UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Genetic Engineering of Cardiac Progenitor Cells for the Treatment of Cardiovascular Disease and Heart Failure

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

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2010

The Dissertation of Kimberlee Marie Fischer is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2010

DEDICATION

This dissertation is dedicated to my family. I thank my mom and dad, Karl and Pamela Fischer, for taking the time to be great parents and helping me become the person I am today. To my little brother Kurt, I love you, and you may now refer to me as "Doctor". And finally I want to thank my husband and best friend, Sebastien Burel. Your love and support has helped to keep me calm, clean, and fed over these last four years. You've helped me to see the best in everyone and I for that I will always be grateful. Thank you for always believing in me.

EPIGRAPH

"Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

Mark Twain

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LIST OF ABBREVIATONS

AIV	Akt inhibitor V
CAD	Coronary Artery Disease
CPC	Cardiac progenitor cell
CPCe	EGFP+ Cardiac Progenitor Cell
CPCeA	Nuclear Akt+ and EGFP+ Cardiac Progenitor Cell
CPCeP	Human Pim-1+ and EGFP+ Cardiac Progenitor
CSC	Cardiac stem cell
CVD	Cardiovascular Disease
Dex	Dexamethazone
dP/dT	Change in Pressure over Change in Time
GFP-NLS	Nuclear targeted GFP
GFP-Pim1	Nuclear targeted Pim-1
ICF	Immunocytofluorescence
IHC	Immunohistochemistry
Lv+egfp	Egfp Lentivirus
Lv+egfp+Pim1	Pim-1 Lentivirus
Lv+egfp+Akt-nuc	Nuclear Targeted Akt Lentivirus
LVEDP	Left Ventricular End Diastolic Pressure

LVDP	Left Ventricular Developed Pressure
MI	Myocardial Infarction
Mito-GFP	Mitochondrial targeted GFP
Mito-Pim1	Mitochondrial targeted Pim-1
MSC	Mesenchymal Stem Cell
NRVCM	Neonatal Rat Ventricular Cardiomyocytes
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
STS	Staurosporine

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ACKNOWLEDGEMENTS

It is with deep gratitude that I would like to thank Dr. Mark Sussman for his support over these last 4 exciting years. Truly, it was an honor to be mentored by such a brilliant scientist who took the time from his incredibly busy schedule to mentor me into the scientist that I am today. He taught me to be persistent, go after what you want, and eventually you *will* get it.

I would like to acknowledge the Rees-Stealy Research Foundation and the ARCS Foundation, in particular the Legler Benbough Foundation, for their continued support of my research over the years. Also thank you to the Inamori Foundation for their support of my academic achievements.

I would like to thank my laboratory and all my committee members Dr. Feuer, Dr. Glembotski, Dr. Spitzer, and Dr. Traver for all their help and support. It is with their help and encouragement that made this thesis possible.

Finally, I would like to thank my family for believing in me and supporting me. It is their encouragement that helped me to begin and finish the quest for the PhD.

The introduction, in part, has been submitted for the publication of the material as it may appear in Heart Failure Clinics, 2010, Fischer, KM; Sussman, MA. The dissertation author was the primary investigator and author of this paper.

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Chapter 2, in full is a reproduction of the material as it appears in Circulation, 2009. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. Fischer KM; Cottage CT; Wu W; Din S; Gude NA; Avitabile D; Quijada P; Collins BL; Fransioli J; Sussman MA. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Genetic Engineering of Cardiac Progenitor Cells for the Treatment of Cardiovascular Disease and Heart Failure

by

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Doctor of Philosophy in Biology

University of California, San Diego 2010

San Diego State University 2010

Professor Mark A. Sussman, Chair

Cardiovascular disease (CVD) afflicts an estimated one in three people in the United States, accounting for more deaths annually than all other causes of death combined. The most prevalent form of CVD is coronary artery disease (CAD). CAD often leads to myocardial infarction (MI) with subsequent

death of the underlying cardiac tissue. While current pharmaceutical treatments help to alleviate the increased demands placed on the damaged heart, they do nothing to regenerate and repair damaged tissue.

Recently, the use of stem cells to regenerate cardiac tissue is being explored. Unfortunately, only modest improvements in myocardial function after stem cell transplantation have been observed, raising concerns over the retention and viability of transplanted stem cells once in the damaged heart. To this end, the field of cardiac regeneration is evolving to include genetic manipulation of stem cells using cardioprotective genes to increase survival and proliferation *in vivo*.

Herein, we evaluate the ability of cardiac progenitor cells (CPC), genetically modified with the known cardioprotective genes, nuclear-targeted Akt or Pim-1, to improve cardiac structure and function after infarction in mice. The results presented in this thesis demonstrate CPCs modified with either nuclear Akt or Pim-1 significantly enhance proliferation *in vitro* and *in vivo*. However, overexpression of nuclear Akt in CPCs abrogates lineage commitment. Lack of terminal differentiation resulted in a lack of significant functional and structural improvements in the hearts of mice receiving injections after MI. In contrast, CPCs modified with Pim-1 kinase exhibited lineage commitment *in vitro* and *in vivo*. Mice receiving Pim-1 modified CPCs after infarction, therefore, had significant improvements in cardiac function and regeneration, compared to mice receiving unmodified

CPCs. Preliminary studies indicate cellular localization of Pim-1 kinase may contribute to its ability to regulate stem cell proliferation and survival.

Taken together, this study demonstrates that CPC commitment is an essential component of the regenerative response. For cardiac stem cell therapies to be effective, cellular survival and proliferation must be promoted without inhibiting lineage commitment.

INTRODUCTION OF THE DISSERTATION

Cardiovascular Disease and Heart Failure

Cardiovascular disease (CVD) is the number one killer among men and women in the United States. Annually it takes more lives than all other diseases combined, including cancer, and is now becoming a worldwide epidemic. The number of people afflicted with the disease as well as the cost to society is staggering. The American Heart Association (AHA) estimates that cardiovascular disease affects one in three people and costs the public over $400 billion dollars annually. The leading causes of heart disease stem almost entirely from obesity and associated side effects. Inactivity and lack of physical activity lead to obesity, high cholesterol, high blood pressure, and diabetes, all factors associated with increased risk of developing cardiovascular disease. In the United States it is estimated that 34% of adults age 20 and over are obese. Even more alarming is that 31% of children 2-19 years old are obese (AHA statistics update 2010). Clearly, in a society where rates of childhood obesity continue to rise, CVD is sure to be a disease lasting well into future generations.

Over the last 50 years there have been significant gains in the way we diagnose and treat CVD, including a barrage of surgical and pharmaceutical treatments that significantly improve survival after a major cardiac event. However, although we have come a long way in preventing the onset of heart failure, it remains for those that are diagnosed that more than half will die within 5 years from heart failure.

Myocardial Infarction and Associated Effects

The most common form of cardiovascular disease is referred to as coronary artery disease (CAD). Death of the myocardial tissue generally occurs through a narrowing of the arteries (arteriosclerosis) by which the main artery of the heart, the left anterior descending coronary artery, becomes blocked by plaque, and thus results in a myocardial infarction (MI). The underlying tissue and myocytes below the blockage are starved of oxygen and nutrients, become ischemic, and often death ensues. Once damaged, both the structure and function of the myocytes, as well as the entire organ are compromised. In order for the heart to maintain function, the surviving myocardium enters a period of compensatory hypertrophy. During this phase of hypertrophy, existing myocytes expand in order to compensate for the damaged and lost tissue in an effort to help the heart to pump. Pharmaceutical treatments are advantageous and are often prescribed during this phase. Pharmaceutical treatments work in a variety of ways by lowering arteriolar resistance, increasing venous capacity, and decreasing blood volume, all, which help the damaged heart to maintain function. However within just a few years, most patients transition into a state of pathological hypertrophy, characterized by increased apoptosis, or cell death, of the surviving myocyte population and thinning of the myocardial wall. At this point, heart function is severely compromised and can no longer keep up with the demands it must meet in order to sustain life. Heart

transplant presents the only remaining viable treatment option for these patients, many of whom die waiting for donor organs.

Cardiovascular Therapeutics and Interventions

Fortunately, over the last 10 years, a revolution in the way we currently treat heart disease has been sweeping through the field of cardiovascular medicine. Spurred on by the regenerative capacity of stem cells, scientists have been trying to regenerate damaged cardiac tissue using stem cells from a variety of both adult and embryonic tissue sources. Various innovative strategies using stem cells have been proposed including intramyocardial injection, heart grafts composed of stem cells, as well as attempts to regenerate the entire organ using stem cells, and offer hope for the discovery of a novel therapy to treat heart disease. This last experiment has recently been reported in the news as scientists at the University of Minnesota have been able to rebuild both a rat and pig heart using a process known as "whole organ decellularization". This technique involves removing the cells from the heart but leaving the extracellular matrix intact, upon which new stem cells are placed. All of these pioneering experiments are leading scientists and clinicians into a new frontier of cardiovascular medicine.

In the last 5 years several human clinical trials have investigated the regenerative potential of a variety of stem cells from various tissues, resulting in somewhat varied outcomes (1). Accounting for some of this discrepancy may be that the current studies have not been directly compared in regards to cell number, cell type, time of transplantation, or the ability of transplanted cells to

differentiate into cardiogenic lineages. This last point remains one of the chief areas of intense research focus for all stem cells (2-4). It is critical for improving heart function that transplanted stem cells be able to functionally integrate into the surviving myocardium, differentiate, and replace the various cardiac lineages lost during pathological stress. Although a plethora of cell types have been shown to mitigate cardiac damage to some extent, the cell type best suited to repair the heart remains elusive.

The most studied stem cells used for repair currently are from the bone marrow (5, 6), mesenchyme (7-14), and cardiac tissue (15-18). Until recently, bone marrow stem cells have been the cell type of choice given the ease of collection and well-characterized isolation. Of late, however, cardiac stem cells have begun to offer new hope as a novel cell population for cardiac regeneration (15-21). Although the heart has long been considered a post-mitotic organ, a subset of resident cycling c-kit+ progenitor cells (CPCs) have been recently identified and found to reside within particular "niches" of the heart (3, 22, 23). C-kit+ CPCs play a role in the normal maintenance and repair of the heart, and have been shown to mobilize to damaged cardiac tissue in response to myocardial injury (3, 21, 24-26). Currently, c-kit+ CPCs have been isolated and used with moderate success to treat and regenerate damaged heart tissue after myocardial infarction (1, 4, 20, 24) Importantly, CPCs are hypothesized to possess an increased ability to differentiate into cardiac cell types as they initially express markers of early cardiac lineages such as NKX2.5, Gata4, and MEF2C (25).

The excitement of these initial reparative studies has been tempered by the somewhat marginal regenerative capacity of adoptively transferred stem cell populations. Experimental data routinely suggest that the vast majority of transferred stem cells die or vanish shortly after delivery. Therefore, it is likely that modest benefits gained in myocardial structure and function are mediated by a small population of surviving cells. Thus it seems that despite the cell type, the ability of adoptively transferred stem cells to effectively repair the myocardium in the face of catastrophic damage falls short. Although initial experiments are optimistic, dramatic improvements necessary for long term cardiac tissue regeneration and improved function essential for clinical implementation remain as an unmet goal. Given these substantial drawbacks, it is clear that stem cell therapies must be optimized for successful execution in the myocardium.

One such hypothesis for improving stem cell therapy for the heart focuses on improving the homing, survival, and proliferation of stem cells in the ischemic and/or damaged myocardium. Ideally, to enhance the regenerative process, adoptively transferred stem cells may benefit from genetic modification to promote cellular proliferation and survival without inhibiting lineage commitment and differentiation. Experimental designs where stem cells are harvested and manipulated *ex vivo* are currently underway (27-29).

Genetic Modification of Stem Cells

Modification of stem cells is in its infancy and a variety of methods to increase the *in vivo* efficacy of these cells in the heart are currently being investigated. Consensus on the most effective and safe way to manipulate stem cells is, however, a subject of intense debate. Strategies ranging from treatment of stem cells with cytokines or paracrine factors to permanent genetic modification of the cells are being explored; each with its own set of pros and cons.

Injection of cytokines into the infarcted myocardium induces the homing of the endogenous stem cells to sites of injury. In particular, endogenous cardiac stem cells have been shown to respond to injection of hepatocyte growth factor (HGF) (30-39) and mediate protective effects. While these endogenous stem cells are amenable to cytokine manipulation, there are not enough cells available to effectively repair the damaged myocardium. *Ex-vivo* treatment of a greater number of stem cells with cytokines to enhance their proliferation and survival prior to injection has, therefore, been proposed as an alternative. As continued local stimulation of these cells with cytokines once delivered to the myocardium would prove to be difficult, it remains to be seen if cytokine stimulated stem cells can perpetuate the preliminary activation of protective signaling initially induced, beyond a few days. Given these potential problems, a more long-term solution has been proposed. Genetic modification would allow permanent integration of cardioprotective genes into stem cells, inducing proliferative

and survival signaling that provides for long term cardiac functional improvement (Figure I) (27).

