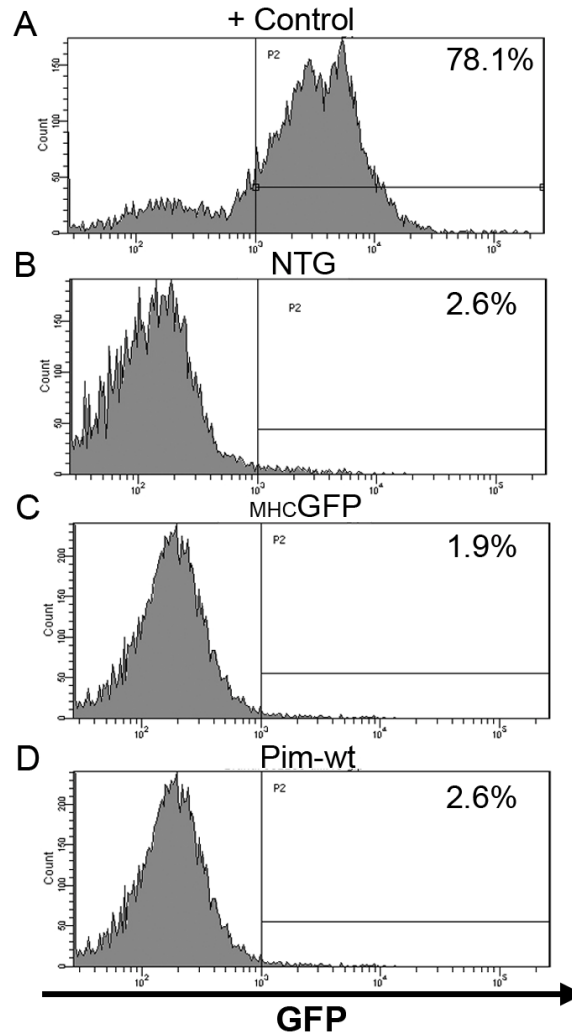


Figure 1. 5: Cells in the bone marrow do not express alphaMHC promoter driven GFP.

A) Whole bone marrow from Sca-1 Knock-In mouse expressing GFP under control of the endogenous Sca-1 promoter is GFP positive by FACS analysis. Whole bone marrow from B) non-transgenic C) $MHC-GFP$ and D) $MHC-Pim-wt$ do not express GFP by FACS analysis.

Figure 1. 6. Post-fixation incubation of NRCMs in media causes failure of NRCM nuclei to stain with Topro.

A) NRCM culture fixed and incubated in 4% PFA and stained for α -sarcomeric actinin (red) and Topro (teal). GFP fluorescence was scanned as a control (green). All NRCMs are Topro positive. B) NRCM culture fixed in 4% PFA then incubated in media for 6 days at 37°C and stained for α -sarcomeric actinin (red) and Topro (teal). GFP fluorescence was scanned as a control (green). None of the NRCMs are Topro positive. C) NRCM culture fixed in 4% PFA then incubated in media with GFP+ CSCs for 6 days at 37°C then stained for α -sarcomeric actinin (red) and Topro (teal). GFP fluorescence was scanned (green). None of the GFP negative NRCMs are Topro positive (white arrows) and all of the GFP positive CSCs are Topro positive (yellow arrows).

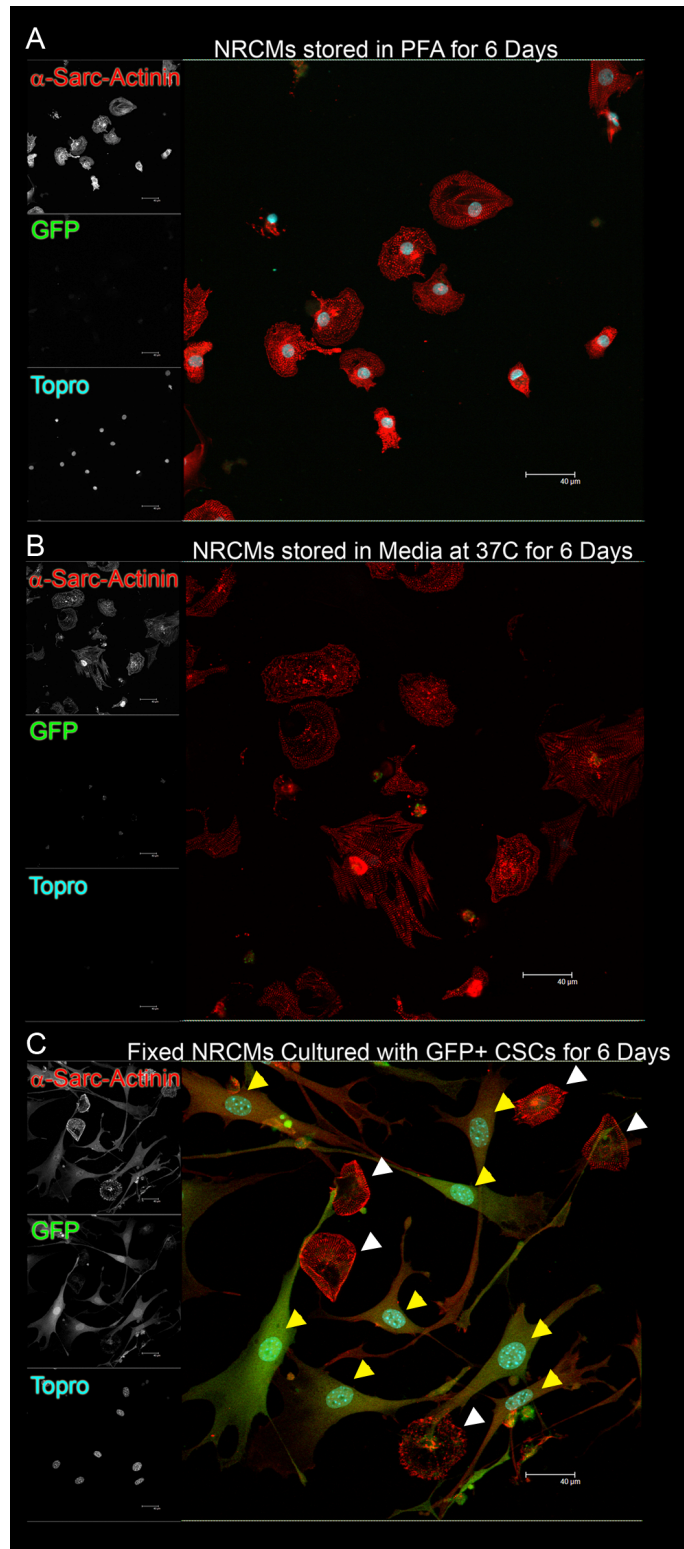
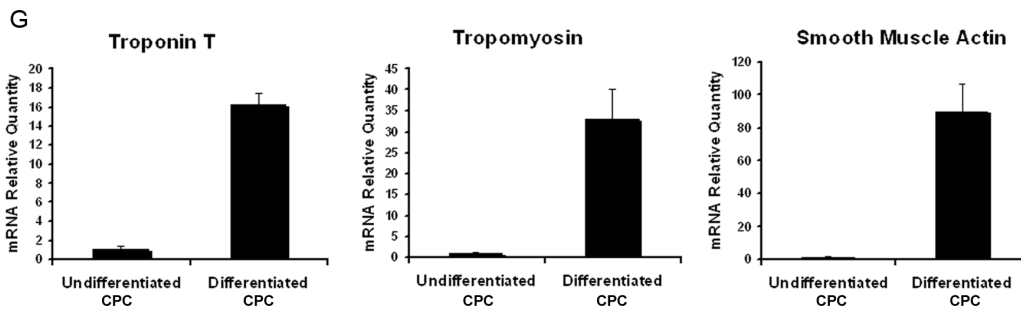
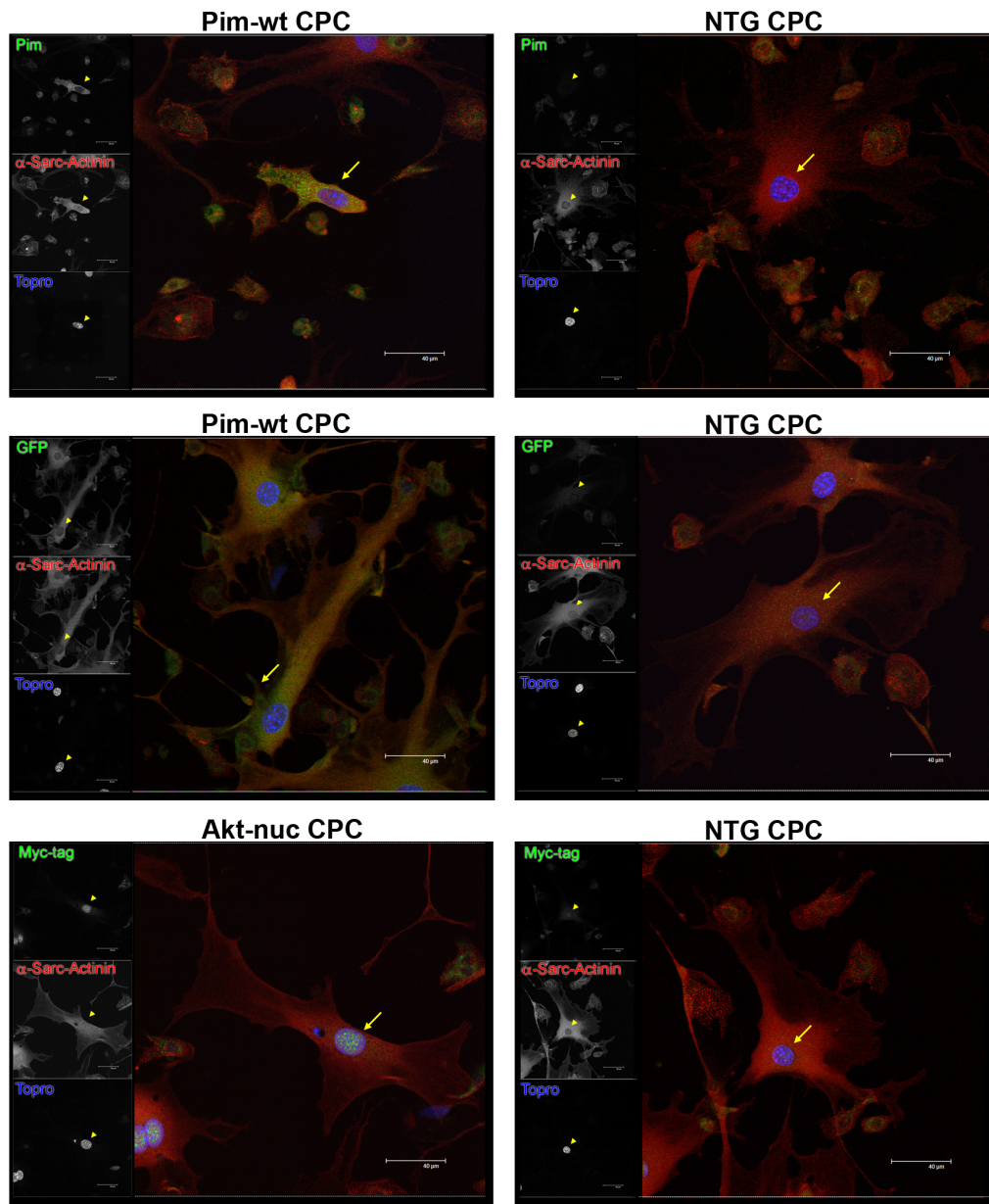


Figure 1. 7: Cardiomyogenic differentiation allows for persistent alphaMHC driven transgene expression.

A) _{MHC}Pim-wt and B) NTG CPCs differentiated on fixed NRCMs for 7 days, fixed and immunolabeled for Pim (green), α -Sarcomeric Actinin (red) and Topro (blue). _{MHC}Pim-wt differentiated CSCs express more Pim protein. C) _{MHC}Pim-wt and D) NTG CPCs differentiated on fixed NRCMs for 7 days, fixed and immunolabeled for GFP (green), α -Sarcomeric Actinin (red) and Topro (blue). _{MHC}Pim-wt differentiated CPCs express GFP. E) _{MHC}Akt-nuc and F) NTG CPCs differentiated on fixed NRCMs for 7 days, fixed and immunolabeled for myc-tag (green), α -Sarcomeric Actinin (red) and Topro (blue). _{MHC}Akt-nuc differentiated CPCs express increased nuclear-localized myc-tag. Scale bar is 40 μ m. G) RT-qPCR analysis of NTG CPCs post-differentiation on live NRCMs shows an increase in relative mRNA levels of troponin T (left), tropomyosin (middle) and α -smooth muscle actin (right) compared to undifferentiated CPCs.

CPCs Differentiated on Fixed NRCMs



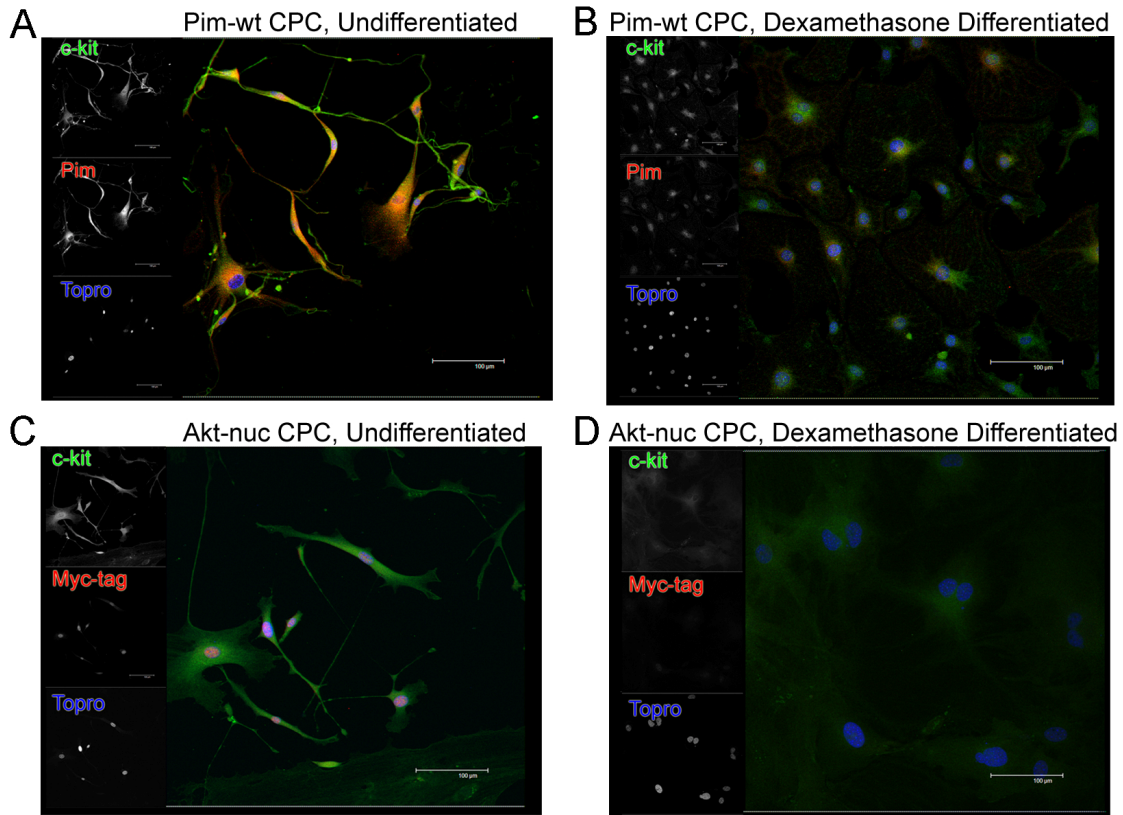


Figure 1. 8: Differentiation of CPCs by exposure to dexamethasone results in loss of transgene expression.

A) MHC^{Pim-wt} CSCs cultured in growth media, fixed and immunolabeled for c-kit (green), Pim (red) and Topro (blue). B) MHC^{Pim-wt} CSCs treated with dexamethasone containing media for 7 days, fixed and immunolabeled for c-kit (green), Pim (red) and Topro (blue) showing decreased c-kit as well as Pim expression. C) $MHC^{Akt-nuc}$ CSCs cultured in growth media, fixed and immunolabeled for c-kit (green), myc-tag (red) and Topro (blue). D) $MHC^{Akt-nuc}$ CSCs treated with dexamethasone containing media for 7 days, fixed and immunolabeled for c-kit (green), Myc-tag (red) and Topro (blue) showing decreased c-kit as well as myc-tag expression. Scale bar is 100μm.

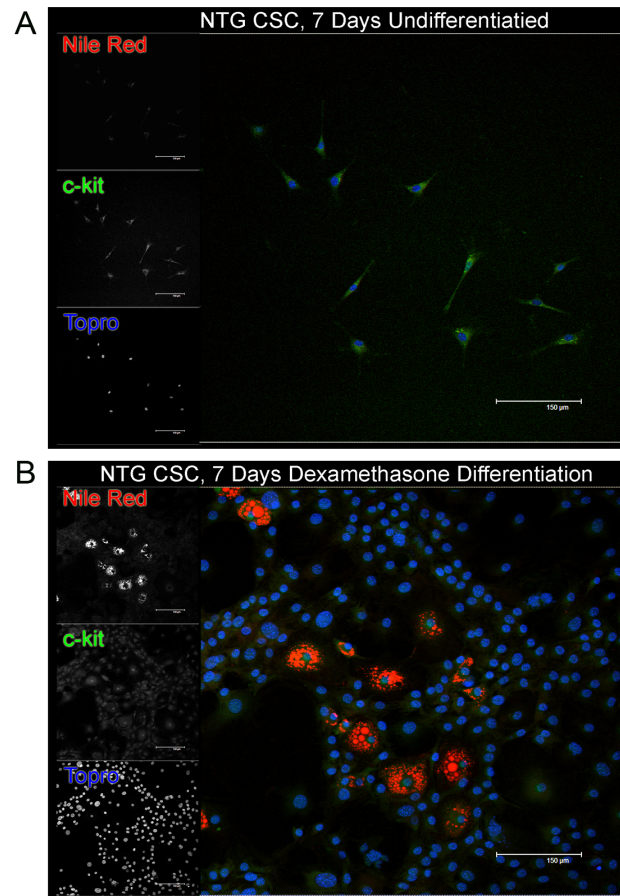


Figure 1. 9: Dexamethasone differentiation of CSCs induces an adipocyte-like phenotype.

A) NTG CSCs stained with Nile Red (red), a red fluorescent lipid dye, c-kit (green) and Topro (blue). NTG CSCs are c-kit positive and show very tiny punctuate staining with Nile Red. B) NTG CSCs that have been differentiated in Dexamethasone media for 7 days stained with Nile Red (red), c-kit (green) and Topro (blue). Some differentiated cells have an adipocyte-like phenotype with large inclusions of lipid showing Nile Red staining.

Table 1. 1: Chapter I RT-Primer Sequences

Gene	F/R	5' - Primer sequence - 3'	Product Length	Annealing Temperature
Akt-myc	F	GAAGACATTCTGCGGAACGCCG	634 bp	55°C
Akt-myc	R	GAGTTTTTGTCTGCGGCCGTATC	634 bp	55°C
Pim	F	GCACTCGAGTGCCCATGGAAGT	310 bp	55°C
Pim	R	TCGATGAGCTTGAGCTCGCC	310 bp	55°C
GFP	F	ATATCATGGCCGACAAG	190 bp	55°C
GFP	R	TCTGATCGCGCTTCTCGTT	190 bp	55°C
GAPDH	F	CAATTCAACGCCACACTCAA	150 bp	55°C
GAPDH	R	CCTTCTCCATGCTGGTGAA	150 bp	55°C

Table 1. 2: Chapter I Primary Antibodies and Dilutions

Primary Antibody	Species	Dilution	Amplification (paraffin only)	Marker	Manufacturer
c-kit	Goat	1:50	Yes	Stem cells	R&D Systems
GFP	Rabbit	1:200	Yes	Tag	Molecular Probes
Pim-1	Rabbit	1:50	Yes		CST
α -sarcomeri c-actinin	Mouse	1:100	No	Cardiomyocyte	Sigma
Desmin	Mouse	1:100	No	Cardiomyocyte	Biosource
Myc-tag	Rabbit	1:50	No	Tag	CST
Flag-tag	Mouse	1:100	No	Tag	Stratagene
HA-tag	Mouse	1:100	No	Tag	Santa Cruz

CHAPTER II:

Sca-1 Knockout Impairs Myocardial and Cardiac Progenitor Cell Function

INTRODUCTION

Identification of multi-potent stem cells in adult tissues such as the myocardium rests with stem cell markers originally developed to define cells of hematopoietic lineage. The c-kit cell surface receptor is commonly used to mark stem/progenitor cells. c-kit has been identified on small non-myocytes within the myocardium which are commonly referred to as cardiac progenitor cells (CPCs). These CPCs have been shown to contribute to myocardial repair following ischemic injury^{2, 43}. CPCs also express the cell surface marker Stem cell antigen-1 (Sca-1) both *in vitro*²⁷ and *in vivo*¹⁹. Sca-1 is an 18 kDa glycosyl phosphatidylinositol-anchored cell surface protein with cell signaling and adhesion properties⁴⁴. It is a member of the Ly-6 antigen family and is present on the surface of tissue-specific stem cells in the hematopoietic¹⁶, mammary gland⁴⁵, and vascular niche⁴⁶ in the mouse. Although typically associated with tissue stem or progenitor cells, Sca-1 is also expressed on a variety of fully differentiated cell types⁴⁷.

Roles of Sca-1 protein in stem cell development and function were primarily defined in the hematopoietic lineage as first described in the mouse by Gumley *et al.* in 1995⁴⁸. Sca-1 has since been used to mark and enrich stem cells. Functions of Sca-1 include promotion of adhesion and proliferation that are critical for optimal hematopoietic activity¹⁶. Furthermore, Sca-1 is a part of a family of proteins that contain a urokinase plasminogen activator receptor (UPAR) domain involved in cellular adhesion, migration by modulating integrin function,

and degradation of the extracellular matrix¹⁶. An additional suggested role of Sca-1 is involvement in signaling cellular differentiation⁴⁹. Isolated mesenchymal stem cells positive for both Sca-1 and c-kit differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells upon injection into the heart⁵⁰. Recent studies reveal that the number of Sca-1 positive cells increases in the left ventricle of the heart after myocardial infarction⁵¹. Progenitor cells from bone marrow also migrate to injured myocardium following infarction and contribute to repair⁵². This finding suggests that Sca-1+ cells contribute to regeneration and repair of a heart following a myocardial infarction (MI). Collectively, these characteristics make Sca-1 a critical protein for evaluating stem cells in the heart.

In this study we assess the effects of Sca-1 knock-out upon the functional properties of myocardial CPCs and myocardial performance. Sca-1 reporter mice with a single allele GFP knock-in, Sca-1^{GFP/+} accurately label Sca-1+ cells in peripheral blood with GFP⁵³. Results presented in this report use the Sca-1 Knock-In (ScaKI) mouse model which has had both alleles of the Sca-1 protein coding sequence replaced with GFP downstream of the endogenous Sca-1 promoter, generating a mouse with a genetic deletion of ScaKI possessing the genotype of Sca-1^{GFP/GFP}. Prior studies reveal the ScaKI mouse exhibits serial bone marrow stem cell repopulating defects but has not been previously studied for a cardiac phenotype¹⁶. Genetic deletion of the well known Sca-1 protein that marks stem cells revealed important insights into the function of this molecule in cardiac maintenance and repair.

METHODS

Transgenic Animal Models

Sca-1 knock-in transgenic mice were a generous gift from Dr. Timothy Graubert^{16, 53}. A construct encoding GFP was inserted into the second exon of the Sca-1 gene at the start codon, functionally eliminating Sca-1 protein expression and creating a mouse in which GFP was expressed under the control of the endogenous Sca-1 promoter. These animals were then back-crossed into a C57/Bl6 background to create the ScaKI mice used in these experiments which are homologous for the recombined genes resulting in a transgenic line which is negative for Sca-1, but positive for GFP wherever Sca-1 promoter is expressed. ScaKI transgenic mice were created using the C57/Bl6 background, thus non-transgenic control animals are denoted as C57. All experimental procedures were designed in accordance with NIH guidelines and approved by the San Diego State University and the Institutional Animal Care and Use Committees.

Surgical Methods

Echocardiography was performed under minimal (1.25%) isoflurane anesthesia with 2.5% supplemented oxygen using an Acuson Sequoia C256 instrument. Myocardial infarctions were carried out under 1.5% isoflurane anesthesia by ligating the left descending coronary artery with 8-0 suture. To confirm adequate ligation, cyanosis and akinesia of the affected left ventricle were observed. Sham operations were executed by opening and closing the chest. Post-

operative palliative care was administered immediately and at 12 hours. Hemodynamic measurements were accomplished with a closed chest by inserting a pressure/volume catheter through the aortic valve into the left ventricle under chloral hydrate sedation. The data was collected and analyzed using a Scisense microtip pressure transducer attached to an A/D converter. To generate paraffin embedded cardiac samples, mice were heavily sedated with chloral hydrate, and the hearts were arrested in diastole by catheterizing the abdominal aorta and flushing the heart with a high potassium chloride/cadmium chloride solution. Neutral buffered formalin (NBF) fixative was perfused into the coronary arteries at systolic pressure while the left ventricle was filled with NBF at diastolic pressure. Retroperfused hearts were then removed from the chest cavity and placed in NBF overnight, followed by processing for paraffin embedding using a Leica TP1050 automated tissue processor.

Immunofluorescence Microscopy

Paraffin embedded tissue sections, cut at 5 μ m, were prepared from mouse hearts that had been processed as described above. Sections of were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was then performed in 10mmol/L citrate, pH 6.0, using 1100-W microwave oven for 3 minutes at high power and 12 minutes at 50% power. The slides were allowed to cool for 15 minutes at 4°C, then washed in 3 times in TN buffer (NaCl 150 mM, Tris 100mM, pH 7.5) and quenched with 3% hydrogen peroxide in TN buffer for 20 minutes to remove endogenous

peroxidase activity. Slides were then washed 3 times in TN buffer and blocked for 1 hour in TNB (TN buffer containing 0.5% Blocking Buffer, proprietary formula from TSATM kit, Perkin Elmer/NEN). Slides were incubated at 4°C overnight with primary antibodies diluted in TNB. Slides were then washed 3 times for 5 minutes per wash in 1X TN and incubated for 2 hours at room temperature in the dark with species-specific secondary antibodies conjugated to fluorophores in TNB. After secondary labeling, slides were washed 3 times in TN. Nuclei were stained for 20 minutes with either To-pro-3-Iodide (Topro) at 1/10,000 in TN or Sytox Blue at 1/200 in TN. Slides were mounted for viewing in Vectashield medium. Detection of some antigens required signal amplification using biotin-streptavidin and/or horseradish peroxidase (HRP) conjugated antibodies followed by reaction with a fluorescent tyramide substrate according to manufacturer's instructions. Antibody information is detailed in Supplemental Table 1. Confocal images were acquired using a Leica TCS SP2 confocal microscope.