In order to deliver genes to cells on a more long-term basis, vectors to deliver these genes are being designed. The most recent strategies involve the use of viral vectors, as viruses are uniquely suited to deliver their genes to cells within the mammalian host. Several different viral vectors have been employed and depending on the virus, genes can be delivered transiently or permanently. To date adenoviral vectors have been used successfully to deliver cardioprotective genes to the heart, however adenoviral delivered genes do not integrate into the host genome and, therefore, are not expressed permanently and are not passed on to cell progeny. To circumvent this problem, retroviruses, and in particular lentiviruses, have been utilized to deliver genes that stably integrate into the host genome. Benefits regarding the use of lentiviral vectors include the ability to infect both dividing and non-dividing cells and permanent integration of the gene of interest into the host genome. Permanent integration allows for the gene to be stably passed on and expressed through all future generations of daughter cells. Additional benefits also include the ability to pseudo-type lentiviruses allowing a wide range of tissue tropisms to be amenable to lentiviral gene delivery.

Delivery of cardioprotective genes to treat heart failure ultimately relies on the modification of signaling pathways in stem cells that regulate proliferation and inhibit apoptosis, without inhibiting lineage commitment, thereby enhancing survival and engraftment of these cells *in vivo*.

Furthermore, rapid ex-vivo expansion of stem cells has critical clinical implications. By increasing the rate at which the stem cells proliferate, the pool of available cells to be used for transplantation can be amplified, ultimately leading to decreased expansion time necessary to obtain adequate numbers of cells to treat patients. Modification of signaling pathways, which promote cardiac regeneration, may therefore be the key to enhancing stem cell regeneration in the heart.

Some of the first studies to prompt investigation into the modification of signaling pathways for adoptive cellular therapy arose from the discovery of an endogenous population of self-renewing CSCs that were found to be lineage negative and c-kit positive (lin^-, kit^+) (23, 40). These cells have since been shown not only to provide normal maintenance and repair for the heart, but also proliferate and migrate to damaged myocardium in response to pathological injury (15-18, 24, 25). Additionally, it has been shown as CSCs age they become increasingly senescent and their capacity to mitigate damage becomes impaired. Experimental studies demonstrated that this age-associated deterioration could be attenuated through administration of IGF (41). Specifically, IGF transgenic mice displayed a delayed aging phenotype in which progenitor cells and myocytes had decreased amounts of senescence associated markers and maintained proliferation. These results implicated the involvement of the cardioprotective serine threonine kinase Akt, which is activated downstream of IGF. These results further suggest that

potentiating the ability of stem cells to proliferate in either the damaged or aged myocardium could drastically improve their abilities to repair the heart.

Survival Kinases

Within the context of the heart, Akt kinase has become one of the most studied cardioprotective molecules. Molecular interactions between Akt and downstream targets have become increasingly important as the field begins to examine the signalling responsible for the beneficial effects observed in the heart.

Akt is a nodal kinase responsible for a number of proliferative and anti-apoptotic effects within a variety of cell types. Akt/PKB is a serine/threonine protein kinase, central to the PI3K/AKT/mTOR pathway. Akt activation has been shown to play roles in the regulation of cell proliferation, cell survival, and inhibition of apoptosis. Downstream targets include members of the p53 pathway such as MDM2, as well as cell cycle targets such as mTOR and GSK-3, to name a few. In the heart, Akt plays a major role in both physiological and pathological hypertrophy. During stress, Akt is activated through receptor tyrosine kinases and PI3K, by which Akt can exert potent anti-apoptotic and survival signals through inhibition of Bad, caspase 9, GSK3, and activation of mTOR (42-44). Although prolonged activation of Akt in transgenic mice leads to cardiac hypertrophy and onset of heart failure, more recent studies have shown that the protective roles exerted by Akt in the heart are mediated through its nuclear localization (43, 45-50). Transgenic mice with cardiac-specific overexpression of nuclear-targeted Akt have hypercellular hearts,

enhanced myocyte contractility, and increased calcium reuptake (43). Additionally, nuclear Akt promotes the cycling of cardiomyocytes and expansion of the progenitor cell pool in transgenic mice (43).

Given the multitude of protective affects afforded by Akt in the heart, several studies have addressed the potential of potentiating this signaling cascade in the myocardium to improve structure and function after pathological injury (51-60). To date, paracrine factors induced by Akt, as well as genetic modification of stem cells, have been used to attenuate myocardial damage after infarction or ischemic damage. Bone marrow mesenchymal stem cells modified to overexpress Akt minimize ventricular remodeling and restore cardiac function within 72 hours post implantation (55). Continued studies by the same group went on to identify key paracrine factors (VEGF, FGF-2, HGF, IGF-I, and TB4) induced by Akt that are believed to be responsible for mitigation of cardiac damage, arguing that improved cardiac performance in such a short time frame seems unlikely to be achieved through engraftment and transdifferentiation of injected stem cell populations (57). While there is no doubt that activation of the Akt induces cytokines that are critical for both early survival of the injected stem cell population and cardiac improvement, rare studies exist to show that these beneficial effects maintain cardiac improvement beyond a short time. To this end, several laboratories have shown longer term success in both the structure and function of the heart after delivery of stem cells genetically modified with Akt (49, 58, 61). These results demonstrate that genetically enhanced stem

cells have the potential to repair the damaged myocardium. Other laboratories have also demonstrated beneficial effects from genetically modified stem cells with alternative cardioprotective genes, many of which are downstream targets of Akt (62, 63).

Recent data demonstrate that some of the protective effects previously ascribed to Akt in the heart may be, in part, due to the action of Pim-1, a downstream target of Akt (64, 65). Although previously identified in the hematopoietic system, Pim-1 kinase was recently identified as a novel downstream target of Akt in the heart. Given that Pim-1 has been found to play a key role in multiple cellular environments, ranging from cancer to inflammation to heart disease, an understanding of the mechanisms governing Pim-1 regulated proliferation and cellular survival will have a broad impact on a variety of diseases, leading to a better understanding of the effects that manifest by manipulating Pim-1 expression.

Pim-1, a conserved serine/threonine protein kinase, originally described as the proviral integration site of the Moloney murine leukemia virus (66), is well known in the hematopoietic system. Like Akt, downstream targets phosphorylated by Pim-1 include proteins involved in cell cycle, proliferation, and survival (66-69). Through both phosphorylation and direct association, Pim-1 can stabilize pro-proliferative proteins and/or influence their subcellular localization, contributing to regulation of proliferation (69-73). Pim-1 phosphorylation of the cyclin dependent kinase inhibitors, p27 and p21, induces their nuclear export and subsequent degradation(72, 73), while Pim-1-

mediated phosphorylation stabilizes and inhibits the degradation of c-myc (69). In addition to promotion of proliferation, recent studies have delineated the protective role Pim-1 plays in cardiomyopathic injury. Transgenic mice overexpressing Pim-1 in the heart were shown to be resistant to myocardial damage and hypertrophy (64, 65). Additionally, Pim-1 transgenic mice subjected to trans-aortic constriction (TAC) banding, demonstrated increased levels of both anti-hypertrophic proteins and anti-apoptotic proteins. Furthermore, hearts from Pim-1 mice exhibited a hyperplasic phenotype and were refractory to hypertrophy. Conversely, hearts from Pim-1 dominant negative mice readily transition into hypertrophy resulting in dilated cardiomyopathy after TAC banding.

Consistent with the pro-proliferative function in the hematopoietic system, recent studies have identified a similar role for Pim-1 in c-kit-positive CPCs. CPCs from Pim-1 transgenic mice showed elevated levels of the nucleolar protein, nucleostemin, the expression of which is associated with increased proliferation in stem cells (74). Like Akt, protective effects of Pim-1 governing cellular proliferation and survival may be dependent upon localization. Pim-1 expression has been shown to be elevated in the nuclei of proliferating neonatal hearts while expression diminishes and localizes to the cytosol upon aging (65). Although the expression of Pim-1 has been linked to both survival and proliferation, the exact mechanisms of this interaction are currently under investigation.

Currently, it appears that unmodified stem and progenitor cells offer an acute ameliorative response after pathological injury; however, their ability to effectively repair and maintain the function of the heart appears to be short-lived. Preliminary data in support of genetic modification of stem cells with cardioprotective genes is beginning to gain ground as studies begin to demonstrate genetically modified stem cells provide early enhanced improvements in myocardial structure and function over unmodified stem cells (Table I). However, many unanswered questions remain in regards to the long-term efficacy of genetically modified stem cell populations.

For real-world clinical implementation, stem cell therapies must mediate long-term functional improvement, and while early benefits likely involve paracrine factors, true sustained functional and structural benefits are unlikely to be gained without the engraftment of stem cells and their integration and differentiation into the surviving myocardium. The data presented herein addresses these concerns, and investigates the long-term protective effects offered by cardioprotective kinases, nuclear Akt and Pim-1.

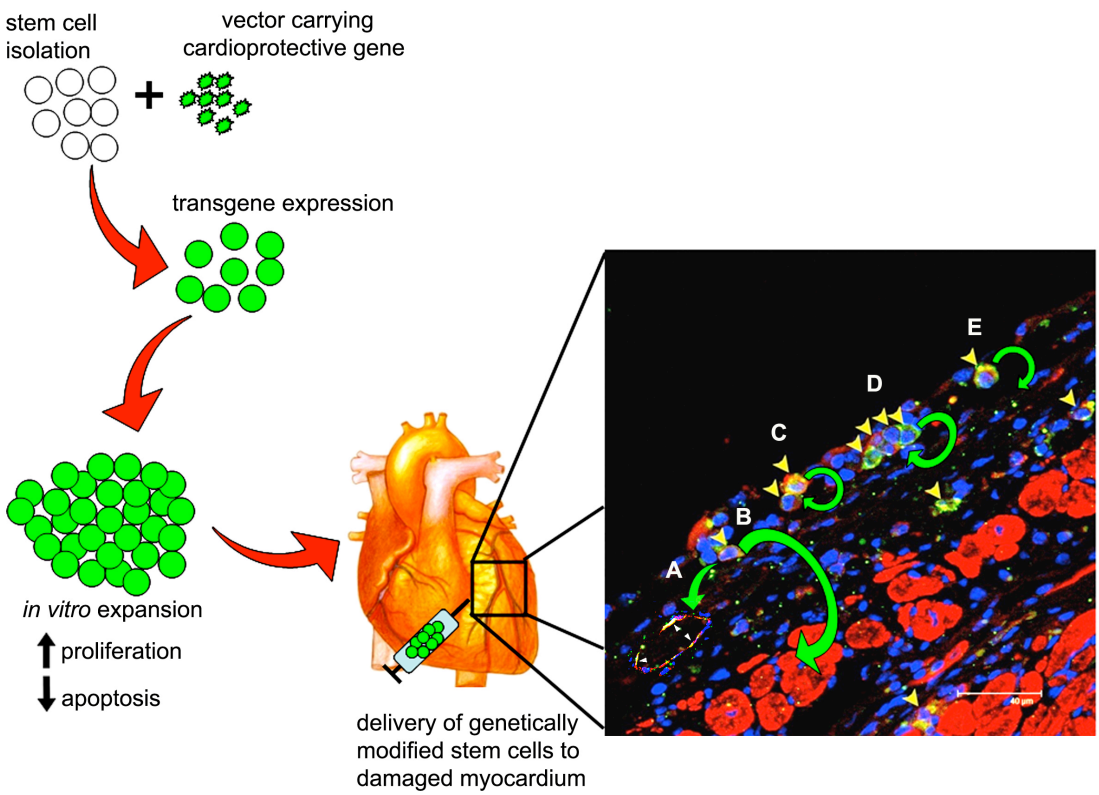


Figure I: Gene Therapeutic Approach

Cardioprotective genes using viral vectors are delivered to stem cells affording the stem cell population with and increased ability to survive and proliferate *in vitro*. After expansion, genetically modified stem cells are injected into the damaged myocardium where they elicit a multitude of protective effects: Differentiation into cardiogenic lineages such as (**A**) vasculature and (**B**) myocytes, (**C**) recruitment of endogenous stem cells to help repair the heart, (**D**) induced proliferation and inhibition of apoptosis for the injected population, and (**E**) secretion of paracrine factors to enhance the survival of the surrounding myocardium.