Vascular Casting

The microfil injection was accomplished according to previously described protocol⁵⁴. Briefly, the mouse was sedated with ketamine/xylazine solution and the right common carotid artery was cannulated and injected slowly with microfil solution. The hearts were placed into PBS overnight and then dehydrated in increasing ethanol solutions and cleared in methyl salicylate according to manufacturer's instructions.

Paraffin Embedded Cell Counts

Hearts were harvested at the specific developmental time points described in the text and paraffin embedded. At least three non-sequential sections were cut and stained per heart. The area included within the count was measured using the Leica LCS Lite (Leica AG) software.

Cardiac Stem Cell Isolation and Culture

CPCs were isolated and cultured as previously described². Briefly, two mice per preparation were anesthetized using ketamine-xylazine solution and the heart was cannulated through the aortic arch and perfused at 37°C in oxygenated basic buffer (J-MEM, 0.7 g/l Hepes, 1.25g/l taurine, 20units/l insulin, Pen/Strep/Glutamine, Amphotericin, Gentamicin, pH 7.3) on a Radnotti apparatus. The heart was then digested for 12 minutes at 37°C in 320units/ml collagenase II in oxygenated basic buffer. Afterwards, the heart was minced in basic buffer containing 0.5% BSA and the cardiomyocytes pelleted for 1 min at 100g and discarded. Remaining cells in the supernatant were passed through a 25µm filter and pelleted. The cell pellet was resuspended and incubated with anti-c-kit (CD117) Miltenyi beads in PBS + 0.5% BSA; washed and isolated on a Miltenyi magnetic column to extract c-kit⁺ CPCs according to manufacturer's instructions. CPCs were cultured according to standard tissue culture protocols in CPC media (DMEM/F12, 10% Embryonic Stem Cell-Grade FBS, PSG, Insulin-Transferrin-Selenium, 1000u/ml LIF, 40ng/ml EGF, 20ng/ml bFGF).

Flow Cytometry and Apoptosis Analysis

Cells were grown in complete media and analyzed by flow cytometry after non-enzymatic removal from the culture plates. The cells were resuspended at 100,000 cells in 100 μ L PBS+2% FBS, stained with directly conjugated antibodies and analyzed following two wash steps. For apoptosis treatment and analysis, CPCs were seeded into 6 well plates in CPC media for at least 12 hours before treatment. Treated cells received the indicated amount of hydrogen peroxide (H_2O_2) for 5 hours. After peroxide incubation, treated cells and untreated controls were labeled with Annexin V-APC Apoptosis Detection Kit (BD Pharmingen) according to manufacturer's instructions. Briefly, cells were suspended at 100,000 cells per 100 μ L in Annexin V Binding Buffer. Each set of treated and untreated cells was incubated for 20 minutes at room temperature in the dark with 0.75 μ L Annexin V and 2 μ L 7-AAD per 100,000 cells. 200 μ L of Binding Buffer was added post incubation and the cells were analyzed by flow cytometry with a BD FACS Aria instrument. Unstained and single stain controls were used to establish baseline fluorescence levels for the samples.

Trypan Blue and Cyquant Assay

Cell cultures were counted after passage using 2% trypan blue solution by hemocytometer determination. For trypan blue assay, 15,000 cells per well were seeded at Day 0 in a 6 well plate and counted at the indicated days post plating. For Cyquant analysis, 1000 cells per well were seeded in triplicate Day 0 early in the morning in 100 μ L of full growth media. In the evening of Day 0 through Day 5,

the growth media was gently removed from the appropriate wells and replaced with 1xHBSS containing Cyquant NF reagent diluted 1:500. Fluorescence was measured 30 minutes later on a Tecan Spectraflour Plus plate reader at an Excitation/Emission of 485/535 with a standard gain of 60.

qPCR

mRNA was isolated from cells using Zymo Research quick RNA miniprep according to manufacturer's instructions. Briefly, at least 500,000 cells per sample were collected in ZR RNA buffer, applied to a binding column and centrifuged at 12000 rpm for 1 minute. Flow through was discarded and washed once with RNA Wash Buffer. The RNA was eluted in DNase/RNase free water and quantitated by spectrophotometer. RT-PCR was accomplished using the Bio-Rad iScript cDNA synthesis kit according to manufacture's instructions. Briefly, 1 μ g of RNA per sample was added to 4 μ l of 5X Master Mix, 1 μ l of 20x reverse transcriptase and RNase free water up to 20 μ L. The rtPCR reaction conditions were 5 min at 25 $^{\circ}$ C, 30 min at 42 $^{\circ}$ C followed by 5 min 85 $^{\circ}$ C. cDNA was diluted at least 1:60 in RNase/DNase free water. qPCR was accomplished using 20-30 μ g of cDNA, 3 μ M forward and reverse primers and 2x Bio-Rad Sybr Green master mix in 15 μ l. qPCR was performed on a Bio-rad/MJ research Opticon instrument with amplification protocol: 10 min at 95 $^{\circ}$ C; then 15 sec at 95 $^{\circ}$ C, 60 sec at 60 $^{\circ}$ C, then plate read for 50 cycles followed by a melt curve analysis for product specificity. qPCR primer sequences are detailed in Supplemental Table 2. Fold difference analysis was calculated using the $2^{-\Delta\Delta Ct}$

method using GAPDH as housekeeping gene control. qPCR arrays were accomplished according to manufacturer instructions by adding 25 μ l per well of 2x Bio-Rad Sybr Green master mix and 25 μ L per well of cDNA. SA Biosciences qPCR array primer sequences are proprietary information.

siRNA

CPCs derived from FVB/N mice were plated in 10ml full growth media for 12 hours at 500,000 cells per 10cm plate. The media was replaced with Transfection Media consisting of CPC growth media containing 0% FBS. siRNA for Sca-1 (Ly6A) was obtained from Bioneer (110454, proprietary sequence), and resuspended at 100 μ mol/l in DNase/RNase free water. Transfection mix containing 425 μ l of Transfection Media, 50 μ L of HiPerfect (Qiagen) transfection reagent and 15 μ l of 20 μ mol/l siRNA was added to the 10cm plate and incubated for 24 hours. The media was then changed to DMEM/F12 with 2.5% FBS and incubated for an additional 24 hours. Cells were then passaged and plated in full CPC media for the indicated assays

Bone Marrow Isolation

Bone marrow cells (BMC) were flushed from both tibias and femurs of each mouse using PBS + 1%FBS buffer. BMCs were separated from red blood cells by density centrifugation using Histopaque reagent according to manufacturer's instructions. The BMCs were assayed by FACS analysis fresh from isolation.

Statistical Analysis

All p values were determined using a paired, two-tailed Student's t-test with an N of at least three samples per group. Significance for qPCR analysis was determined by comparing ΔC_t values of experimental versus control samples using a paired, two-tailed Student's t-test with an N of at least three samples per group.

RESULTS

Vascular endothelial layers in the myocardium express Sca-1 antigen or Sca-1 driven GFP

In addition to serving as a hematopoietic stem cell marker, Sca-1 also labels vasculature in several organ systems^{20, 47}. Due to possible presence of Sca-1 on vascular structures in the heart, the genetic deletion of Sca-1 in ScaKI mice may manifest an altered vasculature. The gross vascular pattern, as assessed by latex perfusion of the heart, appears similar in ScaKI and C57 hearts (Figure 2.1A). Sca-1 is indeed expressed on the vascular endothelial surface as evidenced by colocalization adjacent to the vascular marker α -Smooth Muscle Actin (α SMA) (Figure 2.1B, left) and colocalization with von Willebrand's Factor (vWF) (Figure 2.1C, left) in myocardial sections of nontransgenic mice. In comparison, GFP expression occurs throughout the vascular cell and colocalizes with α SMA in ScaKI myocardial sections (Figure 1B, right). Comparable to Sca-1⁺/vWF⁺ cells in nontransgenic controls, GFP⁺/vWF⁺ cells are observed in ScaKI mice (Figure 2.1C, right). Thus, Sca-1 expression is associated with vasculature of the myocardium.

To examine whether absence of Sca-1 affected vascular density, CD31⁺ vessels in both nontransgenic controls and ScaKI myocardial sections were assessed and quantitated by immunohistochemical staining. Overt appearance of vasculature marked by CD31 immunoreactivity is comparable between nontransgenic controls and ScaKI (Figure 2.1D). Assessment of capillary density

revealed no significant difference in the myocardium between the control and the ScaKI groups (Figure 2.1E).

Depressed cardiac function in ScaKI mice, hypertrophy in older animals and poor survival after myocardial infarction challenge

In the absence of evident vascular anomalies in the ScaKI line, further phenotypic characterization was performed to assess defects in myocardial structure and function by echocardiography using gender matched ScaKI and C57 control mice at 12-18 weeks (males) as well as 12-14 months (male and female groups). Both young and old ScaKI mice exhibit significantly larger End Systolic Diameter (ESD) measurements relative to nontransgenic controls (Figure 2.2A and 2.2B). *In vivo* hemodynamic measurements confirm poor cardiac function in ScaKI hearts as measured by change in pressure over change in time (dP/dT) where maximum pressure was significantly lower and minimum pressure was significantly higher in ScaKI hearts (Figure 2.2C and 2.2D). Fractional shortening (FS) and ejection fraction (EF) are both significantly lower in 1 year old ScaKI mice, which also exhibited significantly larger hearts as measured both by echocardiography of Anterior Wall Dimension (AWD), Posterior Wall Dimension (PWD) (Figure 2.2B) and heart weight/body weight ratio (Figure 2.2F). Increased weight of old ScaKI hearts and size can be attributed to cardiomyocyte hypertrophy due to an increase in average cardiomyocyte cross-sectional area (Figure 2.2G). Despite relatively modest remodeling in the young ScaKI hearts, significantly fewer ScaKI mice survive

myocardial infarction (MI) for 14 days relative to nontransgenic controls as shown by Kaplan-Meier analysis (Figure 2.2H). Postmortem investigation indicates cardiac rupture as the primary cause of death (4 cardiac rupture, 2 undetermined) in ScaKI mice after infarction challenge.

Diminished number of CPCs in ScaKI mice

c-kit positive CPCs are present in both C57 and ScaKI hearts (Figure 2.3A). However, significantly fewer total c-kit⁺ CPCs are present in ScaKI myocardial sections from normal tissue ($1.5 \pm 0.6/\text{mm}^2$) relative to the level observed in nontransgenic controls ($2.6 \pm 0.8/\text{mm}^2$; Figure 2.3C). CPC migration to the zone of pathological damage increases in response to MI (Figure 2.3B), peaking between 4-10 days post-MI²⁰. In order to obtain ScaKI 7 day survivors, infarct size was gradually reduced in both groups by ligating the LAD further toward the apex of the heart. As true for normal myocardium, significantly fewer CPCs are present in the infarct region of ScaKI hearts ($2.4 \pm 1.0/\text{mm}^2$) relative to nontransgenic controls ($8.7 \pm 1.2/\text{mm}^2$; Figure 2.3C). A significant difference in infarct size is not evident as measured by percent of involved left ventricular free wall (Figure 2.3D). CPC proliferation, assessed using proliferating cell nuclear antigen⁵⁵ (PCNA), revealed c-kit⁺/PCNA⁺ cells in the infarct and border region (Figure 2.3E) with significantly fewer c-kit⁺/PCNA⁺ cells in the ScaKI infarct region compared to nontransgenic controls (Figure 2.3F).

12-14 month old ScaKI mice have fewer stem cells as labeled by c-kit expression in the bone marrow

In order to further characterize the effects of Sca-1 knock out on another stem cell population, the levels of stem cell marker c-kit⁺ cells were analyzed from both C57 and ScaKI bone marrow at the ages of 16 weeks and 12 months of age. Whole bone marrow was isolated and red blood cells were removed. Staining and FACS analysis (Figure 2.4A) of Sca-1 and c-kit markers in C57 mice and GFP fluorescence and c-kit in ScaKI mice revealed that although the levels are c-kit⁺ cells are equal in younger mice, older ScaKI mice have fewer c-kit⁺ cells in the bone marrow (Figure 2.4B). The data indicate that ScaKI mice have more GFP⁺ cells in the bone marrow than normal controls probably due to the longer turnover rate for GFP protein compared to Sca-1⁵⁶ (Figure 2.4A).

Developmental expression pattern of Sca-1 promoter-driven GFP in the heart

The relationship between cardiac development, CPCs, and Sca-1 driven GFP expression in the heart was determined by following these parameters in a developmental time course encompassing 2 day, 1 week, 2 weeks and 12 weeks. Both c-kit⁺ only and c-kit⁺/GFP⁺ cells were observable at age two days and one week (Figure 2.5A, top) in myocardial sections. Incidence of c-kit⁺/GFP⁻ decreases whereas GFP⁺ cell numbers increase and coincide with vascular structures as mice mature beyond 2 weeks into adult age, (Figure 2.5B, right). Both c-kit⁺/GFP⁻ cells and c-kit⁺/GFP⁺ cells persist in the myocardium into adulthood (Figure 2.5B). The number of cells and the area of each heart were

measured to determine the number of c-kit-/GFP+, c-kit+/GFP+, and c-kit+/GFP- cells per mm² of myocardium (Figure 2.5C). Frequency of c-kit+/GFP- decreases significantly between 2 days ($2.6/\text{mm}^2 \pm 1.0$) to 2 weeks ($0.23/\text{mm}^2 \pm 0.1$) but remains similar thereafter into adulthood (0.41 ± 0.1). Dual positive c-kit+/GFP+ cell incidence remains relatively consistent throughout development at 2 days (0.19 ± 0.17), 1 week (0.63 ± 0.12), 2 week (0.27 ± 0.07), and 12 weeks of age (0.47 ± 0.01). The c-kit-/GFP+ population progressively increases significantly from 11.4 ± 2.3 at 2 days and 20.8 ± 12.6 at 1 week to 141 ± 2 at 2 weeks and 115 ± 19 at 12 weeks (Figure 2.5C). Collectively, these results demonstrate the continuous presence of c-kit+/Sca-1 driven GFP+ cells in the myocardium from a neonate through adult and the increasing presence of Sca-1 driven GFP in the maturing vasculature.