Table I: Experiments using gene therapy to treat heart failure

NAME	Type of cell	Vector/ Delivery	Protein	# of cells	Time assessed	Cardiac Benefits	Cardiac function vs control
Duan, et al. (2003)	MSC	Adenovirus	HGF	unknown	2 weeks	decreased collagen	improved
						decreased risk area	
						increased capillary density	
Mangi, et al. (2003)	MSC	Retrovirus	Akt	5.00E+06	3 weeks	decreased inflammation	normalized systolic
						decreased collagen	and diastolic function
						decreased hypertrophy	
						cardiac like: expressed	
						MHC, Ctnl, alpha-SA and MLC	
Wang, et al. (2004)	n/a	Tail Vein Injection	HGF	n/a	7 days	increased capillary density	improved LV systolic
						decreased apoptosis and fibrosis	and diastolic function
						decreased infarct size	
Gnecchi, et al. (2005)	MSC	conditioned media	Akt	n/a	72 hours	decreased apoptosis	not reported
						decreased infarct size	
Iwasaki, et al. (2005)	n/a	microbubbles	HGF	n/a	2 weeks	decreased dilation	improved contractile
							function
Davis, et al. (2006)	n/a	Nanofibers and	IGF	n/a	28 days	decreased caspase 3 activation	improved systolic
		cardiomyocytes				increased Troponin I	function
Jiang, et al. (2006)	MSC	Adenovirus	Akt and Ang-1	3.00E+06	14 days	myogenic differentiation	increased ejection fraction
						increased vessel density	increased fractional shortening
						enhanced cell survival	
Li, et al. (2006)	n/a	Adenovirus	iNOS	n/a	2 months	decreased infarct size	no adverse effects
Noiseuz, et al. (2006)	MSC	Retrovirus	Akt	5.00E+05	28 days	decreased infarct size	increased ejection fraction
Xie, et al. (2007)	ES cells	Lentivirus	VEGF	5.00E+05	21 days	increased cell survival	increased ejection fraction
						increased vessel density	
Yang, et al. (2007)	n/a	Adenovirus intracoronary	HGF	n/a	3 weeks	increased angiogenesis	increased ejection fraction
		injection				decreased apoptosis	
						decreased fibrosis	
						recruitment of stem cells	
Guo, et al. (2008)	MSC	adenovirus	HGF	5.00E+06	8 weeks	increased angiogenesis	increasd systolic and
						decreased apoptosis	diastolic function
Haider, et al. (2008)	MSC	adenovirus	IGF	1.50E+06	1 week	decreased infarct size	increased ejection fraction
						increased angiogenesis	increased fractional shortening
						increased stem cell mobilization	
Shujia, et al. (2008)	MSC	adenovirus	Akt and Ang-1	3.00E+06	3 months	myogenic differentiation	increased ejection fraction
						increased vessel density	increased fractional shortening
						enhanced cell survival	
Zhu, et al. (2009)	Adipose derived	lentivirus	HGF	1.00E+08	28 days	increased blood flow	increased cardiac function
	stem cells					differentiation into endothelial cells	
						decreased fibrosis	
Fischer, et al. (2009)	Cardiac stem cells	lentivirus	Pim-1	1.00E+05	8 months	increased cell survival	increased ejection fraction
						decreased fibrosis	increased fractional shortening
						increased engraftment and	improved hemodynamics
						differentiation	

CHAPTER I:

Cardiac Progenitor Cell Commitment is Inhibited by Nuclear Akt Expression

INTRODUCTION

Akt/PKB is a pivotal regulatory kinase with various effects on growth, metabolism, and survival. In the heart, Akt has become one of the most studied cardioprotective kinases with various roles in preventing cardiomyopathic injury (43, 45, 46, 49, 58, 60, 65). Activation of Akt is initiated by growth factor dependent stimulation of receptor tyrosine kinases, which in turn stimulate a cascade of signaling events beginning with the activation of PI3 kinase (PI3K) at the plasma membrane. Subsequent activation of PDK1/2 phosphorylates and activates Akt. Downstream targets of Akt are numerous and include both pro-proliferative and anti-apoptotic substrates (43, 44, 65, 74, 75).

In order to investigate the mechanisms governing the cardioprotective effects of Akt, a variety of systems including cardiac specific overexpression and viral infections have been employed. Numerous studies attribute short-term Akt activation to the profound protective effects seen in post ischemic injury models, whereby Akt drastically increases cell cycle and inhibits apoptosis in cardiomyocytes (52, 55, 56, 58-61, 76-79). Additionally, Akt activation stimulates neoangiogenesis and vasculogenesis (58, 80-90) in part accounting for the dramatic improvements seen in pathologically challenged Akt transgenic mice. However, constitutive activation of Akt can have detrimental effects upon the myocardium, including hypertrophic growth and abnormal vascular remodeling (76, 85, 91, 92). Thus, the protective roles

provided by Akt have been demonstrated to be governed by its nuclear localization (16, 43, 45, 46, 93, 94). Previous studies by our group demonstrate that cardiac-specific overexpression of nuclear Akt allows for expansion of the progenitor cell pool, as well as enhanced protection of the myocardium against pathological injury without induction of hypertrophic remodeling (43).

Recently, stem cell therapies are being explored as a novel way to treat heart failure (4, 21, 23, 24, 26). Unfortunately to date, only modest improvements in cardiac structure and function have been observed, due to poor stem cell proliferation and viability after delivery. To improve the benefits of stem cell therapy, mechanisms promoting proliferation and survival of the stem cell population without inhibiting lineage commitment have become an area of intense research focus (43, 56-61, 64, 79).

The current study evaluates the ability of cardiac progenitor cells (CPCs) modified to overexpress nuclear-targeted Akt (CPCeA) to mediate cardioprotection in infarcted hearts. Results presented herein demonstrate that CPCeA are highly proliferative, resulting in a rapid expansion of the progenitor cell pool *in vitro and in vivo*. However, CPCeA are resistant to differentiation, compared to CPC controls. Further data provided in this study demonstrate a potentially novel mechanism governing CPC differentiation downstream of nuclear Akt, while highlighting the importance of understanding effects survival signaling has on progenitor cell lineage commitment.

METHODS

Lentiviral vectors and generation of lentivirus

Bicistronic lentiviral vectors were generated as previously described (95), however briefly, murine AKT cDNA was isolated and fused to 3X nuclear localization sequence as well as a myc tag. The control construct CPCe, expresses eGFP off an internal ribosomal entry site (IRES), while the nuclear targeted AKT construct termed CPCeA, expresses nuclear targeted AKT from a **m**yeloproliferative sarcoma virus LTR-**n**egative control region **d**eleted (MND) promoter as well as eGFP off an IRES. Lentivirus was generated as previously described (95).

Cardiac Progenitor Cell Isolation, Cell culture, and Lentiviral Infection

CPCs were isolated from 10-12 week old male FVB mice and cultured in cardiac stem cell (CSC) media: DMEM F-12, 10% FBS, 1% PSG, 1X ITS (Lonza, 17-838Z), .4mg/ml EGF (Sigma, E9644), .02ng/ml bFGF (Peprotech, 100-18B), and 1000U/ml LIF (Chemicon #ESG1107). CPCs were plated in 96-well flat bottom plates and transduced with lentivirus (eGFP or nuclear AKT) at an MOI of 10 overnight. CPCs were washed 18 hours later and fresh CSC media was added. Cells were expanded and analyzed by flow cytometry to determine the percentage of eGFP+ cells. Cells were incubated with Akt inhibitor V (10μM) for 7 days with and without dexamethazone (Dex) where indicated.

Trypan blue and CyQuant Assays

Uninfected, CPCe, and CPCeP, were plated in quadruplicate (10,000 cells/well) in 24 well plates. Viable cells were determined by trypan blue exclusion. For MTT assays, 5000 cells/well were plated in 96-well plates, incubated 4-5 hours with 50mg of MTT reagent (Fluka), treated with 100ml stopping reagent (.01N HCl + 10% SDS) overnight at 37°C, and analyzed on a spectrophotometer at 570nm. For CyQuant (Invitrogen) assays, CPCe and CPCeA were plated in quadruplicate in 96-well flat bottom plates at 4000 cells per well. CyQuant reagent was added to the media at day 1 and day 3, as per manufactures protocol. CyQuant reagent was incubated for 45 minutes and read on a spectrophotometer at 530nm. AKT inhibitor V was added to the wells where indicated and used at 10uM.

Western blots

CPCe and CPCeA were plated in 6-well dishes at 50,000 cells per well. Cells were harvested the next day in sample buffer, boiled, and sonicated before running on 4-12% Bis-Tris gels. Primary antibodies were used at appropriate concentrations and incubated overnight at 4°C in 7% milk. Secondary antibodies were used at 1:4000 dilutions and incubated at room temperature for two hours. Membranes were washed in TBST and scanned on a Typhoon.

Myocardial Infarctions, CPC Injections, Echocardiography and Hemodynamics

Infarctions and injections were performed as previously described (95), briefly 10-12 week old female FVB mice were anesthetized under isoflurane, intubated, and ventilated. A thoracotomy was performed and the LAD ligated. At five minutes post ligation a blinded surgeon delivered vehicle, CPCe, or CPCeA at 5 sites surrounding the border zone with a total of 100,000 cells per heart. Infarction size was standardized by echocardiography performed on animals imaged along a parasternal short-axis view by M-mode recorded at 3 days post-infarction and injection. Lack of anterior wall motion in conjunction with at least 40% decrease in EF and FS were required for study inclusion.

Immunohistochemistry and confocal microscopy

Heart sections were deparaffinized and antigen retrieved in 1mM Citrate (pH 6.0), followed by one-hour block in TNB. Antibody stains were performed as previously described; however briefly, primary antibody was incubated overnight at 4 degrees followed by secondary antibody incubation for 2 hours at room temperature. Tyramide amplification was performed as necessary.

SuperArray and qRT-PCRs

To obtain mRNA and cDNA from CPCe and CPCeA Triazol (Invitrogen) was used as per manufacturers protocol. Cell proliferation (PAMM-020) array was obtained from SuperArray and ran per manufacturer's protocol. For qRT-PCR,

RNA was harvested as per manufacturer's protocol using the RNA isolation kit from Zymo Research. cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad, #170-8891) and qRT-PCR run using the iQ SYBR Green Supermix (Bio-Rad, #170-8882). Troponin T forward primer sequence: ACCCTCAGGCTCAGGTTCA; Troponin T reverse primer sequence: GTGTGCAGTCCCTGTTCAGA; 18S forward primer sequence: CCCCCTCGATGCTCTTAGCT; 18S reverse primer sequence: GGGCCTGCTTTGAACACTCTA.

Statistics

Statistics were calculated using Prism software. One-way ANOVA and two-way repeated measures ANOVA for echocardiography with Tukey's post-hoc test were calculated. Values with $p<.05$ were considered statistically significant. Generalized estimating equations with empirical (robust) covariance estimation were used to confirm two-way ANOVA results. Where appropriate Student's T test was utilized.

Animal studies

All animal studies were approved by IACUC.

RESULTS

Delivery and Expression of Nuclear-Akt to Cardiac Progenitor Cells (CPCs) using Lentiviral Vectors

cDNA from murine Akt was fused to a 3X nuclear localization sequence (NLS) and a myc-tag in order to target Akt to the nuclear compartment of the cell. Bicistronic lentiviral vectors were designed to express enhanced green fluorescent protein alone (Lv-egfp) or in combination with nuclear Akt kinase (Lv-egfp+Akt-nuc) in order to stably deliver cDNA constructs into c-kit+ CPCs isolated from male nontransgenic (NTG) FVB mice (Figure 1.1A). CPC populations modified with Lv-egfp (CPCe) or with Lv-egfp+Akt-nuc (CPCeA) were subjected to immunoblot analysis to confirm stable integration of the gene and overexpression of the target protein. CPCeA demonstrate overexpression of Akt and myc-tag protein, while both CPCeA and CPCe express eGFP protein (Figure 1.1B). GAPDH is shown as a loading control.

Nuclear Akt increases CPC proliferation

CPCeA have a significantly increased proliferation rate ($p<.01$) over a four day time course as determined by Trypan blue exclusion measuring total number of viable cells (Figure 1.2A). Additionally, CPCeA have an increased proliferation rate at day three ($p<.05$) as determined by CyQuant assay (Figure 1.2B, dark colored bars). The effect was abrogated by day three with addition of Akt kinase inhibitor V (Figure 1.2B, light colored bars). A cell cycle array demonstrated CPCeA regulate gene expression of several cell cycle

genes (Figure 1.3A). Additionally, CPCeA down-regulate protein expression of Cyclin D1 (Figure 1.3B), and significantly increase protein expression of Chk1 and CDC2 (Figure 1.3C).

Nuclear Akt modified CPCs maintain c-kit expression after differentiation

CPCeA were treated with and without dexamethazone (Dex) for seven days and evaluated for c-kit protein expression by immunocytochemistry. After seven days of Dex treatment, CPCeA remained c-kit positive, in contrast to unmodified CPCe cells whereby c-kit expression was lost (Figure 1.4).