Stem cell marker expression, decreased proliferation and survival in adult ScaKI CPCs

CPCs were isolated using the c-kit+ marker from both ScaKI and nontransgenic control mice to determine phenotypic properties *in vitro*. Resulting cultures exhibit activation of the Sca1 promoter as evidenced by GFP expression in ScaKI (64%) or Sca-1 protein in nontransgenic controls (78%) by flow cytometry (Figure 2.6A). Internal controls confirm that nontransgenic CPCs are GFP- whereas ScaKI CPCs are Sca-1- (Figure 2.6A). CPCs from both nontransgenic and ScaKI lines are comparable in morphology and appearance under normal culture (Figure 2.6B), but proliferation of ScaKI CPCs is depressed relative to normal CPCs

(Figure 2.6C) and ScaKI CPCs lack Ki67 immunoreactivity (Figure 2.6B). ScaKI CPCs also show increased sensitivity to oxidative stress induced by H₂O₂ exposure leading to apoptosis (Figure 2.6D).

Decreased TCF/LEF1 controlled gene expression in ScaKI CPCs

The mechanism of Sca-1 signaling and specific genes activated by Sca-1 has yet to be described. However, phenotypic deficiencies in proliferation and survival in ScaKI CPCs (Figure 2.3 – 2.5) suggest an underlying molecular basis. Screening assays by qPCR analysis using cDNA derived from each cell line under optimal growth conditions assess activation of multiple signaling cascades (Figure 2.7A). The canonical Wnt Pathway exhibited the greatest number genes significantly changed between ScaKI CPCs versus nontransgenic controls. Down regulation of three separate genes in the Wnt family were highly significant: WNT1 inducible signaling pathway protein 1 (Wisp1), Cadherin 1 (Cdh1), and Lymphoid enhancer binding factor 1 (Lef1). Other relevant genes significantly down regulated include vascular cell adhesion molecule 1 (Vcam1) and NLR family, apoptosis inhibitory protein 1 (Naip1/Birc1a). Downregulation of these genes was confirmed by qPCR of 3 additional cDNA samples from C57 and ScaKI CPCs performed in duplicate (Figure 2.7B, left). Curiously, Axin2 transcript is approximately 13-fold induced in ScaKI CPCs compared to C57 (Figure 2.7B, right). Axin2 is an upstream inhibitor of TCF/Lef activation that is transcribed by TCF/Lef usually resulting in a negative feedback loop⁵⁷. To confirm the transcriptional and proliferative phenotypes generated by genetic deletion of Sca-1, siRNA to Sca-1

was used to knockdown Sca-1 in normal CPCs generated from wil-type FVB/N mice. The number of Sca-1⁺ CPCs in the culture was reduced from an average of approximately 55% in siScramble transfected cells to 10% in siSca-1 transfected cells from Days 1-3 post plating (Figure 2.7C). Analysis of RNA samples for Sca-1, Lef1 and Axin2 expression collected on Days 1, 2 and 3 post plating reveals significant knockdown of Sca-1 was observed at all timepoints; however, there was only a transient reduction in Lef1 transcripts at Day 1 and Day 2, which was recovered by Day 3. This could reflect a disparity in severity of acute versus chronic Sca-1 depletion. Although Lef1 transcript reduction was not as severe as in the genetic Sca1 deletion model, a significant reduction in proliferation was still observed in siSca-1 cells compared to siScramble cells by both Trypan Blue and Cyquant analysis (Figure 2.7E and 2.7F).

DISCUSSION

Maintenance of myocardial structure and function depends upon CPCs, so understanding the molecular biology of CPCs will be essential for expanding our repertoire of methodological approaches for enhancing cellular therapeutic approaches. Although Sca-1 is a widely accepted marker of stem cells in the hematopoietic system, downstream molecular signaling and consequences of Sca-1 dysregulation are poorly understood in the hematopoietic context and essentially unknown in the myocardial environment. Therefore, this study was undertaken to characterize Sca-1⁺ cells and Sca-1⁺/c-kit⁺ CPCs in particular with respect to development, function, maintenance and repair of the heart. Sca-1 has been previously used by other groups to mark mouse²⁷ and human⁵⁸ CPCs. Lack of Sca-1 in the adult mouse heart results in a minor developmental contractile defect, age associated hypertrophy and a reduced ability to survive infarction (Figure 2.2). Immunohistochemical analyses of Sca-1 in the heart throughout development reveals that Sca-1 is present on approximately half of all c-kit⁺ progenitor cells at all ages above 2 days and vascular endothelial cells only in the mature (>2 week old) myocardium (Figure 2.5).

The cardiac phenotype of the ScaKI mouse and pervasiveness of Sca-1 on vascular endothelial surfaces prompted the hypothesis that vascular anomalies would be present in the ScaKI line. However, our data indicate that the number of CD31⁺ major vessels, the number of IsolectinB4⁺ capillaries and the gross vasculature structure of the heart is similar between C57 control and

ScaKI mice (Figure 2.1). Therefore, vascular insufficiency is not likely to be responsible for the cardiac phenotype of the ScaKI mice.

Although Sca-1 expression in the mouse model is not as precise as c-kit labeling for CPC identification (Figure 2.1) that does not diminish importance of Sca-1 as a stem/progenitor cell surface protein. Prior publications examining the hematological system of the ScaKI mice failed to reveal an overt phenotype, instead characterized only a mild immunological impairment as measured by a deficiency in serial bone marrow repopulation ability¹⁶. We hypothesized that the consequences of Sca-1 genetic deletion would have an effect upon stem cells, but only manifest in adult animals and be exacerbated by substantial aging or pathological challenge. Deficiency in c-kit⁺ cells in the bone marrow was apparent in one year old ScaKI mice but not young adult animals (Figure 2.4) indicating that lack of Sca-1 results in an age-related stem cell defects. The age associated increase in average myocyte size within the ScaKI heart is possibly due to decreased new myocyte formation by CPCs⁵⁹.

The most salient phenotype of the ScaKI heart is reduced ability to for mice to cope with infarction challenge, as evidenced by diminished capacity of ScaKI mice to survive MI challenge (Figure 2.2). Loss of Sca-1 is associated with functional deficiencies in mounting a CPC response to infarction challenge (Figure 2.3) consistent with the premise that the ScaKI heart harbors a functionally compromised stem cell population. Although there are significantly fewer c-kit⁺ cells in the ScaKI myocardium, a CPC line was successfully established from ScaKI mice. However, these ScaKI CPCs possess deficiencies

in growth and survival (Figure 2.6), corroborating earlier studies in the non-cardiac context using Sca-1 knock-down to reveal impaired growth kinetics⁶⁰. The poor growth potential of ScaKI CPCs observed *in vitro* (Figure 2.6) parallels the lack of CPC response to infarction *in vivo* (Figure 2.3).

Genetic screening supports a role for canonical Wnt signaling in the pathogenesis observed for Sca-1 deletion, wherein TCF/Lef is the primary transcriptional effector for growth and survival (Figure 2.7). TCF/Lef also has non-Wnt associated functions⁶¹. Wnt signaling has been well associated with adult stem/progenitor cell proliferation, survival and differentiation in many cell types⁶². Wnt signal cascades are also crucial during cardiac development in the embryogenesis^{63, 64}. Logically, it would not be surprising for a mouse with a canonical Wnt signaling defect specifically within the progenitor cell population to have impairment of repair and regeneration capabilities. Association between Sca-1 protein expression and the Wnt pathway is intriguing and future studies should examine a role for Sca-1 in CPC lineage commitment, since a crucial role for Sca-1 in lineage commitment and c-kit expression has been reported for hematopoietic stem cells¹⁶. Investigation, perhaps in a hind limb ischemia model, of a vascular repair defect in ScaKI mice would also be an important contribution.

The function of cardiac progenitor cells as described in the literature is to replace aged or damaged myocardium with newly synthesized and differentiated cells. The data from this study demonstrate a requirement for functional Sca-1 protein expression to support optimal cardiac progenitor cell function in the roles of heart development and repair.

SUMMARY POINTS

- Sca-1 is present on both the surface of CPCs and vascular endothelial cells in the heart.
- Knockout of Sca-1 does not seem to cause baseline vascular insufficiency.
- Knockout of Sca-1 impairs CPC proliferation and survival *in vitro* and proliferation in response to MI *in vivo*.
- Decreased MI survival in the context of Sca-1 knockout may be due to CPC impairment.
- Sca-1 knockout appears to disregulate the canonical Wnt signaling pathway via Lef1 transcription factor reduction.
- Humans have several surface proteins described as analogous to Sca-1 which may be targets of interest in the manipulation of human CPCs.

FIGURES

Figure 2. 1: Sca-1, or Sca-1 driven GFP, is expressed on the vascular endothelial surface and lack of Sca-1 does not alter vessel density in the heart.

(A) Representative latex vascular castings of C57 and ScaKI adult hearts. “Left” indicates left ventricle with the LAD featured prominently. “Right” indicates right ventricle. (B) Sca-1 in C57 (left) and GFP in ScaKI (right) animals co-localize with α -Smooth Muscle Actin in the adult mouse myocardium; α -SMA (white), GFP or Sca-1 (green), Tropomyosin (“Tmyo”, red), and Sytox Blue (“Sytox”, blue). (C) Sca-1 in C57 (left) and GFP in ScaKI (right) animals co-localize with vonWillebrand’s Factor in the adult myocardium; vonWillebrand’s Factor (white), GFP or Sca-1 (green), Tropomyosin (red), and Sytox Blue (blue). Scale bar is 100 μ M. (D) C57 (left) and ScaKI (center) heart sections stained with CD31 (green), Desmin (red) and Topro (blue). Quantitation (right) of CD31 vessels per mm² in both C57 and ScaKI hearts. (E) C57 (left) and ScaKI (center) heart sections stained with IsolectinB4 (green), Desmin (red) and Topro (blue). Quantitation (right) of isolectinB4+ vessels per mm² in both C57 and ScaKI hearts. Scale bar is 40 μ m. Error bars represent standard error of the mean (SEM).

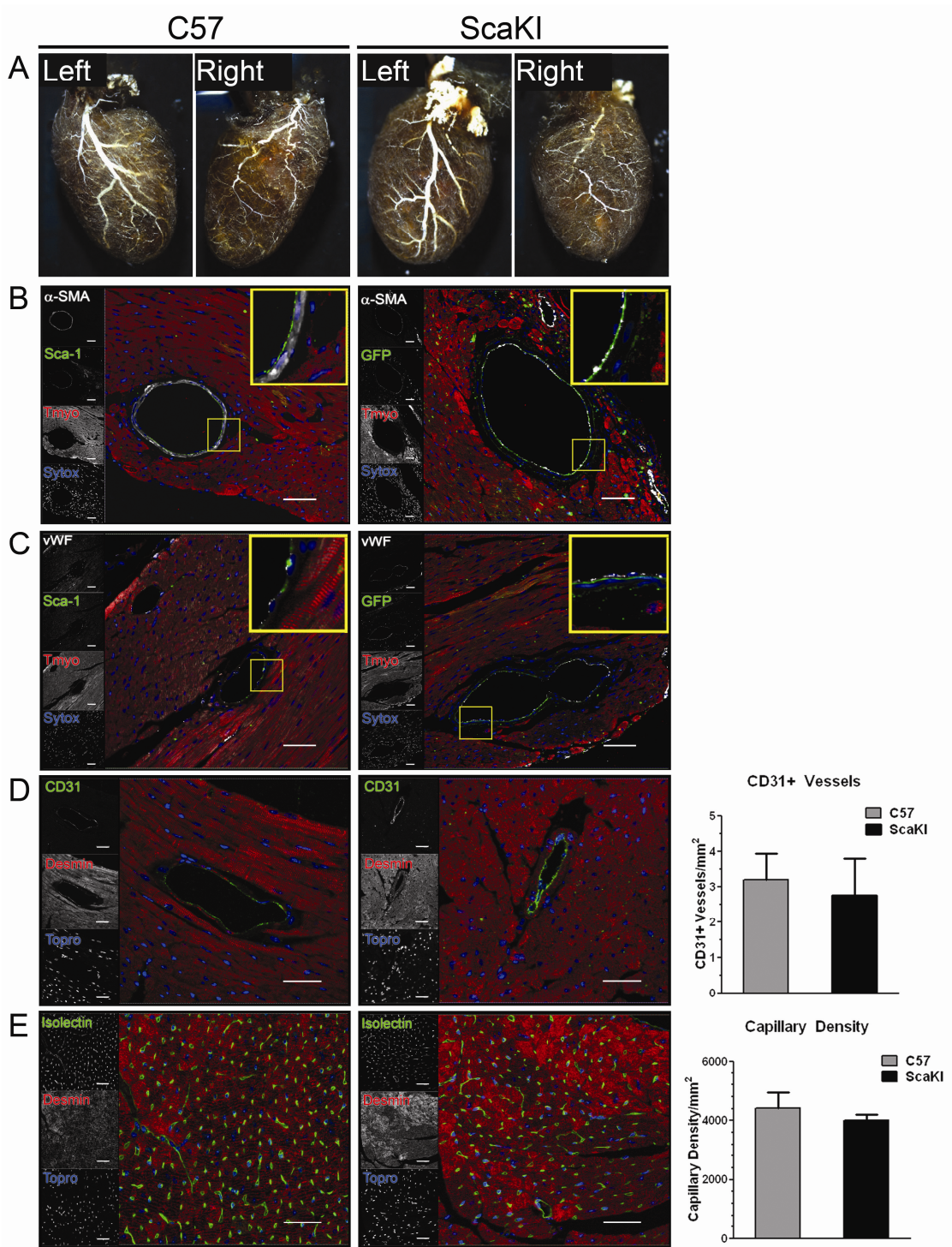


Figure 2. 2: ScaKI mice have depressed cardiac function in younger and older animals, hypertrophy in older animals and do not survive cardiac insult.