Elevated levels of phospho-CREB in CPCeA

Activated Akt has been shown to induce the phosphorylation and activation of the transcription factor CREB. Elevated levels of phospho-CREB have been previously demonstrated to promote proliferation in progenitor cell populations. CPCeA were immunoblotted to assess the phosphorylation status of CREB before and after Dex treatment (Figure 1.4) to determine if a similar mechanism was involved in the CPCeA population. Undifferentiated CPCeA had a statistically significant ($p=.007$) 3.3-fold increase in the level of phospho-CREB over total CREB (Figure 1.5) compared to CPCe. Interestingly, upon differentiation, CPCeA had a 19-fold significant increase in the level of phospho-CREB over total CREB when compared to CPCe controls (Figure 1.5). CPCe had no statistical change in phospho-CREB over total CREB levels before and after differentiation (Figure 1.5).

CPCeA do not provide functional and structural benefits to infarcted myocardium

To assess whether protective benefits are gained from intramyocardial injection of CPCeA, twelve-week old female mice were injected with CPCeA or CPCe after infarction and cardiac function assessed by echocardiography and *in vivo* hemodynamics. Hearts of animals receiving CPCeA did not show a statistically significant advantage over CPCe injected hearts in anterior wall dimension (AWD, Figure 1.6A), fractional shortening (FS, Figure 1.6B), or ejection fraction (EF, Figure 1.6C), at twelve weeks. Hemodynamic assessment further confirmed deterioration of cardiac function in CPCeA injected animals as assessed by left ventricular developed pressure (LVDP, Figure 1.6D), left ventricular end diastolic pressure (LVEDP, Figure 1.6E), and dp/dt maximum and minimum (Figure 1.6F). In fact, as early as four weeks post-injection, cardiac function in CPCeA injected hearts was not statistically different ($p>.05$) from saline injected animals. CPCe injected hearts show a statistically significant ($p<.05$) advantage at early time points, but beneficial effects were not sustained past 4 weeks.

Infarct size was determined in hearts of animals receiving CPCeA and CPCe at twelve weeks post-infarction and injection. Injection of CPCeA did not result in a statistically significant reduction in infarct size compared to CPCe and saline injected controls (Figure 1.7).

Increased number of c-kit+ cells in hearts receiving CPCeA

The number of c-kit+ cells within the infarct was quantified by immunohistochemistry. CPCeA injected animals had a significant ($p<.01$) 2.3-fold increase in the number of total c-kit+ cells compared to CPCe injected controls (Figure 1.8A). Additionally, CPCeA injected hearts had a significant ($p<.005$) 2.7-fold increase in c-kit+ eGFP+ cells (Figure 1.8B) and a 1.8-fold increase in c-kit+ eGFP- cells ($p<.05$) (Figure 1.8C) compared to CPCe injected controls. There was no statistical difference ($p>.05$) in the number of c-kit+ eGFP- cells between CPCe and saline injected hearts. Although a significant number of adoptively transferred c-kit+ progenitor cells were identified in animals that received CPCeA, there was a noticeable lack of GFP+ myocytes (data not shown).

Attenuation of Akt expression increases cardiac lineage commitment

CPCe and CPCeA were treated *in vitro,* with and without Akt inhibitor V (AIV, Akt activity inhibitor) and subjected to Dex-induced differentiation. CPCeA treated with AIV and Dex had a statistically significant ($p<.002$) 2.7-fold reduction in phospho-CREB protein expression (Figure 1.9A) compared to Dex treated CPCeA without AIV treatment. CPCeA and CPCe treated with AIV and Dex were also harvested for RNA and transcript levels of Troponin T (TnT), a marker consistent with cardiac lineage commitment, were analyzed by quantitative real time PCR (qRT-PCR). Before Dex stimulated differentiation, CPCe and CPCeA do not express TnT (Figure 1.9B). Upon induction of differentiation, CPCeA express significantly ($p<.008$) reduced levels of TnT transcript compared to CPCe controls (Figure 1.9B). In order to determine if

overexpression of Akt in CPCeA or CPCe abrogates cardiac lineage commitment CPCeA and CPCe were treated with AIV prior to Dex treatment. CPCeA treated with AIV and Dex had a significant reduction in phospho-CREB protein levels (Figure 1.9A) as well as a statistically significant ($p<.008$) increase in TnT transcript (Figure 1.9C), compared to CPCeA treated with Dex alone. Although the difference was not as dramatic as in CPCeA, CPCe also had significant increases in TnT transcript after treatment with AIV and Dex, compared to CPCe treated with Dex alone (Figure 1.9C). These results are consistent with previous studies demonstrating high levels of Akt expression promote rapid proliferation of progenitor cells which must be attenuated in order to progress through lineage commitment (96).

DISCUSSION

For years treatment of the damaged myocardium has suffered from one major limitation, the inability to regenerate cardiac tissue. Pharmaceutical treatments have prolonged the life of many patients, but ultimately continue to fail as a permanent "fix" for the treatment of heart failure. Recently, with the explosion of stem cell research and tissue regeneration, a long-term solution for the repair of damaged myocardium appears to be just around the corner. Ongoing clinical trials whereby stem cells are delivered to the damaged myocardium are in effect, however results offer only modest short-term improvements in cardiac function. Consistently, studies demonstrate a minority of adoptively transferred stem cells survive in the damaged myocardium, accounting, at least in part, for the lack of substantial improvements in cardiac structure and function. These observations have led to the hypothesis that increasing the ability of adoptively transferred stem cells to survive and proliferate may considerably improve the efficacy of stem cell regeneration in the heart.

Genetic modification of the stem cell population with survival kinases has now been shown to improve the ability of progenitor cells to mitigate cardiac damage and improve regeneration (19, 56-58, 61, 63, 95). One such cardioprotective kinase is Akt. While constitutive activation of Akt leads to hypertrophic growth and abnormal vascular remodeling (76, 85, 91, 92), a plethora of studies have shown short term Akt activation as well as nuclear

localized Akt imparts protective benefits on the pathologically challenged heart including growth, inhibition of cell death, and increased angiogenesis (16, 43, 45, 46, 93, 94). However, it is important consider that successful modification of stem cells relies on the ability to increase proliferation and survival without inhibiting lineage commitment upon appropriate environmental stimulation. The vast majority of experiments involving Akt overexpression in stem cells do not assess for the amount or for the duration of this stimulation within the progenitor cell pool. While protective benefits have been gained through Akt activation, studies demonstrate sustained overexpression can inhibit lineage commitment and terminal differentiation in various progenitor cell populations. The current study evaluated the effect of long-term overexpression of nuclear Akt upon CPCs. Our results demonstrated CPCeA were highly proliferative both *in vitro* (Figure 1.2 and 1.3) and *in vivo* (Figure 1.6). However, after Dex treatment *in vitro,* CPCeA had significantly reduced levels of TnT transcript (Figure 1.9B), compared to CPCe controls, indicating a resistance to lineage commitment (Figure 1.4). Therefore, although significantly more CPCeA were retained after adoptive transfer into infarcted hearts compared to CPCe controls (Figure 1.8), overexpression of nuclear Akt antagonized cardiac lineage commitment and did not lead to improved function (Figure 1.6) or structure (Figure 1.7) of the heart after pathological challenge.

The cyclic AMP response element binding protein (CREB) is a transcription factor and known downstream target of Akt. In the heart, Akt

phosphorylates and activates CREB on serine 133 (97). It has been demonstrated phosphorylation of CREB induces proliferation and elevated levels of phospho-CREB are found in several forms of cancer (98). Consistent with these observations previous studies have shown chondrocyte progenitor cells overexpressing Akt and phospho-CREB were highly proliferative but also refractory to differentiation. Terminal differentiation of chondrocyte progenitor cells was only observed in this study after inhibition of Akt activity(96). In accordance with previous observations, our studies revealed undifferentiated CPCeA have significantly increased levels of phospho-CREB compared to control CPCe (Figure 1.5). Given that CREB is localized to the nucleus, it is likely a target of our nuclear-targeted Akt. Interestingly, upon *in vitro* differentiation with Dex, CPCeA have a 19-fold significant increase in the level of phospho-CREB compared to CPCe (Figure 1.5). In an effort to elucidate the mechanism inhibiting differentiation, CPCeA were treated with an Akt inhibitor prior to Dex treatment. Our results demonstrate inhibition of Akt activity in CPCeA reduced phospho-CREB levels 2.7-fold (Figure 1.9A) and resulted in increased expression of TnT transcript, a marker consistent with cardiogenic differentiation (Figure 1.9B, C). Taken together, our results suggest a previously unknown mechanism underlying CPC differentiation whereby levels of phosphorylated CREB may be critical to controlling CPC differentiation. Further studies using a specific CREB inhibitor are currently underway to determine whether inhibiting activation of CREB targets may elucidate the role activated CREB plays during differentiation of cardiac progenitor cells.

Concurrently, studies are being pursued in order to regulate the expression of nuclear Akt using an inducible system. Rapid expansion of the progenitor cell pool as well as increased survival within the damaged myocardium is critical for clinical implementation; however regulation of gene expression may provide a way to control and induce progenitor cell differentiation after delivery.

Genetic modification of stem cell populations with cardioprotective genes has now been demonstrated in numerous studies to decrease damage to the heart caused by pathological injury. However, successful long-term regeneration and improvement relies not only on short-term paracrine effects to enhance survival signaling but also requires long-term engraftment and differentiation of the progenitor cells into the major myocardial cell lineages. Thus, a thorough understanding of the mechanisms governing progenitor cell differentiation is of paramount importance both in and out of the clinic.

Chapter 1, in part, is a reproduction of the material as it may appear in *Stem Cells,* 2010. Fischer KM, Wu W, Din S, Gude NA, Sussman MA. The dissertation author was the primary investigator and author of this paper.

FIGURES

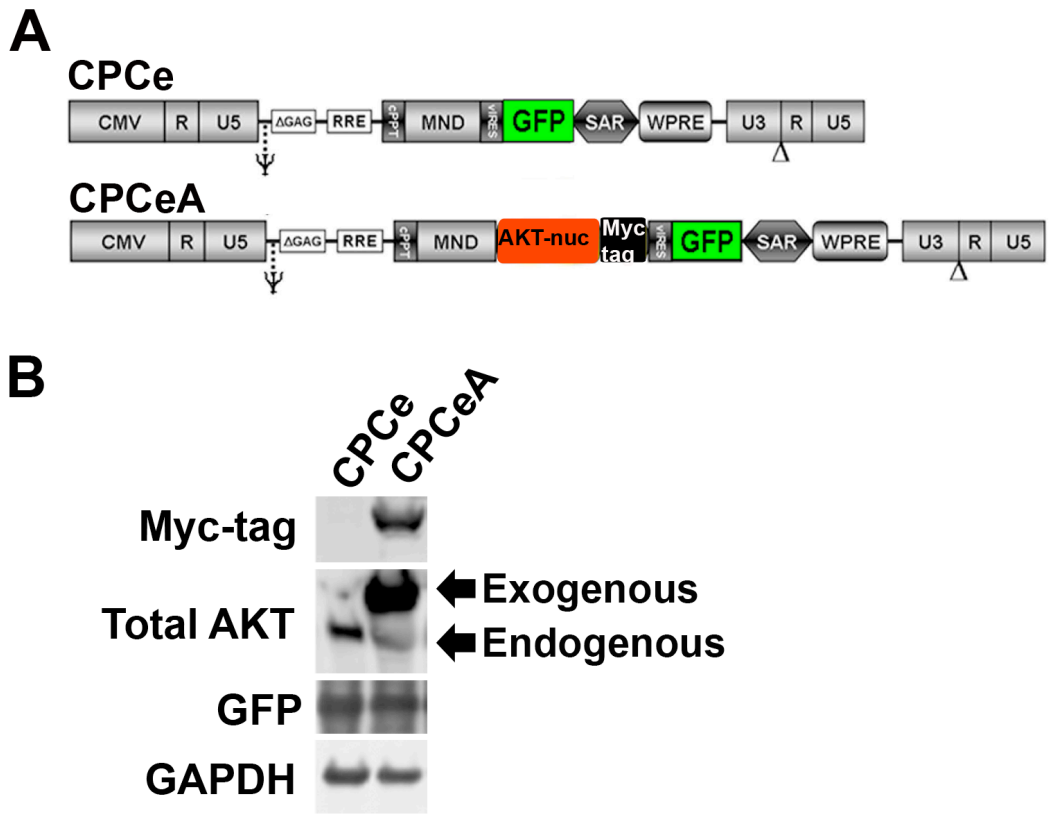


Figure 1.1: Stable Overexpression of Nuclear Akt in c-kit+ CPCs

(**A**) Self-inactivating lentiviral vectors, termed Lv-egfp (GFP control) and Lv-egfp+Akt-nuc. (**B**) Representative immunoblot of CPCe and CPCeA, immunolabeled for myc-tag, Akt1/2, GFP, and GAPDH.

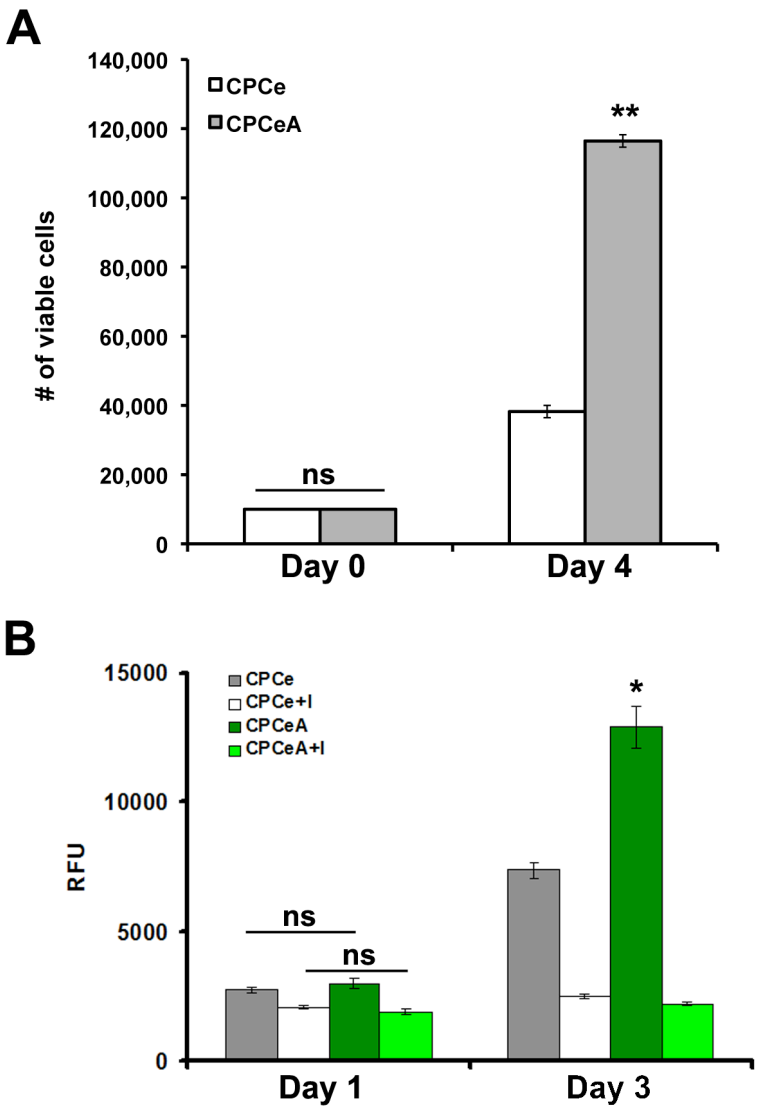
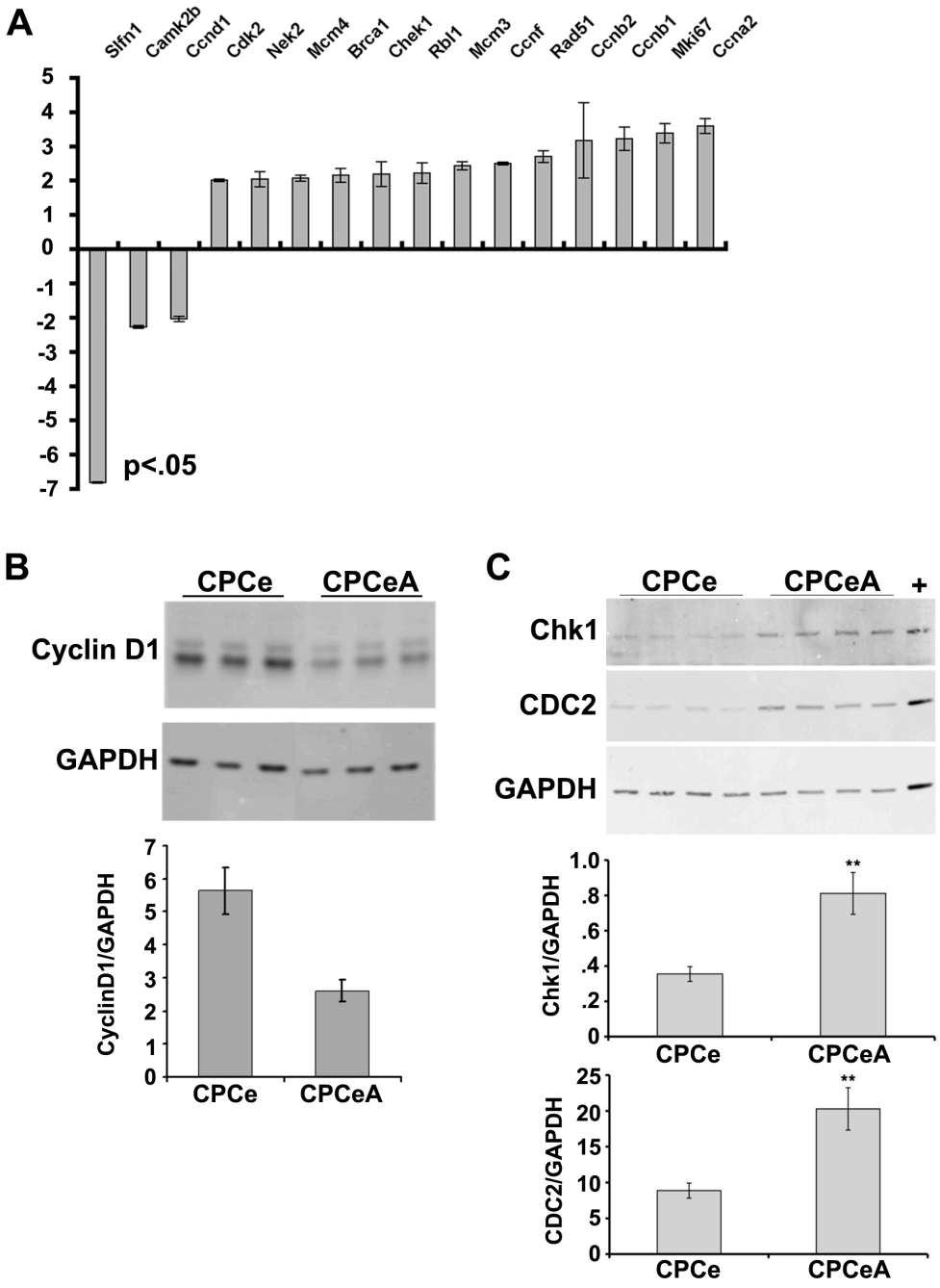


Figure 1.2: Overexpression of Nuclear AKT increases CPC proliferation rate

(**A**) Trypan blue exclusion was used to determine the number of viable cells in CPCe and CPCeA over a four day time course (mean ± SEM, n=4/group). (**B**) Proliferation rate of CPCe and CPCeA treated with and without Akt inhibitor V was determined by CyQuant assay over a three-day time course (mean ± SEM, n=4/group). *p<.05 compared to CPCe at day 3.

Figure 1.3: Nuclear Akt modified CPCs mediate gene expression of cell cycle proteins

(**A**) Total mRNA was extracted from CPCe (n=3) and CPCeA (n=3) and run on a cell cycle RT^2 Profiler Cell Proliferation Array from SuperArray in triplicate. CPCeP and CPCe were normalized to GAPDH. Samples analyzed had ≥2-fold difference from control, with $p<0.05$, (mean ± SEM, n=3). (**B-C**) Protein expression of Cyclin D1 (B), Chk1 and CDC2 (**C**) were examined in CPCe and CPCeA (mean ± SEM). *p<.05, **p<.01 compared to CPCe.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Slfn1
Camk2b
Ccnd1
Cdk2
Nek2
Mcm4
Brca1
Chek1
Rbl1
Mcm3
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Rad51
Ccnb2
Ccnb1
Mki67
Ccna2
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
p<.05
B
CPCe
CPCeA
Cyclin D1
GAPDH
CyclinD1/GAPDH
7
6
5
4
3
2
1
0
CPCe
CPCeA
C
CPCe
CPCeA
+
Chk1
CDC2
GAPDH
Chk1/GAPDH
1.0
.8
.6
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CDC2/GAPDH
25
20
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CPCeA

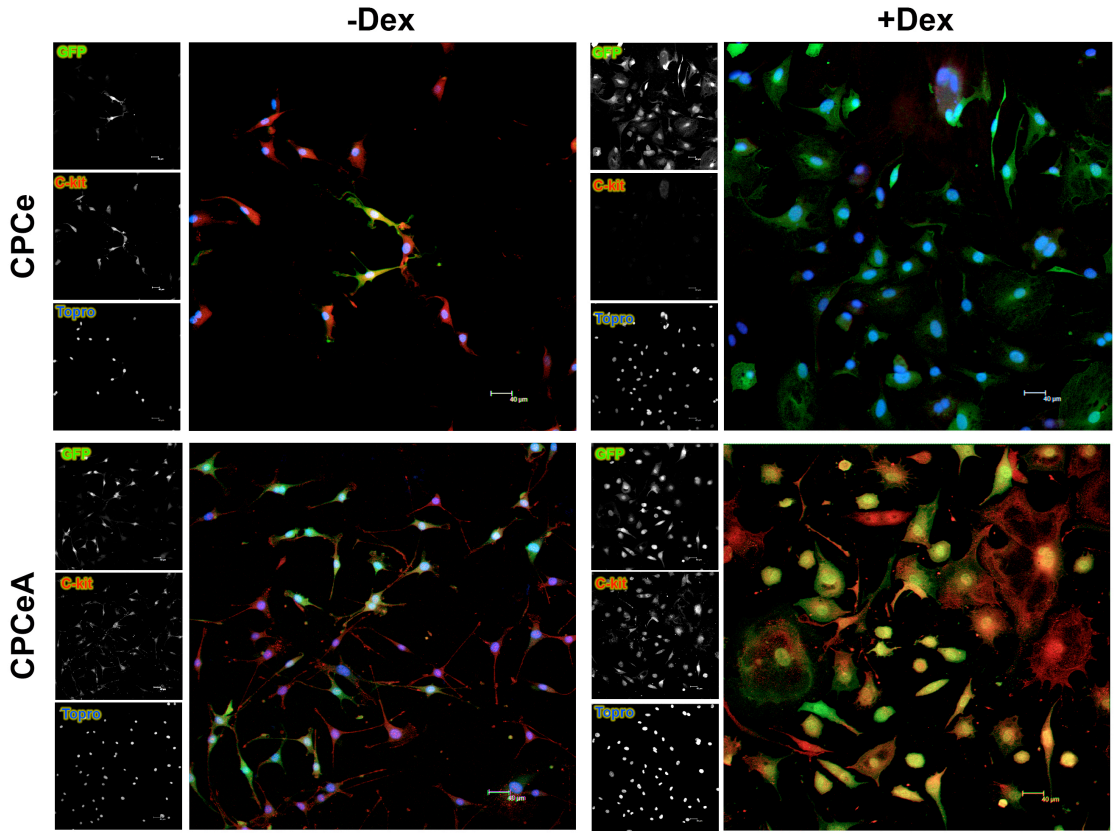


Figure 1.4: Nuclear Akt modified CPCs are refractory to *in-vitro* differentiation

C-kit expression was analyzed in CPCe and CPCeA treated with and without Dex for 7 days. GFP (green), c-kit (red), and nuclear stain Topro (blue).