Echocardiographic measurements of C57 and ScaKI mice at (A) 12-16 weeks (w) old and (B) 12-14 months (mo) old. End Systolic Diameter (ESD), End Diastolic Diameter (EDD), Anterior Wall Dimension (AWD), Posterior Wall Dimension (PWD). Hemodynamic measurements of change in pressure over change in time (dP/dT) in C57 and ScaKI mice at (C) 12-16w old and (D) 12-14m old. (E) Calculated measurements of cardiac function, fractional shortening (FS) and ejection fraction (EF) in younger and older C57 and ScaKI mice. (F) Heart weight to body weight ratios of 12-16w and 12-14mo old C57 and ScaKI mice. (G) Cardiomyocyte cross-sectional area in the left ventricle of 12-14mo old C57 and ScaKI animals. (H) Kaplan-Meyer MI survival analysis of N=6 C57 and ScaKI mice after myocardial infarction. N is at least 3 for all other measurements. Error bars represent SEM. * $p < 0.05$, # $p < 0.02$, NS - not significant compared to age matched C57 controls.

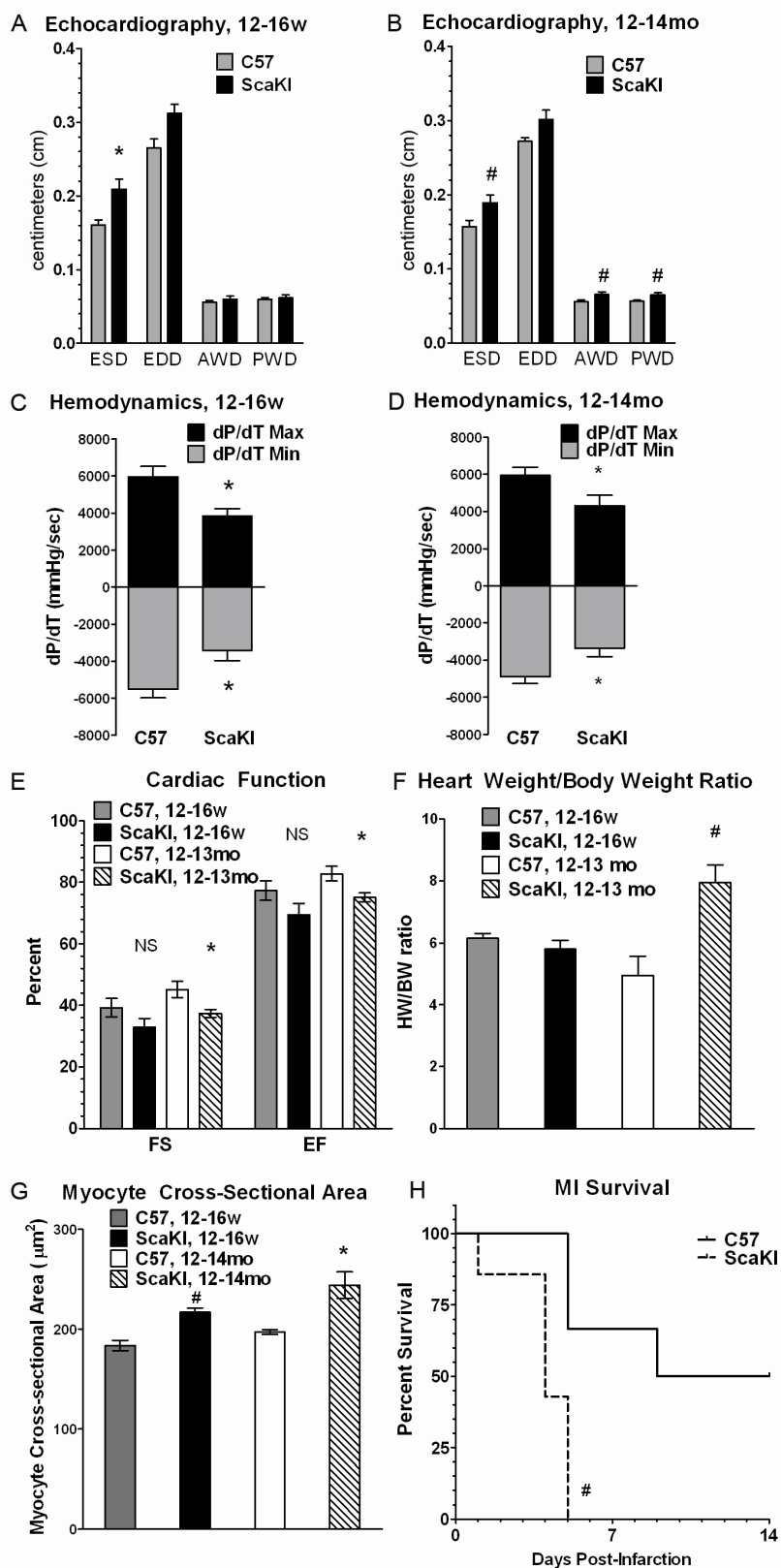
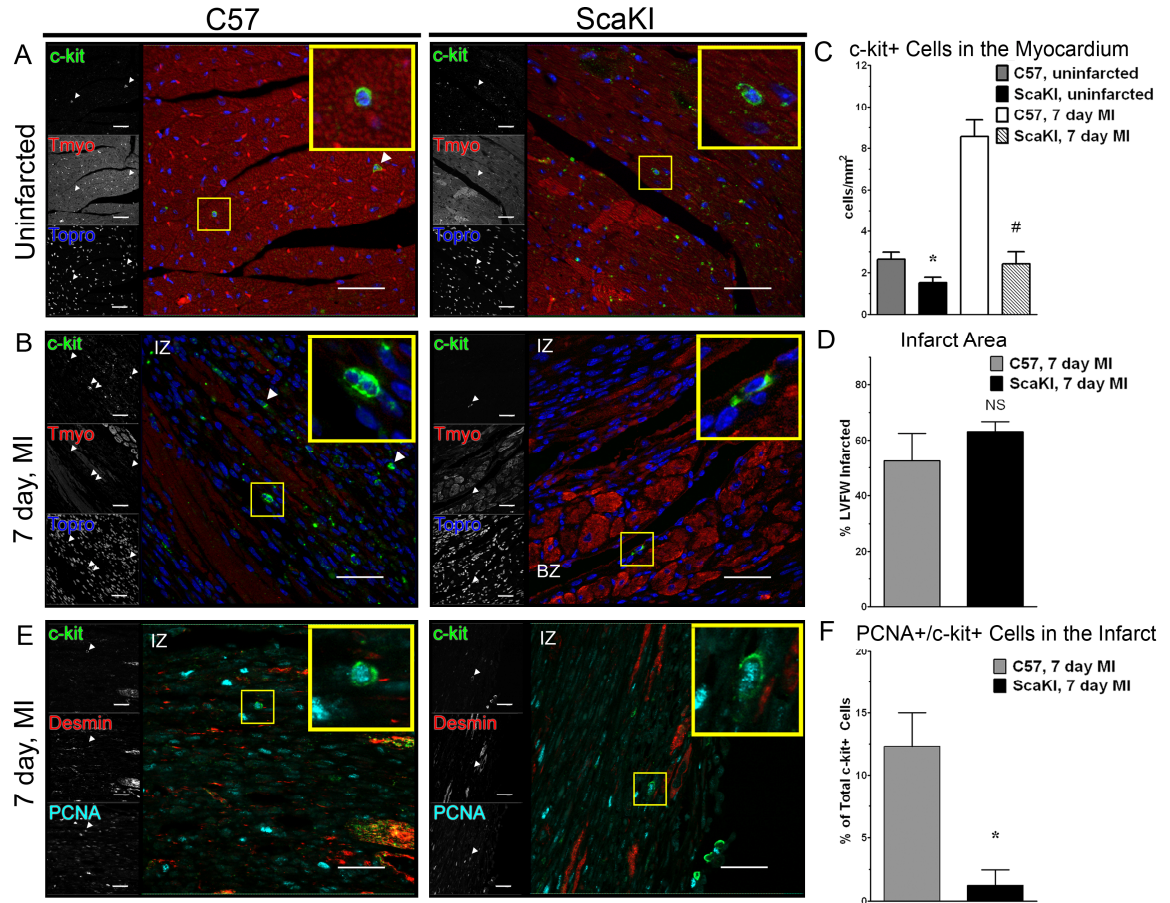


Figure 2. 3: ScaKI mice have fewer c-kit positive cells in normal myocardium and in the infarct and border zone of a 7 day myocardial infarction.

(A) Representative confocal images of c-kit⁺ cells in the normal myocardium of C57 (left) and ScaKI (right) mice. (B) Representative scans in the infarct zone of a 7 day MI in a C57 (left) heart and a ScaKI heart (right); c-kit (green), Tropomyosin ("Tmyo", red) and Topro (blue). (C) Quantitation of c-kit positive cells/mm² in normal and infarcted myocardium of C57 and ScaKI mice. (D) Infarct area as measured by percent of scarred left ventricular free wall (LVFW) at 7 days. (E) Representative confocal images of proliferating c-kit cells at 7 days post MI in C57 (left) and ScaKI mice (right); c-kit (green), Desmin (red) and proliferating cell nuclear antigen (PCNA, teal). Scale bar is 40μm. (F) Quantitation of proliferating c-kit⁺ cells as percentage of PCNA⁺/c-kit⁺ cells over total c-kit⁺ cells. Error bars represent SEM. Infarct Zone (IZ), Border Zone (BZ). * p<0.05, # p<0.02, NS - not significant.



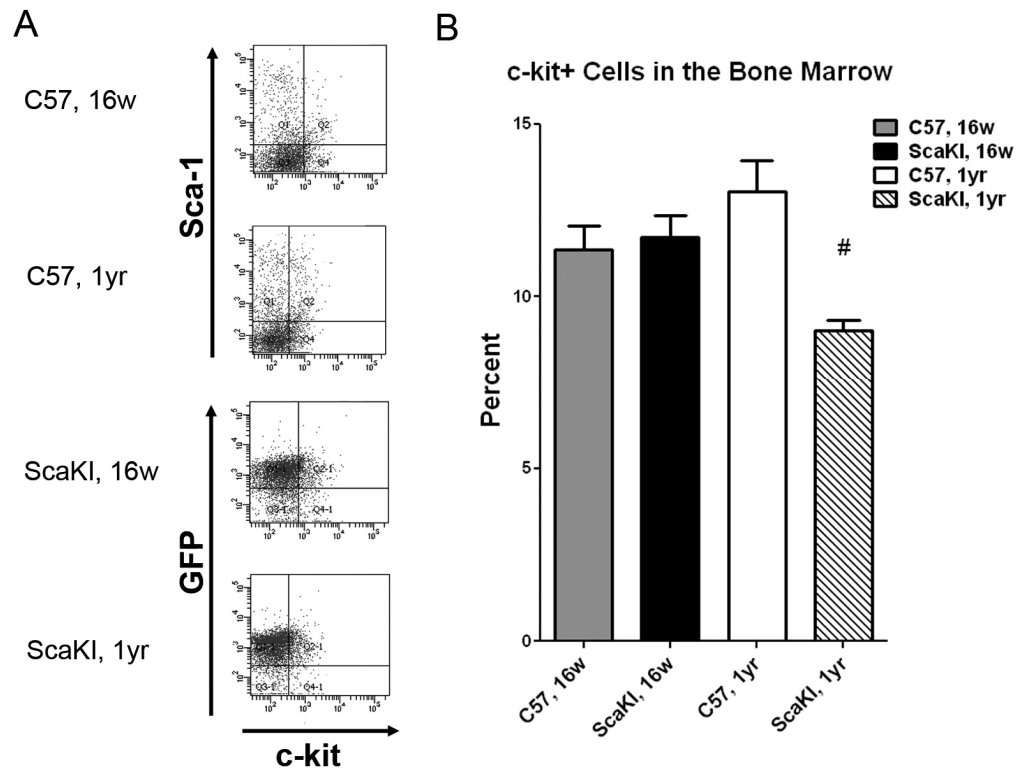


Figure 2. 4: 12-14 month old ScaKI mice have fewer stem cells as labeled by c-kit expression in the bone marrow.

(A) Representative scatter plots of bone marrow isolated from animals at the indicated ages and stained for either c-kit and Sca-1 in C57 animals or c-kit alone combined with Sca-1 driven GFP fluorescence in ScaKI animals. (B) Quantitation of the percent c-kit⁺ cells in the bone marrow of N=3 animals at the indicated ages. Error bars represent SEM. # p<0.02.

Figure 2. 5: Developmental expression pattern of Sca-1 promoter-driven GFP in the heart.

(A) Confocal images of the GFP expression pattern during development at the indicated ages; c-kit (white), GFP (green), Tropomyosin ("Tmyo", red), and Topro (blue). Arrows indicated GFP+/c-kit+ or GFP-/c-kit+ cells. (B) Representative confocal images of c-kit+ (left) and c-kit+/GFP+ (right) cells in the adult ScaKI myocardium. Scale bar is 40 μ m. (C) Quantitation of cells expressing c-kit only, GFP only, or co-expressing c-kit and GFP by confocal microscopy in 2 day, 1 week, 2 week, and 12 week old ScaKI mice; c-kit only (red), GFP only (green), and dual c-kit+/GFP+ (yellow). Error Bars represent SEM. * $p < 0.05$, # $p < 0.02$, NS - not significant; all vs. the same measurement at 2 days.