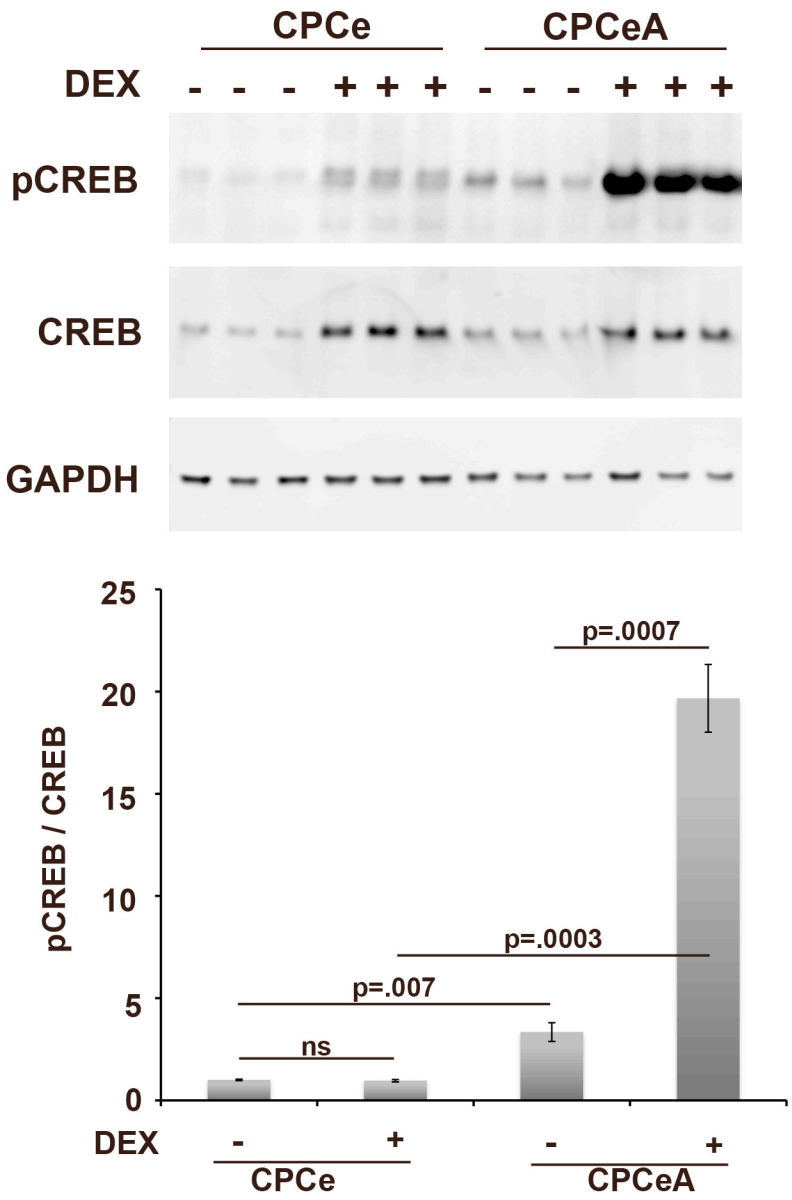
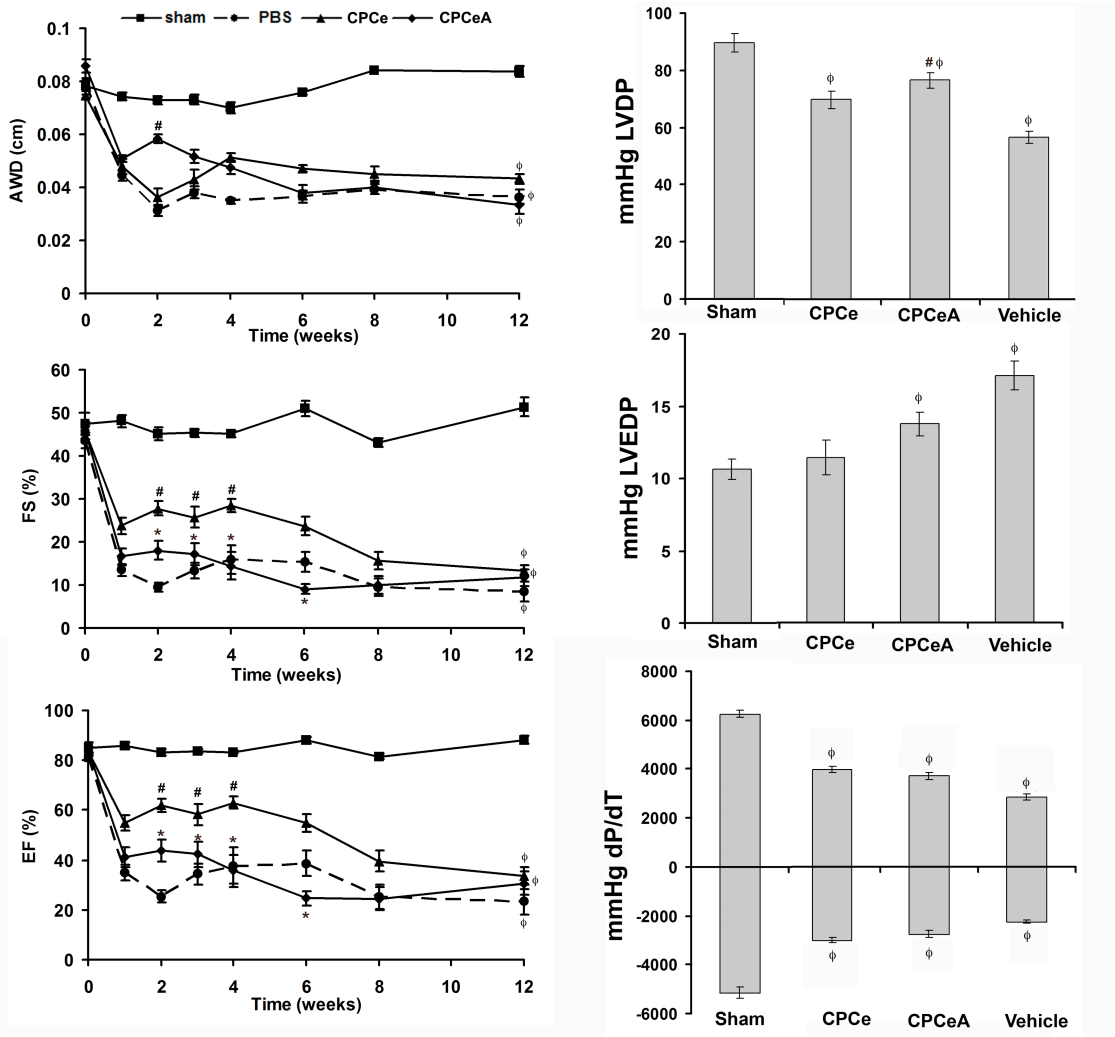


Figure 1.5: Differentiated CPCeA have increased levels of pCREB

Immunoblot and quantitation analysis of phospho-CREB, total CREB, and GAPDH as loading control in CPCe and CPCeA treated with and without Dex.

Figure 1.6: Intramyocardial injection of CPCeA does not improve cardiac function 12-weeks post infarction

(**A-C**) Electrocardiographic assessment of AWD (**A**), FS (**B**), and EF (**C**), in sham (■, n=4), vehicle (●, n=7), CPCe (▲, n=8), and CPCeA (♦, n=7), 12-weeks post-infarction (mean ± SEM). (**D-F**) Cardiac function of sham (n=4), vehicle (n=5), CPCe (n=6), and CPCeA (n=5) were evaluated using *in vivo* hemodynamic measurements of LVDP (**D**), LVEDP (**E**), and dP/dT (**F**) 12-weeks post-intramyocardial injection (mean ± SEM). ϕp<.05, ϕϕp<.01, ϕϕϕp<.001 compared to sham; #p<.05, ##p<.01, ###p<.001 compared to vehicle, * p<.05, **p<.01, ***p<.001 compared to CPCe.


sham
PBS
CPCe
CPCeA
AWD (cm)
FS (%)
EF (%)
Time (weeks)
mmHg LVDP
mmHg LVEDP
mmHg dP/dT
Sham
CPCe
CPCeA
Vehicle

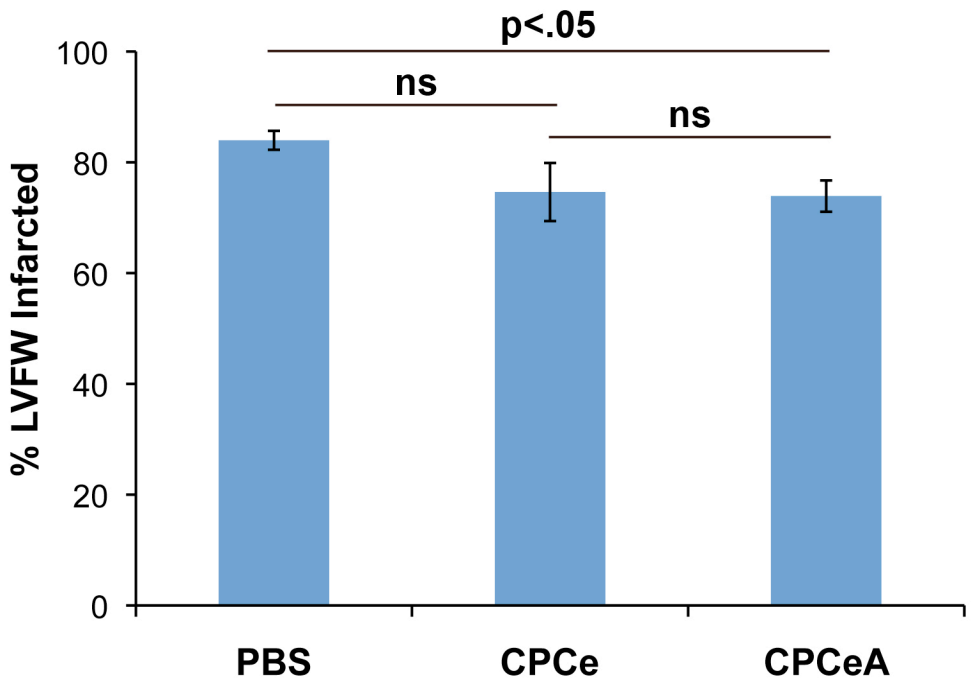


Figure 1.7: Infarct size in CPCeA injected animals is comparable to CPCe injected hearts

Quantitation of infarction area in vehicle (n=3), CPCe (n=3), and CPCeA (n=4) treated hearts 12-weeks post injection (mean ± SEM).

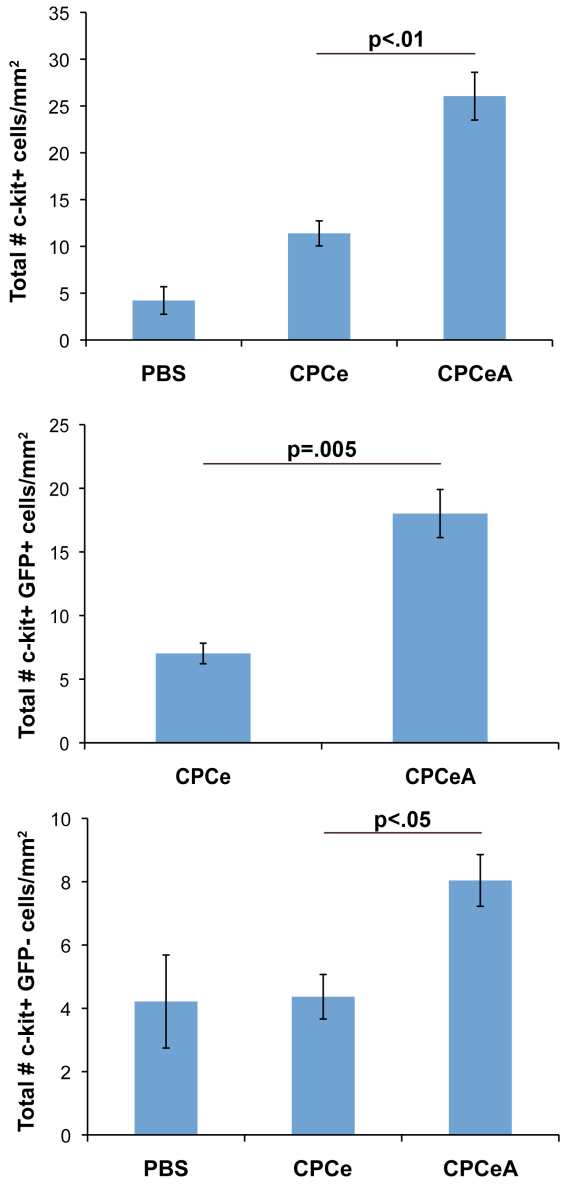
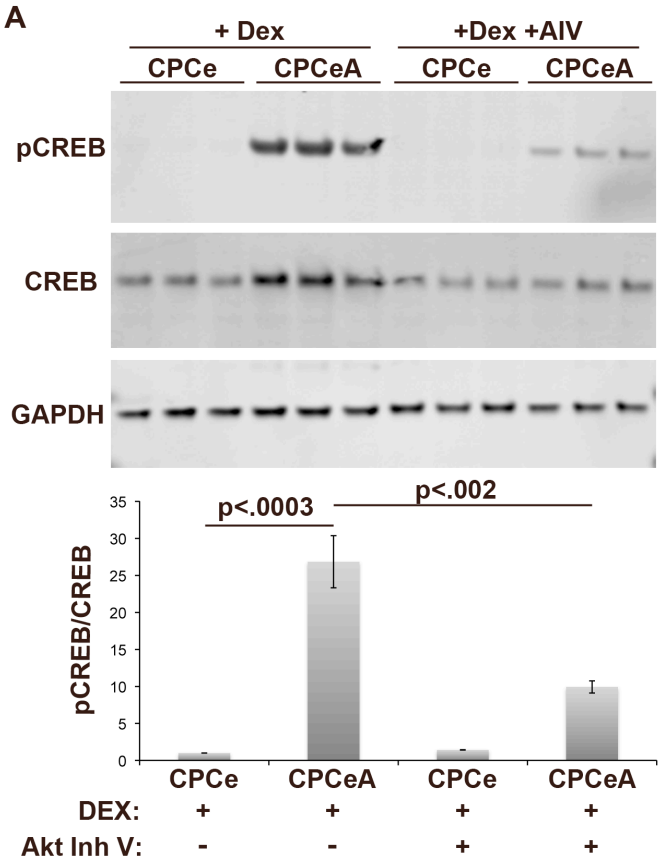


Figure 1.8: CPCeA treated hearts have increased numbers of c-kit+ cells 12-weeks post intramyocardial injection

(**A**) Quantitation of the number of total, (**B**) eGFP+, and (**C**) eGFP- c-kit+ cells in hearts of mice injected with vehicle (n=3), CPCe (n=3), or CPCeA (n=4) (mean ± SEM).