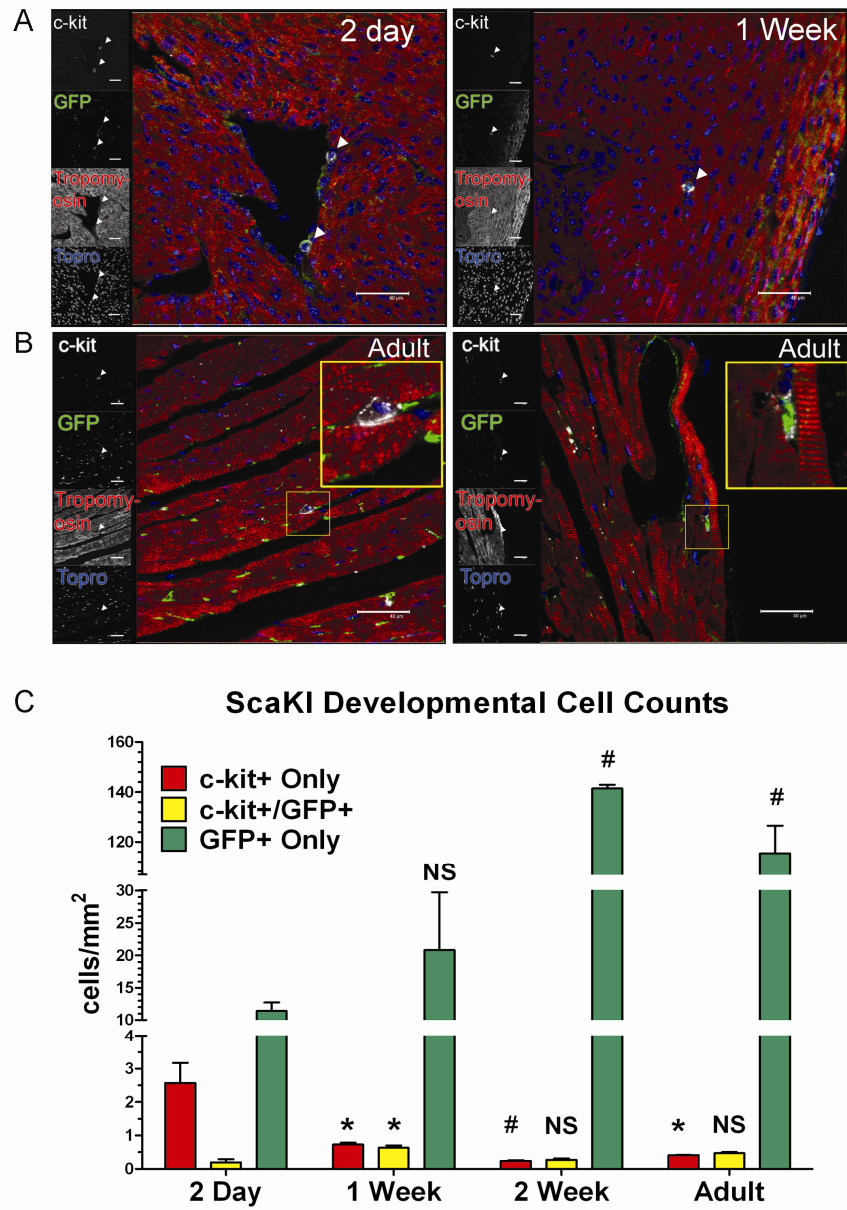


Figure 2. 6: Adult ScaKI CPCs express stem cell markers and demonstrate decreased proliferation and survival *in vitro*.

(A) Flow cytometric analysis of CPCs derived from C57 (left) and ScaKI (right) hearts and analyzed for GFP expression (top) or stained for Sca-1 expression (bottom). (B) Immunocytochemistry images of CPCs isolated from C57 (left) and ScaKI (right) hearts; c-kit (red), Ki67 (green) and Topro (blue), Scale bar is 150 μ m. (C) Representative growth curve of n=3 C57 and ScaKI CPCs using Trypan Blue Exclusion assay. (D) C57 and ScaKI CPCs treated with H₂O₂, stained with AnnexinV/7-AAD and analyzed with flow cytometry, n=4. Error bars represent SEM, * p<0.05.

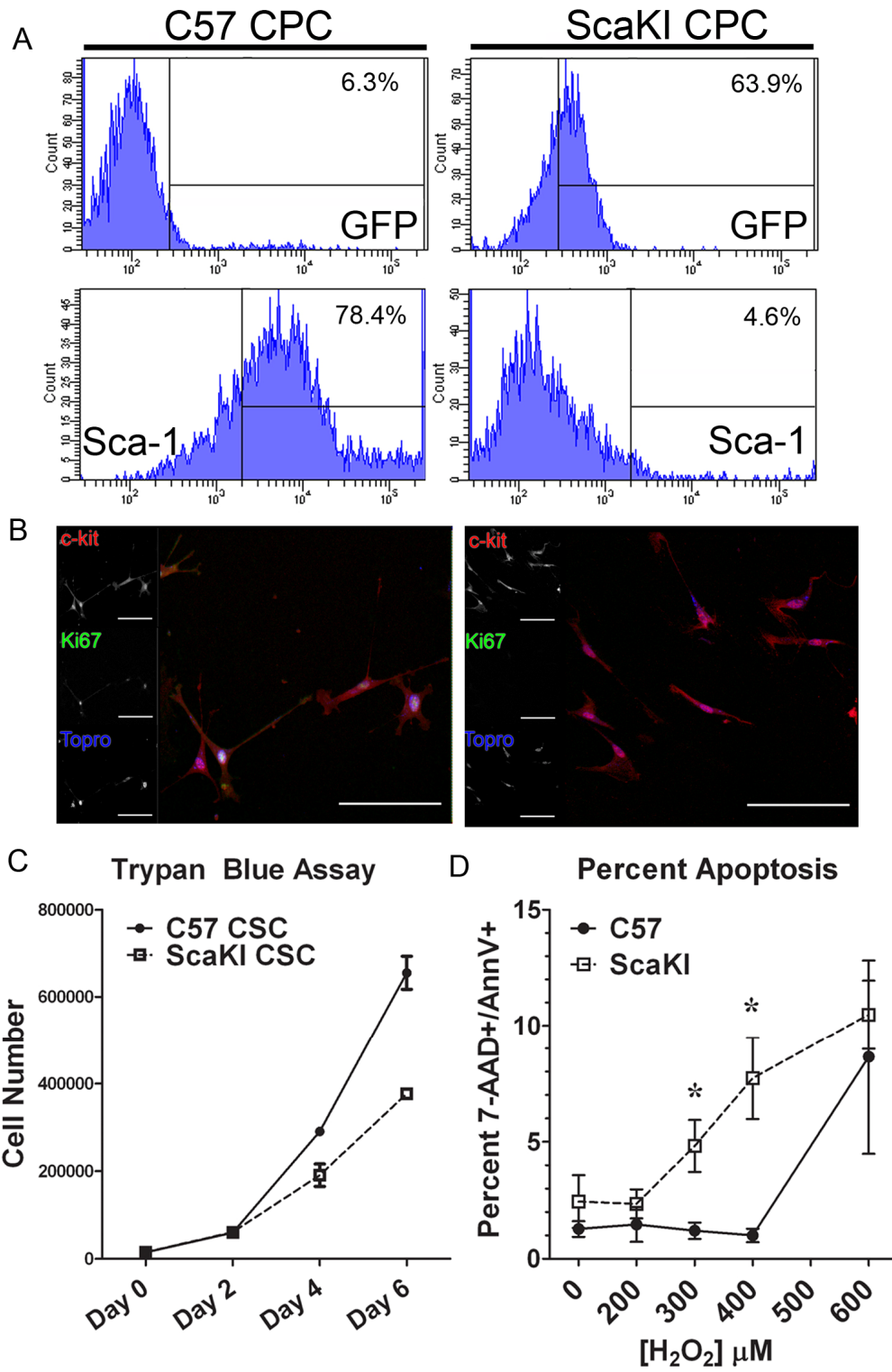


Figure 2. 7: Decreased TCF/LEF1 controlled gene expression in ScaKI CPCs.

(A) Unbiased qPCR gene expression array analysis for activated signaling pathways. Data from samples with greater than two-fold change is expressed as Fold change of ScaKI CPC gene expression compared to C57. (B) qPCR gene expression analysis of significantly depressed (left) and increased (right) genes involved in TCF/LEF signaling and survival signaling from screen depicted in A. (C) Flow cytometric analysis of Sca-1⁺ CPCs after siRNA of Sca-1 or Scrambled RNA at the indicated timepoints. (D) Gene expression analysis of siSca-1 vs. siScramble CPCs at the indicated timepoints after siRNA transfection. (E) Cyquant proliferation assay of siSca-1 vs siScramble CPCs at the indicated timepoints. (F) Representative growth curve of C57 and ScaKI CPCs using Trypan Blue Exclusion assay. * $p < 0.05$, # $p < 0.02$. B through F, n is at least 3 independent experiments.

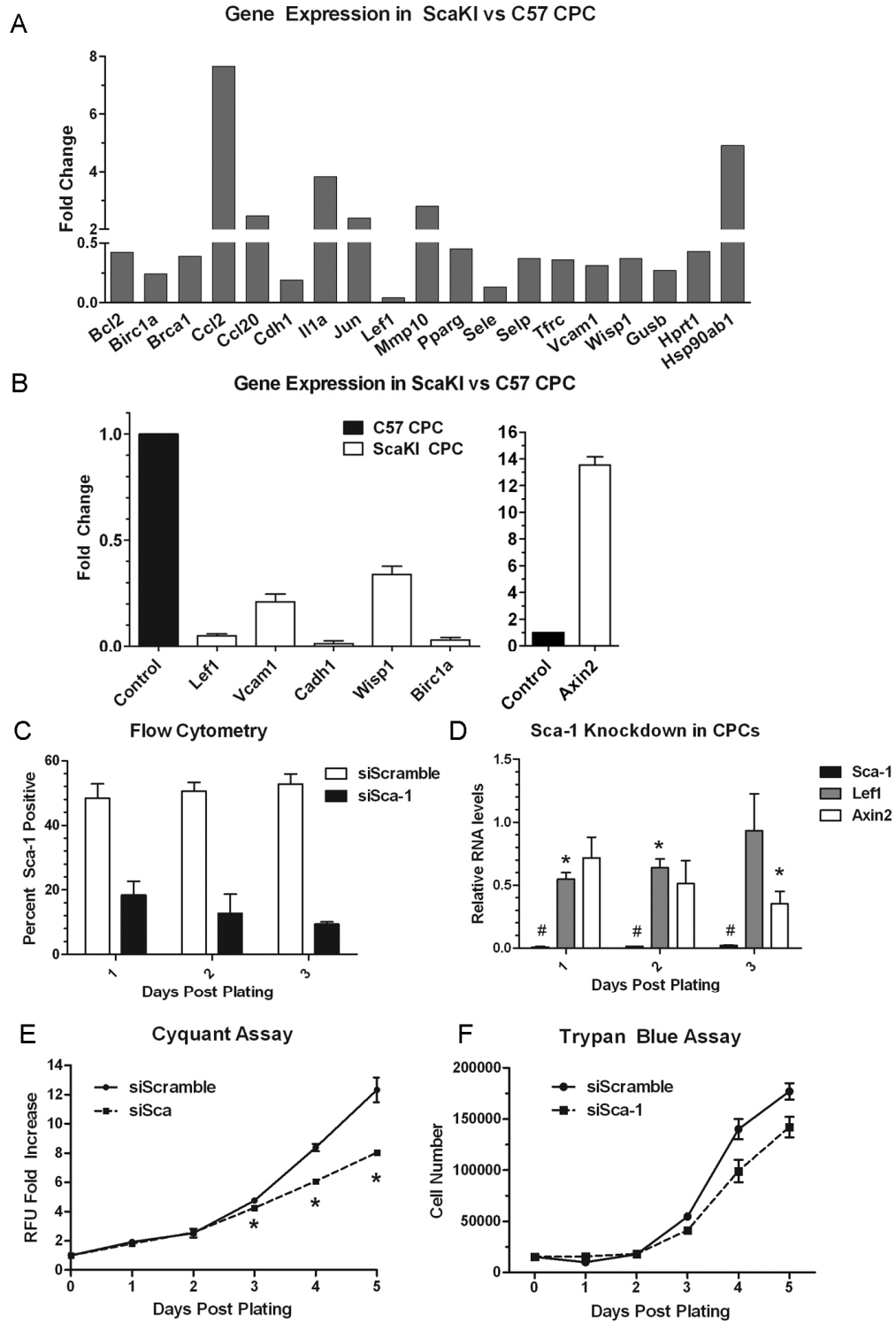


Table 2. 1: Chapter II qPCR Primer Sequences

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
Axin2	TGGTGCATACCTCTTCCGGAC TTT	TCAGGTTTCATCTGCCTGAACCC AT
Birc1a	AAACCGGATGTGCTCTCAGTC CTT	AGCACAGCCATGAGAGAGTCAC AA
Cdh1	TGACTCGAAATGATGTGGCTC CCA	ACTGCCCTCGTAATCGAACACC AA
GAPDH	TTGTCTCCTGCGACTTCAACA	CATACCAGGAAATCAGCTTGAC AAA
Lef1	AGTGACCTAATGCACGTGAA GCCT	TAGCGTGCACTCAGCTACGACA TT
Sca-1	TCAGGAGGCAGCAGTTATTGT GGA	TACATTGCAGAGGTCTTCCTGG CA
Vcam1	TACCTGCCATCGGGATGATC GTTT	AATGCATGGCTTGTTTGTGGA GG
Wisp1	GCGCTTTACTGACATGGCTTG CTT	TGTTACATGACACTGGGCTTCG GA

Table 2. 2: Chapter II Primary Antibody and Dilutions

Primary Antibody	Species	Dilution	Amplification (paraffin only)	Marker	Manufacturer
c-kit	Goat	1:50	Yes	Stem cells	R&D Systems
GFP	Rabbit	Paraffin: 1:300 Cells: 1:50	Yes	Tag	Molecular Probes
Ki67	Rat	1:40	No	Proliferation	Dako
Tropomyosin	mouse	1:100	No	Sarcomeric	Sigma
vonWillebrand's Factor	Rabbit	1:100	No	Endothelial cells	Sigma
α -Smooth Muscle Actin	Rabbit	1:100	No	Smooth muscle	Anaspec
Sca-1	Rat	1:25	No	Stem Cell	Ceder Lanes
PCNA	Rabbit	1:40	No	Proliferation	Santa Cruz
Sca-1 (PE)	Rat	1:700 (FACS)		Stem Cells	BDBiosciences

CHAPTER III:

The Impact of Aging and Disease Upon the Proliferation of Cardiac Progenitor Cells

INTRODUCTION

The paradigm accounting for the mechanism of aging has undergone a profound change following discovery of tissue-specific stem cells which replenish the tissues in the blood, and more recently discovered in brain and heart. The most novel postulate is that aging is a direct consequence of declining function of this resident tissue-specific stem cell population. Organ function, including that of the heart, undergoes an age-associated decline in function. A mechanistic understanding of stem cell function at different age points and during disease will help provide valuable clues as to how the aging process can be slowed and myocardial function preserved.