Figure 1.9: Phospho-CREB Attenuation in CPCeA increases lineage commitment

(**A**) Immunoblot and quantitation of Dex treated CPCe and CPCeA, incubated with and without Akt inhibitor. (**B**) qRT-PCR quantitation of TnT transcript levels in CPCe and CPCeA treated with or without Dex treatment and no AIV, (**C**) qRT-PCR quantitation of TnT transcript levels in CPCe and CPCeA, treated with or without Dex and with AIV. Values were normalized to CPCe treated with Dex.


A
+ Dex
+Dex +AIV
CPCe
CPCeA
CPCe
CPCeA
pCREB
CREB
GAPDH
p<.0003
p<.002
pCREB/CREB
35
30
25
20
15
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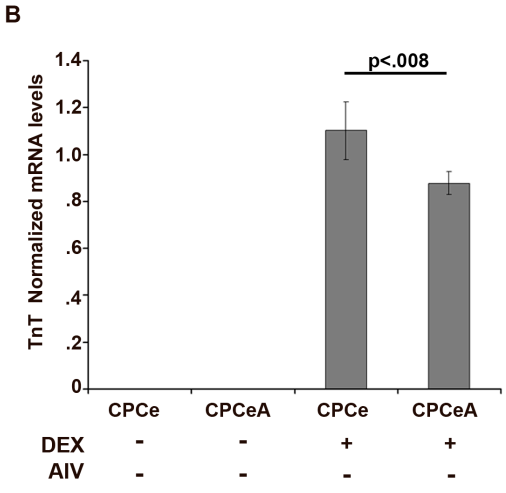
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TnT Normalized mRNA levels
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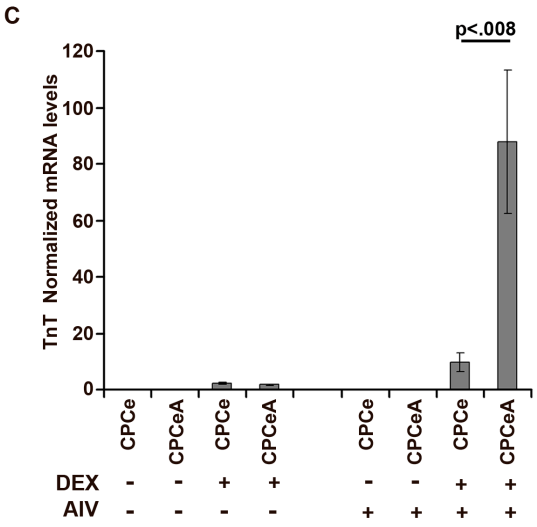
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CHAPTER II:

Enhancement of Myocardial Regeneration through Genetic Engineering of Cardiac Progenitor Cells Expressing Pim-1 Kinase

INTRODUCTION

Stem cell-based interventional approaches for myocardial regeneration have recently generated substantial enthusiasm as a novel treatment for heart failure. Despite initial optimism, application of regenerative medicine in the myocardium has been stymied by the marginal regenerative potential of adoptively transferred stem cell populations. Experimental studies routinely find the vast majority of adoptively transferred stem cells die or vanish shortly after delivery. Thus, benefits observed are likely mediated by a small population of surviving cells, leading to the postulate: improving cardiac progenitor cell (CPC) survival and proliferation will have dramatic consequences for enhancing myogenesis and empower therapeutically relevant implementation of myocardial regeneration.

CPCs expressing the stem cell marker c-kit+ reside within the heart(3, 23), mediating maintenance and repair of damaged cardiac tissue in response to myocardial injury(3, 21, 24-26). CPCs have been isolated and used with modest success for treatment and regeneration of damaged heart tissue after infarction(1, 4, 20, 24). Ideally, to enhance the regenerative process, adoptively transferred CPCs would benefit from modification to promote cellular survival and proliferation without inhibiting lineage commitment and differentiation provided by appropriate environmental cues.

Previous studies by our group have identified a kinase responsible for cardioprotection downstream of Akt signaling named Pim-1(65), a conserved

serine/threonine protein kinase originally described as the proviral integration site of the Moloney murine leukemia virus(66). Primary downstream targets of Pim-1 include molecules responsible for regulation of cellular survival and mitotic activity(66-69, 100). The role of Pim-1 in promoting proliferation and transition through cell cycle has been extensively documented(69-73). Unlike native Akt regulated by phosphorylation, Pim-1 is constitutively activated and is produced in response to stress or pathologic injury in the myocardium. Pim-1 is also expressed in activated stem cells(101) as well as in endothelial(102) and vascular smooth muscle cells(67). While cardioprotective effects of Pim-1 are clear from our work(64, 65), the impact of Pim-1 as a mediator of myocardial regeneration remains to be explored. Results presented herein demonstrate the beneficial capacity of Pim-1 to enhance cardiac regeneration; advancing the concept of *ex vivo* gene therapy with cultured CPCs to enhance cardiogenesis when reintroduced into infarcted myocardium.

METHODS

Generation of Lentiviral Vectors

CPCs were genetically modified using a bicistronic lentiviral vector to deliver human Pim-1 gene under control of a **m**yeloproliferative sarcoma virus LTR-**n**egative control region **d**eleted (MND) promoter and eGFP driven off a vIRES. Lentivirus was made as previously described(103). All constructs are third generation self-inactivating (99) lentiviral vectors and incorporate several elements to ensure long-term expression of the transgene. The MND promoter allows for high expression of the transgene, while the LTR allows for long-term expression after repeated passage (104, 105). The vectors also include (IFN)-b-scaffold attachment region (SAR) element. The SAR element has been shown to be important in keeping the vector transcriptionally active by inhibiting methylation and protecting the transgene from being silenced (106-108). The human Pim-1 cDNA was amplified out using primers containing EcoR1 restriction sites at both ends in order to facilitate cloning into the lentiviral backbone.

Lentiviral preparation

Lv-egfp and Lv-egfp+Pim1 constructs were co-transfected with packaging plasmids pMDLg/pRRE, pRSV-rev, and VSVG into 293T cells. Media changed 16 hours later and viral supernatant harvested at 24 and 48 hours. High titer virus was achieved using ultracentrifugation and titer calculated using limiting dilutions of viral stock.

Cardiac Progenitor Cell Isolation, Culture, and Transductions

CPCs were isolated from syngenic male FVB mice as previously described(25) and cultured for 3-weeks in cardiac stem cell media. CPCs were plated (.2x10^6 cells/well) in 48-well plates, transduced with lentivirus (MOI=10) with 4mg/ml polybrene, expanded, and analyzed by flow cytometry to determine percentage eGFP+ cells. CPCs were differentiated as previously described(25) using 10^{-8}M dexamethasone.

Cardiac Stem Cell Medias

DMEM-F12 with 10% FBS, 1% PSG, .02ng/ml bFGF (Peprotech #100-18B), .4mg/ml EGF (Sigma #E9644), 1000U/ml LIF (Chemicon #ESG1107), and 1X ITS (Lonza #17-838Z). Differentiation media: aMEM, 10% FBS, 1% PSG, .22% sodium bicarbonate, 10nM Dexamethasone (Sigma # D4902), pH 7.2.

Myocardial Infarction, Injections, Echocardiography, and Hemodynamics

Briefly, 10-12 week old female FVB mice were anesthetized under isoflurane, intubated, and ventilated. A thoracotomy was performed and the LAD ligated. Vehicle, CPCe, or CPCeP were injected by blinded surgeon at five minutes post ligation around border zone in five sites with a total of 100,000 cells per heart. Infarction size was standardized by echocardiography performed on animals imaged along a parasternal short-axis view by M-mode recorded at 3 days post-infarction/injection. Lack of anterior wall motion in conjunction with at least 40% decrease in EF and FS were required for study

inclusion. Hemodynamic performance assessed by echocardiography three days post-infarction was not statistically different between infarcted and injected groups (PBS, CPCe, and CPCeP). Closed chest hemodynamic assessment was performed on anesthetized mice prior to insertion of microtip pressure transducer (FT111B, Scisense) into the right carotid artery and advancement into left ventricle. The catheter was connected to an A/D converter (FV892A, Scisense) for data collection. After hemodynamic measurements, hearts were arrested in diastole and perfused with phosphate-buffered formalin.

SuperArrays

mRNA and cDNA from CPCe and CPCeP was obtained using Triazol (Invitrogen) and harvested per manufacture's protocol. Apoptosis (PAMM-012) and cell proliferation (APMM-012) arrays were obtained from SuperArray and ran per manufacturer's protocol.

Trypan Blue Exclusion and MTT assay

Uninfected, CPCe, and CPCeP, plated in quadruplicate (10,000 cells/well) in 24 well plates. Viable cells determined by trypan blue exclusion. For MTT assay 5000 cells/well were plated in 96-well plates, incubated 4-5 hours with 50mg of MTT reagent (Fluka), treated with 100ml stopping reagent (.01N HCl + 10% SDS) overnight at 37°C, and analyzed on a spectrophotometer at 570nm. Quercetagetin (Calbiochem, #551590) was incubated with cells at 10mM where indicated.

Confocal Microscopy

Heart sections were deparaffinized, antigen retrieved in 1mM Citrate (pH 6.0), followed by 1-hour block in TNB. Primary antibodies were incubated overnight at 4°C at appropriate dilutions (see Supplemental Methods). Slides washed in 1X TN followed by secondary antibody incubation, 2 hours at room temperature. Subsequent tyramide amplification was performed as necessary.

Telomere Length

Paraffin sections were prepared for in-situ hybridization and stained for telomere length as per manufacturers protocol (Dako).

Statistics

Statistics were calculated using SPSS software. One-way ANOVA and two-way repeated measures ANOVA for echocardiography with Tukey's post-hoc test were calculated. Values with $p<.05$ were considered statistically significant. Generalized estimating equations with empirical (robust) covariance estimation were used to confirm two-way ANOVA results.

Animal Studies

All animal studies were approved by IACUC.

RESULTS

Pim-1 lentiviral vector expression in cardiac progenitor cells (CPCs)

Bicistronic lentiviral vectors expressing enhanced green fluorescent protein alone (Lv-egfp) or in combination with Pim-1 kinase (Lv-egfp+Pim1) (Figure 2.1A) were used to introduce cDNA constructs into c-kit+ CPCs isolated from male nontransgenic (NTG) FVB mice. CPC populations modified with Lv-egfp (CPCe) or with Lv-egfp+Pim1 (CPCeP) were subjected to immunoblot analyses to confirm protein expression of genomically integrated gene products. eGFP protein expression was present in CPCe and CPCeP and increased protein levels for Pim-1 in CPCeP samples (Figure 2.1B). CPC and CPCe showed low level endogenous Pim-1 protein expression.

Pim-1 increases CPC proliferation

CPCeP exhibit increased proliferation compared to CPCe ($p<.05$) and CPC ($p<.001$) as assessed by Trypan Blue exclusion (Figure 2.2A). In comparison, increased CPCe cell numbers observed at day 4 were not maintained at day 6. CPCeP also possess increased metabolic rates relative to CPCe at time zero ($p<.001$) or 48 hours ($p<.001$) *in vitro* as determined by MTT assay (Figure 2.2B). The increase in CPCeP growth rate was abrogated by addition of Quercetagetin (a specific Pim-1 activity inhibitor) after forty-eight and seventy-two hours ($p<.001$) relative to vehicle treated CPCeP (Figure 2.2C). Several mRNAs encoded by genes responsible for cell cycle arrest are downregulated in CPCeP by array analysis (Figure 2.3). Immunoblot analyses

of CPCeP also show decreased expression of p27, a cell cycle dependent kinase inhibitor (Figure 2.2D). Thus, enhanced proliferation of CPCeP is likely due to altered regulation of cell cycle inhibitors.

CPCeP differentiate into cardiac, endothelial, and smooth muscle lineages

Flow cytometric analyses (Figure 2.4, Figure 2.5) confirmed CPCe and CPCeP had significant ($p<.01$) increases in MEF2C and vWF after treatment with dexamethasone (Dex)(23). CPCeP also had a significant increase ($p<.001$) in percent of cells staining positive for MEF2C compared to CPCe. These results indicate genetically modified CPCs are amenable to lineage commitment.