Current research indicates that autologous transplantation of stem cells is a promising new therapeutic approach for treatment of cardiomyopathic damage resulting from Myocardial Infarction (MI). Previous studies in mice or rats using stem or progenitor cells derived from either cardiac tissue² or bone marrow^{6, 40} have shown that injected stem cells engraft and contribute to tissue regeneration and functional recovery of the heart in the wake of myocardial infarction. An ongoing unresolved issue in the field is the quality and regenerative capacity of the stem cell pool from young versus old individuals or a healthy versus cardiomyopathically damaged heart resulting from chronic stress. Cardiac Progenitor Cells (CPCs) in the elderly possess increased p16 expression indicating senescence²¹, which raises questions regarding the overall therapeutic relevance of CPCs from aged individuals. However, there is a pool of active

stem cells which can be increased by growth factor stimulation²¹. CPCs are active and responsive in mitigating the effects of aging and heart failure, but the regenerative and reparative capacity of these cells naturally declines with advancing age. All previous publications involving post-MI injections of CPCs have utilized CPCs derived from young healthy hearts for therapy. No study has defined the differences between younger and older CPCs in either tissue culture or post-infarct injection models.

It is also important to investigate CPCs in the context of clinical cardiac disease. The Tropomodulin Overexpressing Transgenic mouse (TOT) is a well characterized model of early-onset extreme dilated cardiomyopathy^{65, 66}. The TOT mouse was derived from the FVB line to overexpress tropomodulin under the control of the α MHC promoter. TOT hearts are enlarged with thin walls, poor pumping ability and a high rate of cardiomyocyte apoptosis⁶⁷. It is vital to understand the changes undergone by CPCs in the context of aging and disease in order to understand their role in the organism and to further their development for clinical therapies.

METHODS

Animal Models

All animal protocols and procedures described herein conform to Institutional Animal Care and Use Committee approved protocols regarding appropriate care, facilities, anesthesia and analgesia. FVB/N mice were aged under normal conditions and standard DIET. Tropomodulin overexpressing transgenic mice (TOT) were derived from FVB/N mice and over express tropomodulin under the control of the alpha myosin heavy chain promoter. TOT mice develop an early onset extreme dilated cardiomyopathy with increased myocyte apoptosis as previously described^{66, 67}.

Echocardiography

Echocardiography was performed under minimal (1.25%) isoflurane anesthesia with 2.5% supplemented oxygen using an Acuson Sequoia C256 instrument. The N is at least 4 for all age groups.

CPC isolation and culture

CPCs were isolated and cultured as previously described². CPCs are cultured according to standard tissue culture protocols in CPC media (DMEM/F12, 10% Embryonic Stem Cell-Grade FBS, PSG, Insulin-Transferrin-Selenium, 1000u/mL LIF, 40ng/mL EGF, 20ng/mL bFGF).

Immunofluorescent Microscopy

The hearts were fixed by retroperfusion of 10% neutral buffered formalin and paraffin embedded as previously described²⁰. Paraffin sections of mouse hearts cut at 4 μ m were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed in 10mmol/L citrate pH 6.0 using 1100-W microwave oven for 3 minutes at high power and 12 minutes at 50% power. The slides were allowed to cool to room temperature, washed in 3 times in TN buffer (NaCl 150 mmol/L, Tris 100mmol/L, pH 7.5) and quenched with 3% hydrogen peroxide in TN buffer for 20 minutes to block endogenous peroxidase activity. Slides were then washed in TN buffer and blocked for 1 hour in TNB (1X TN buffer containing 0.5% Blocking Buffer, proprietary formula from Perkin ElmerTM kit). Primary antibodies were diluted in TNB and incubated overnight at 4°C. Slides were then washed 3 times in TN and incubated 2 hours at room temperature in the dark with species-specific secondary antibodies conjugated to a fluorophore in TNB. Nuclei were stained for 20 minutes with Topro. Coverslips were mounted with Vectashield and imaged by confocal microscopy. Confocal images were acquired using a Leica TCS SP2 confocal microscope.

Bromodeoxyuridine Treatment

Bromodeoxyuridine (BRDU) was solvated at 5mg/ml in PBS, sterile filtered for injection and sterile aliquots stored at -20°C. 50mg/kg BRDU was injected intraperitoneally twice a day for 7 days before sacrifice in 3 month and 13 month old animals.

RESULTS

Heart function declines with advanced age in mice as measured by echocardiography

Organ function tends to decline with age in mammals. It has been assumed that this is also true for the heart, however the timing and extent of heart function decline has never been measured in FVB laboratory mice to our knowledge. End systolic diameter (ESD), end diastolic diameter (EDD), anterior wall dimension (AWD) and posterior wall dimension (PWD) were increased but not significantly at middle age (13mo) and again by advanced age (24mo) in the FVB mouse (Figure 3.1A). However, the decrease in calculated values of fractional shortening (FS) and ejection fraction (EF) were significant ($p < 0.05$) only at the advanced age of 2 years (24 mo) in FVB mice (Figure 3.1B). TOT mice had extreme dilated cardiomyopathy as measured by echocardiography (Figure 3.1C) and the calculated values of fractional shortening and ejection fraction (Figure 3.1D). See Table 3.1 for measurement data.

Increased numbers of c-kit⁺ cells in the cardiomyopathic heart

There were significantly more c-kit⁺ CPCs per mm² in myopathic TOT hearts at 13 weeks of age (1.33 ± 0.01) compared to 13 week old FVB (0.47 ± 0.15) control hearts. The numbers of c-kit⁺ CPCs in one year old FVB mice were variable between the minimal range of the 13 week old normal mice and the maximal range of the 13 week old TOT mice. Three animals per group in 13 week FVB and 13 month old FVB as well as 13 week TOT were treated with

50mg/kg BRDU for 1 week. c-kit+/BRDU+ analysis for proliferating c-kit+ cells was performed for all three groups (Figure 3.2D). The percent of proliferating c-kit+ cells was variable from animal to animal but trended higher in older and cardiomyopathic hearts (Figure 3.2B). The number of BRDU+/Tropomyosin+ myocytes in the heart was also analyzed (Figure 3.2E) and found to be significantly higher in TOT hearts (1.35 ± 0.12 per mm^2) compared to FVB hearts at 13 weeks (0.43 ± 0.15 per mm^2) and 13 months (0.24 ± 0.10 per mm^2) (Figure 3.2C).

Decreased proliferation rate in CPCs derived from middle aged myocardium

CPCs derived from both young adult (3 month) and middle-aged hearts were phenotypically similar and both express stem cell markers c-kit and nucleostemin in culture (Figure 3.3A and 3.3B). Although young adult and middle aged CPCs appear similar, middle-aged CPCs did not proliferate as rapidly as young adult CPCs as measured by trypan blue exclusion assay (Figure 3.3C).

DISCUSSION

Aging can be conceptualized as a disease of: metabolic dysfunction and oxidative stress⁶⁸, a decrease in telomere length⁶⁹, and/or a reduction in tissue stem cell efficacy⁷⁰ caused by both of the latter. However, if we can remove tissue stem cells from the milieu of the body, it provides the opportunity to expand and engineer those cells for therapeutic purposes. It has recently been shown that CPCs derived from young adult myocardium will regenerate more myocardial tissue and recover more heart function when engineered to express the cardioprotective gene, Pim-1 kinase⁷¹.

Heart disease is a disease of aging. However, a majority of the current data in the literature describes the CPCs of the young adult animal. The data herein assess the function of the CPCs relative to the age of the mouse and the function of the myocardium. Heart function, as measured by ejection fraction and fractional shortening, starts to decline at 13 months of age and becomes a significant decline at 2 years of age (Figure 3.1 and Table 3.1). Conversely, CPC proliferation is on average higher, if not statistically significantly so, at 13 months (Figure 3.2). These results are consistent with observations that CPCs respond to myocardial injury (Figure 2.3) or in a disease state such as the TOT model (Figure 3.2), which has a significantly higher number of CPCs than a normal adult myocardium.

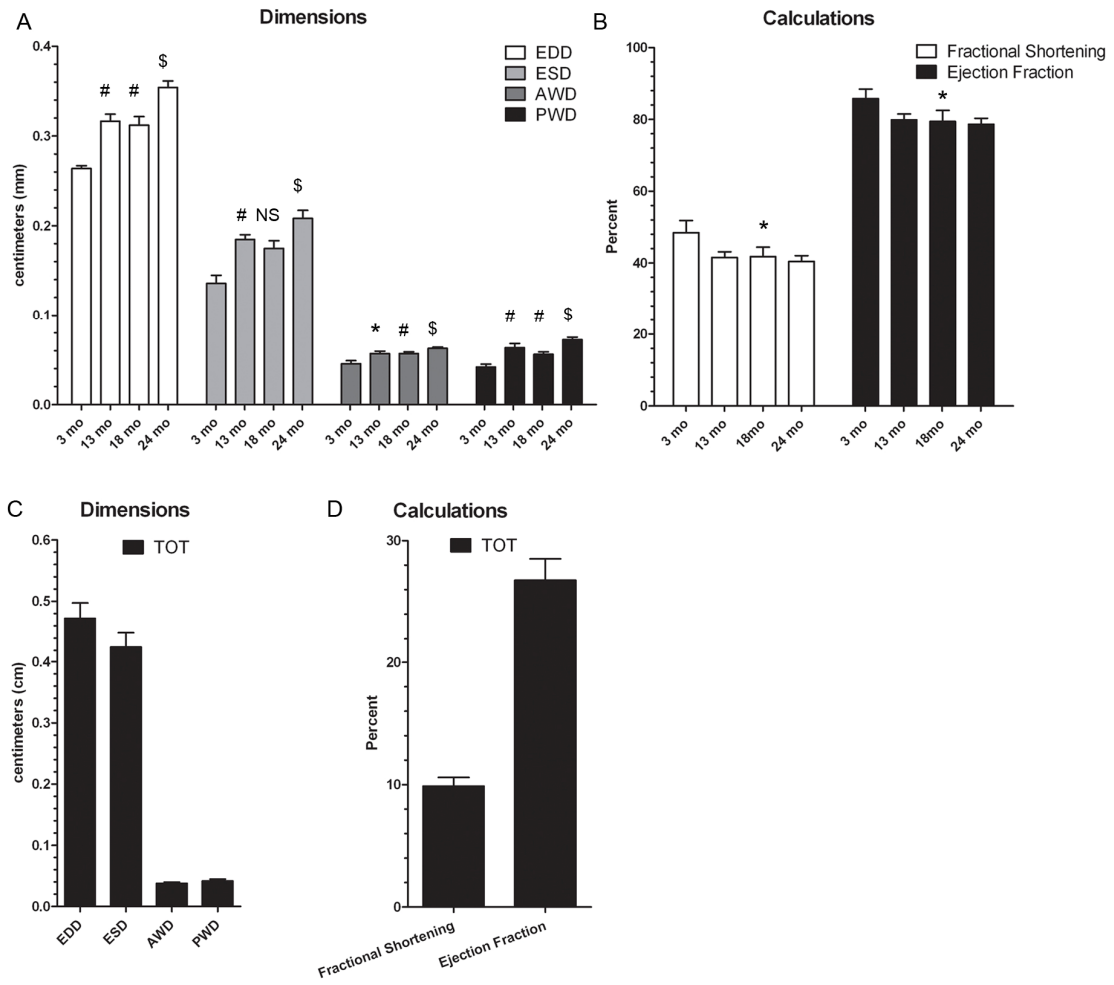
SUMMARY POINTS

- Heart function as measured by fractional shortening and ejection fraction declines with age in the normal FVB mouse and is highly impaired in the TOT mouse.
- CPCs respond to aging and genetically induced cardiomyopathy in a fashion similar to ischemic damage, by increasing cell number and proliferation.
- CPCs from aged or pathological myocardium have impaired growth kinetics in vitro and might benefit from pro-survival, pro-proliferative genetic engineering.

FIGURES

Figure 3. 1: Heart function declines with advanced age by echocardiography.

The heart function of FVB mice of both genders were measured by echocardiography at the indicated ages. A) Average of End Diastolic Diameter (EDD), End Systolic Diameter (ESD), Anterior Wall Dimension (AWD) and Posterior Wall Dimension (PWD) at the indicated ages. B) Calculated measures of fractional shortening and ejection fraction from echocardiography data in A. The heart function of TOT mice of both genders were measured by echocardiography at 16-20 weeks of age. C) Average of End Diastolic Diameter (EDD), End Systolic Diameter (ESD), Anterior Wall Thickness (AWT) and Posterior Wall Thickness (PWT). D) Calculated measures of fractional shortening and ejection fraction from echocardiography data in C. Error bars indicate SEM. * $p < 0.05$, # $p < 0.02$, \$ $p < 0.001$.



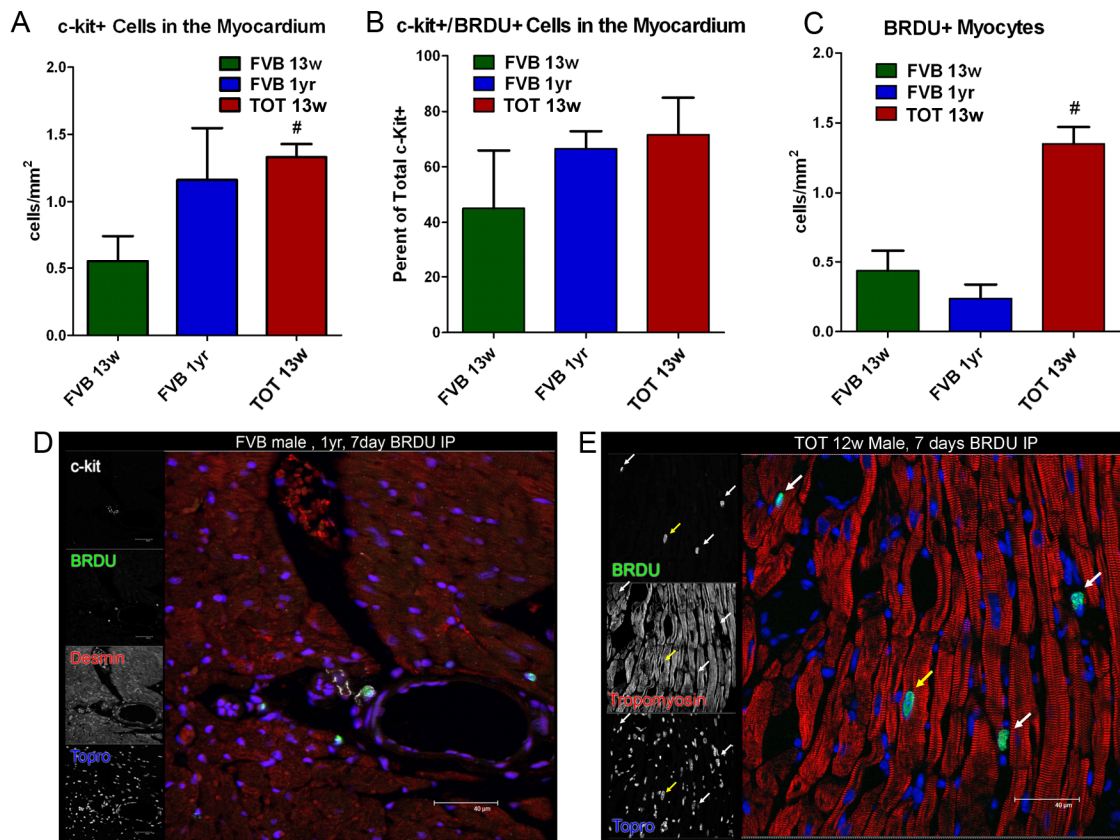


Figure 3. 2: Increased numbers of c-kit⁺ cells in the cardiomyopathic heart.

A) c-kit⁺ cells per mm² in the young (13w) and middle aged FVB hearts as well as young (13w) TOT hearts. B) Percent of c-kit⁺ cells which are also BRDU⁺ after 7 days of IP BRDU injection in young (13w) and middle aged FVB hearts as well as young (13w) TOT hearts. C) BRDU⁺/Tropomyosin⁺ cells per mm² in the young (13w) and middle aged FVB hearts as well as young (13w) TOT hearts. D) Immunohistochemistry of BRDU⁺/c-kit⁺ cells in the myocardium of a 1 year old FVB mouse. E) Immunohistochemistry of BRDU⁺/Tropomyosin⁺ cells in the myocardium of an adult TOT mouse. Error bars indicate SEM. *p<0.05, #p<0.02.

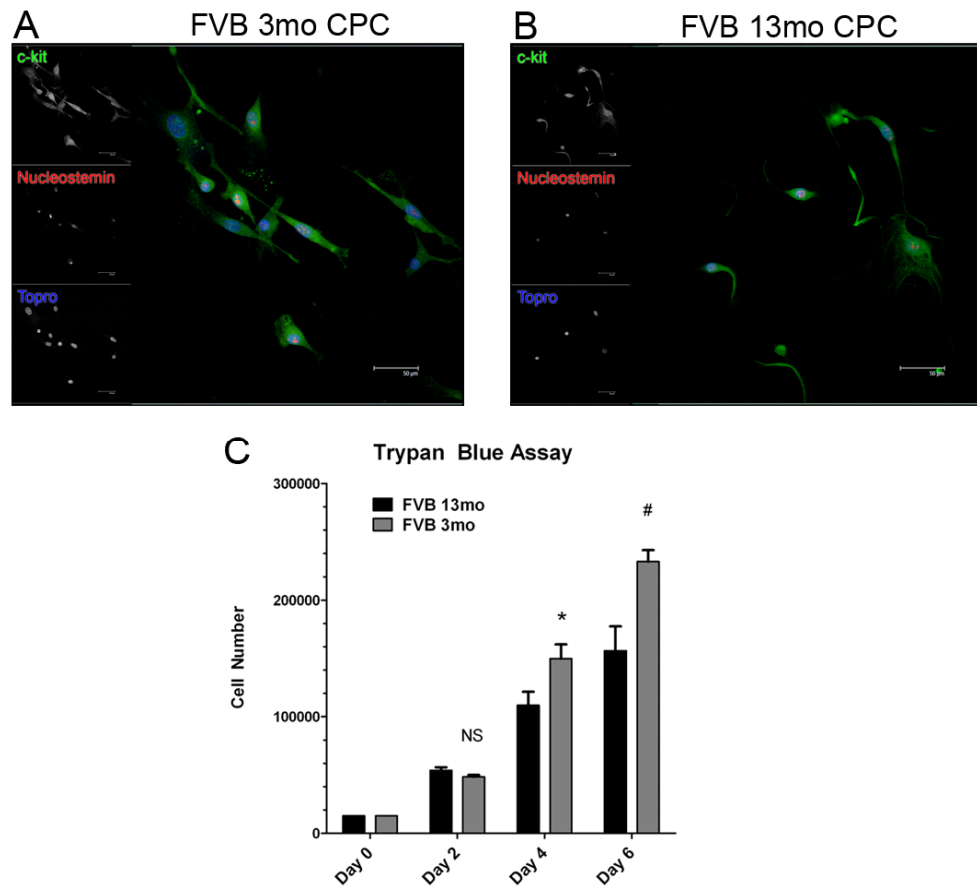


Figure 3. 3: Decreased proliferation rate in CPCs derived from middle-aged myocardium.

Representative confocal images of CPCs derived from A) 3 month (3mo) old FVB mice or B) 13 month old (13mo) mice, c-kit (green), nucleostemin (red), and Topro (blue). C) Average of 3 independent trypan blue proliferation assays of 3mo v 13mo CPCs. Error bars indicate SEM. NS – not significant, * $p < 0.05$, # $p < 0.02$.

Table 3. 1: Echocardiography Measurement Data

Age	EDD (cm)	ESD (cm)	AWT (cm)	PWT (cm)	FS (%)	EF (%)
FVB 3mo	0.264±0.006	0.136±0.018	0.046±0.007	0.042±0.007	48.5±6.8	85.8±5.3
FVB 13mo	0.317±0.017	0.185±0.012	0.057±0.006	0.063±0.011	41.6±3.5	79.9±3.7
FVB 18mo	0.322±0.038	0.189±0.046	0.056±0.005	0.056±0.008	41.8±7.5	79.4±8.7
FVB 24mo	0.354±0.019	0.208±0.023	0.063±0.004	0.072±0.007	40.5±3.8	78.7±4.0
TOT 4mo	0.549±0.067	0.425±0.062	0.038±0.005	0.042±0.008	9.9±1.9	26.8±4.6

Expressed as mean ± standard deviation. Months (mo)

CONCLUSION OF THE DISSERTATION

Heart disease has become a primary public health and medical concern in the United States and around the globe. Mortality from heart disease usually results from heart failure as a consequence of myocardial infarction or chronic pressure overload. Much of the research in modern cellular and molecular cardiology has been expended to understand the pumping mechanism of the heart and what pharmacological agents improve, regulate, hasten or slow the contractions of the cardiomyocytes. This research has led to fruitful drug development to mitigate heart disease but has not led to a patient ever being treated to the point where they no longer have the disease.

The next phase of cardiac research in the age of the gene has been to dissect protein or gene specific contributions to hypertrophy, cell survival and apoptosis in the heart. A major biological tool to dissect these complicated pathways has been the transgenic mouse which was made to overexpress a gene of interest specifically in cardiomyocytes. Gene products successful in mitigating the effects of ischemia and reperfusion injury would then be candidates for new gene or protein based therapies. Several genes were highly successful in preserving myocardial function, decreasing infarct size and/or increasing survival after myocardial infarctions including: myristolated-Akt⁷², nuclear-targeted Akt³⁵, Pim-1^{36, 37, 73}, IGF-1^{74, 75}, iNOS⁷⁶, SirT1⁷⁷ and Catalase⁷⁸. The promoter construct controlling these genes, α -myosin heavy chain, was

intended to express the genes in only mature cardiomyocytes. However, I have shown (Figure 1.3) that an unintended but potentially beneficial side effect was the expression of these genes in the cardiac progenitor cells. Most of the effect of these genes to decrease infarct size was attributed to preservation of the cardiomyocytes. However, some of the impact could have been due to CPC associated regeneration of the myocardium. This effect was confirmed in the IGF-1^{74, 75} and nuclear-targeted Akt transgenic mice³⁵. Moreover, α -myosin heavy chain driven overexpression of IGF-1 and Catalase was found to increase the average and maximum lifespan of the transgenic animals.

Gene or protein based therapies for heart failure or infarction continue to be subjects of intense research. However, stem cell therapy, which has the potential to generate new cardiac tissue to support heart function, would be the next logical step in advancing therapy for heart disease. The best kind of stem cells (embryonic, bone marrow derived, or cardiac tissue derived) to use to treat heart disease is a matter of vociferous and sometimes antagonistic scientific debate. However, the most recently discovered class of stem cells with the potential to treat heart disease is the cardiac tissue derived or cardiac-resident progenitor cells. Autologous transplantation of cardiac progenitor cells is one of the most promising candidates for stem cell therapy for the heart. Pre-clinical large animal studies of CPCs for the treatment of myocardial infarction are underway with positive results⁷⁹.

Cardiac progenitor cells are the most recently (2003)² discovered of the tissue stem cell class and still the most controversial. Many important scientific

questions remain to be addressed about the signaling pathways and contribution to cardiac function of cardiac resident progenitor cells. Recent data indicated that at least half of the cells in the heart are turned over in the course of the human lifetime^{80, 81}. In the mouse, impairment of the resident CPCs by deletion of the stem cell marker, Sca-1 (Figure 2.3 and 2.6) resulted in only a minor baseline impairment of contractile function (Figure 2.2). However, impairment of CPCs in mice caused a major repair defect after myocardial infarction resulting in increased mortality (Figure 2.2) and characterized by decreased progenitor cell presence in the infarct zone (Figure 2.3). CPCs lacking Sca-1 have decreased proliferative capabilities and increased sensitivity to toxic stimuli (Figure 2.6) which may result from disrupted Lef1 transcription (Figure 2.7). If a disruption in CPC function results in increased mortality from myocardial infarction, then it follows that increased CPC function could mitigate the effects of cardiac injury.

The possible decline in effectiveness of CPCs derived from older adult tissue or cardiomyopathic tissue must also be addressed. Human CPC functional impairment with age, if any, will vary from individual to individual. However, the general functionality of CPCs in relation to the age of the animal and the function of the heart can be estimated using the mouse model at various ages from young adult to the average lifespan of 2 years (Chapter 3). *In vivo*, CPCs responded to aging or cardiomyopathy in a fashion similar to “damage”. There are increased numbers of c-kit⁺ CPCs in the myocardium of TOT mice and slightly more depending on the animal in 1 year old mice (Figure 3.2). *In vitro*, however, CPCs derived from older myocardium in a rich media environment

proliferate more slowly and may be more contact inhibited than control cells from younger myocardium (Figure 3.3). This leads to an interpretation that although more is demanded of CPCs in the older myocardium, they may have less maximal proliferative capability.

Researchers are now attempting to synergistically combine gene therapy with stem cell therapy by modifying the CPCs to overexpress protective genes before injection. The genes identified which enhanced CPC function *in vivo* (Akt, Pim-1, IGF-1, iNOS, SirT1 and Catalase) are all excellent candidates for CPC modification. There is a great deal of evidence, both presented here and in the literature, that CPCs are important for cardiac repair. In our Sca-1 knock-in model, there was a baseline defect that is, at least in part, attributable to CPC impairment. Finally, CPCs increased proliferation in response to aging and cardiomyopathy. Taken together, these data indicate that CPCs and proper CPC function contribute to the optimal performance and repair of the mammalian heart.

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