Additional phenotypic characterizations for cell markers consistent with cardiovascular lineages by immunolabeling confirmed CPCe and CPCeP co-expressed c-kit and MEF2C, a myocyte-specific transcription factor. Additionally, CPCeP also expressed VWF, an endothelial cell marker (Figure 2.6A). The endothelial nature of CPCeP was supported by uptake of acetylated-low density lipoprotein (Ac-LDL) showing a small percentage of CPCeP is Ac-LDL+, whereas Ac-LDL was undetectable in CPCe (Figure 2.6B). The capacity of genetically engineered CPCs to express cardiogenic markers consistent with lineage commitment was examined *in-vitro* by treatment with Dex. Immunostaining for cardiogenic lineages was performed using antibodies to c-kit, MEF2C, VonWillebrands Factor (vWF), and Gata6 to identify progenitor, cardiac, endothelial, and smooth muscle cells,

respectively. Markers of all three lineages were detected by immunostaining following Dex treatment of CPCe or CPCeP (Figure 2.6C).

CPCeP improve cardiac function 12-weeks post myocardial infarction

Transgenic mice with cardiac-specific Pim-1 expression are resistant to infarction challenge(64, 65), therefore CPCeP should ameliorate pathological damage following adoptive transfer into infarcted myocardium. Twelve-week-old female mice, subjected to myocardial infarction, were treated with CPCe or CPCeP injected directly into the peri-ischemic border zone. Hearts of mice receiving CPCeP possessed thicker anterior wall dimension (AWD) compared to vehicle ($p<.001$) or CPCe ($p<.01$) injected mice (Figure 2.7A) by echocardiography at 2-weeks. Both groups receiving CPCs showed significant improvements in ejection fraction (EF) and fractional shortening (109) at 4-weeks post-delivery ($p<0.05$) relative to vehicle injected mice ($p<.05$) (Figure 2.7B-C). However, EF, FS (Figure 2.7B-C), left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP), and maximum dp/dt (Figure 2.8A-C) were not significantly different ($p>0.05$) between CPCe and CPCeP groups.

CPCeP injections were compared to mice receiving CPCe injections over an extended 12 week time course following delivery into infarcted hearts to assess long lasting beneficial effects. Injection of CPCe conferred improved function at one week after delivery, however transitioned into decompensation at 6-weeks, becoming indistinguishable from vehicle controls by 8-weeks (Figure 2.7B-C). In comparison, CPCeP injected mice

maintained EF and FS and had significantly improved function relative to CPCe injected mice at 6-weeks following delivery. At 12-weeks, CPCeP treatment preserved EF and FS, whereas CPCe treated hearts suffer from a 2-fold and 1.6-fold decrease in function, respectively (Figure 2.7B-C). Significantly enhanced cardiac function in mice receiving CPCeP was confirmed by measurement of LVDP, LVEDP, and changes in ±dp/dt (Figure 2.7D-F) relative to mice receiving CPCe.

Injection of CPCeP leads to infarct reduction, de novo myocyte formation, and neovascularization

Hearts from CPCe and CPCeP injected animals were immunostained with sarcomeric tropomyosin to detect surviving myocardium at 12-weeks post-infarction. CPCeP injected hearts showed significant reductions in infarct size (1.5-fold; $p<0.05$; Figure 2.9A) and fibrosis (Figure 2.10) relative to CPCe treated. In CPCeP injected hearts 39.8% of the surviving myocardium within the infarct was eGFP+ (Figure 2.9B). This corresponded to a significant ($p<.001$) 4-fold increase compared to CPCe injected hearts.

Persistence and cellular phenotype of donated CPCs was examined at 12-weeks post-delivery by immunostaining myocardial sections with antibodies to eGFP co-localized with sarcomeric tropomyosin to detect myocytes as well as with connexin-43 suggesting formation of functional gap junctions with neighboring cells (Figure 2.9C). Coincident localization of eGFP and striated tropomyosin labeling is indicative of myocytes presumptively derived from the donated cell population in hearts receiving CPCeP. Interestingly, while de

novo myocardium is formed in the central area of the infarcted region, the majority of regeneration from transplanted cells is at or adjacent to the border zone where cells are initially injected.

Together with myocyte labeling, vessels coincident with eGFP were observed by colocalization with antibodies to VonWillebrands Factor (vWF) (Figure 2.9D) or smooth muscle actin (Figure 2.9E) from vehicle treated, CPCe, and CPCeP injected hearts 12-weeks post-infarction. Quantitative assessment of lineage markers demonstrated that in CPCeP injected hearts 32.8% and 51.6% of the eGFP+ population stained positive for vWF and SMA (Figure 2.9F), respectively. This correlated to a significant 1.8- and 2.3-fold increase compared to CPCe, further implicating the CPCeP population in formation of new vasculature.

Telomeric length preservation in myocytes derived from CPCeP

Telomere length was measured in myocytes of mice receiving CPCeP in the border zone at 12-weeks after delivery. Presumptive de-novo myocytes derived from CPCeP as evidenced by coincidence of eGFP and tropomyosin labeling showed relatively greater telomeric length compared to resident eGFP- myocytes (Figure 2.11A). Additionally, cross sectional area of eGFP+ myocytes with long telomeres was significantly smaller compared to those cells with shorter telomeres (Figure 2.11A). Together, small eGFP+ myocytes with long telomeres suggests these cells are newly formed young cells presumably originating from the donated cell population.

CPCeP origin for these small eGFP+ myocytes was supported by both immunohistochemical and PCR analyses. Small eGFP+ myocytes show coincident labeling for increased Pim-1 expression. The level of myocyte-specific Pim-1 immunoreactivity in sections from CPCeP injected hearts was clearly greater than that observed for sections from receiving CPCe (Figure 2.11B). In addition, presence of genomic DNA of male origin was detected in cells (Figure 2.11C), as well as within the infarct region of female hearts (Figure 2.11D) by PCR for SRY, a gene located on the Y chromosome. Demonstration of DNA for eGFP encoded by insertion of the Lv-egfp or Lv-egfp+Pim1 sequences into the cellular genome corroborates the presence of cells derived from donor origin in the infarcted region (Figure 2.11D).

Increased number of c-kit+ cells in hearts receiving CPCeP show enhanced proliferation

Presence of c-kit+ cells within the infarct area was quantitated to assess enhanced recruitment of endogenous stem cells by CPCeP (20, 26, 110). CPCeP treated hearts had a 2.6-fold increase in total c-kit cells relative to CPCe at 12-weeks (Figure 2.12A and B). Quantitation of the number of eGFP+ c-kit+ cells in CPCeP injected hearts demonstrated CPCeP had a 6-fold increase compared to CPCe (Figure 2.12C). The number of eGFP- c-kit+ cells was not statistically different between CPCeP and CPCe (Figure 2.12D). Furthermore, at 5-weeks post infarction CPCeP injected animals maintain cardiac function while CPCe injected controls begin to display signs of cardiac failure determined by echocardiography. CPCe or CPCeP injected

animals were given BrdU at 5-weeks post-infarction to assess whether sustained cardiac performance in CPCeP animals may be attributed to increased numbers of proliferating cells. Quantitation of the BrdU+ eGFP+ population demonstrates CPCeP injected animals have a significant ($p<.001$) 3-fold increase in the number of proliferating cells at 5-weeks (Figure 2.13A). At the 12 week time point co-expression of PCNA and eGFP were quantitated. Results were similar to that obtained at 5-weeks, whereby CPCeP injected animals had a significant 1.9-fold increase in the number of PCNA+ eGFP+ cells (Figure 2.13B).

CPCeP and CPCe injected mice were stained with TUNEL and eGFP to address CPCeP resistance to cell death sections from. At 5-weeks no TUNEL+ eGFP+ cells were found in CPCeP or CPCe injected mice. Interestingly CPCeP hearts had a significant 4-fold decrease in TUNEL+ eGFP- cells (Figure 2.13C). These data suggest injection of CPCeP has a protective effect on surrounding myocardium; however it is not yet known if CPCeP are refractory to apoptosis at time points before 5-weeks. Collectively, these results indicate CPCeP have an increased ability to survive and proliferate in the damaged myocardium compared to CPCe.

Persistent improvement in myocardial performance is afforded by CPCeP

Beneficial effects of adoptively transferred cell populations are often measured within days or a few weeks after delivery (20, 26, 111, 112), but genetic modification of CPC with Pim-1 may allow for long-term functional improvements not observed with normal cells. A 32-week longitudinal study

was performed to determine myocardial function in infarcted mice receiving vehicle, CPCe, or CPCeP. Validity of infarction was verified at three days by statistically equivalent decreases in EF, FS, and AWD (Figure 2.14A-C) of all three infarcted experimental groups. CPCeP injected mice showed improved FS and EF by one week following delivery sustained throughout the course of the study (Figure 2.14A-B). CPCeP injected hearts also had significantly increased AWD relative to CPCe injected hearts at 32-weeks (Figure 2.14C). *In vivo* hemodynamic analysis confirmed improved myocardial performance following CPCeP treatment, evidenced by decreased LVEDP, increased DP, and ±dp/dt (Figure 2.14D-F) relative to mice receiving CPCe. Heart weight to body weight ratios in CPCeP treated mice were comparable to sham operated controls and significantly smaller relative to CPCe and vehicle-injected controls (Figure 2.14G; $p<.05$). Cardiac remodeling as determined by Law of Laplace revealed the ratio of left ventricular diameter (r) to wall thickness (h) was significantly decreased in mice receiving CPCeP at 36-weeks post-delivery (Figure 2.14H). Results from the 32-week time course validate results obtained in the 12-week study (Figure 2.7) and demonstrate persistent and significant improvement in myocardial performance and remodeling afforded only by CPCeP.

CPCeP engraftment and commitment persists up to 32-weeks post intramyocardial injection

Myocardial samples from mice receiving CPCe and CPCeP were examined for presence of adoptively transferred cells as evidenced by

presence of immunoreactivity for eGFP coincident with markers of cardiogenic lineages at 32-weeks post-delivery. Myocytes expressing eGFP were detected in hearts receiving either CPCe or CPCeP. Similar to observations at 12-weeks (Figure 2.9A), hearts receiving CPCeP showed a significant 1.3-fold reduction in infarct size (Figure 2.15A). In CPCeP injected hearts 32.8% of the surviving myocardium was eGFP+ (Figure 2.15B,C). This corresponded to a significant ($p<.001$) 6-fold increase compared to CPCe injected hearts. CPCeP injected animals also had significant increases in total c-kit+ cells (2.9-fold) at 32-weeks relative to CPCe injected hearts. CPCeP injected hearts had a significant 7.9-fold increase in the number of eGFP+ c-kit+ cells compared to CPCe, while number of eGFP- c-kit+ cells was not statistically different between the two groups (Figure 2.15D-G). Hearts of mice receiving CPCeP showed a 71% increase in vascular density in the infarct region visualized by immunostaining for smooth muscle actin, with 74% more vessels characterized by small diameter of 1-2 cells evident in the circumference (Figure 2.15H-J).

DISCUSSION

A revolution in the treatment of heart disease has begun, initiated by discovery of stem cell-mediated myocardial regeneration (23, 113, 114). As the field matures key observations and recurrent themes continually emerge from experimental and clinical studies: 1) myocardial structure and function are consistently and reproducibly improved although degree of benefit is modest, 2) the vast majority of adoptively transferred stem cells are lost due to death or poor retention after delivery, 3) long-term efficacy of the regenerative process mediated by adoptively transferred cells remains debatable, due in part to their short-lived nature, 4) therapeutically relevant implementation of stem cell-based myocardial repair ultimately depends upon enhancing the modest regenerative effects currently observed, and 5) *ex vivo* modification or treatment of adoptively transferred stem cells significantly enhances reparative and regenerative processes. The first four observations point out present day stem cell-based myogenesis and regeneration approaches fall short of the efficacy desired for reconstitution of myocardial tissue following cardiomyopathic injury. However, the last point offers hopes in the form of engineering stem cells to enhance their regenerative capacity.

Several laboratories have demonstrated impressive gains in blunting infarction damage through use of stem cells modified by genetic engineering (58, 60, 115, 116) or exposure to environmental, chemical, and biological

treatments prior to delivery (57, 115, 117, 118). A consistent theme from these studies is that the *ex vivo* manipulation of the stem cell population has salutary effects for the adoptively transferred cells but also for the host myocardium upon reintroduction. The mechanistic basis of these treatments has been linked to enhanced cellular survival, secretion of paracrine factors, activation of endogenous repair processes, and contribution of the donated cells to formation of *de novo* myocardium. Among studies of *ex vivo* stem cell modification, activation of survival kinases such as Akt, is an effective strategy for potentiating stem cell regeneration, but severely limited or complete lack of direct participation in *de novo* tissue formation and marginal long-term engraftment of engineered cells remains a serious limitation (57-59, 61, 119, 120). Downstream of nuclear Akt signaling we identified Pim-1 (65) kinase that stabilizes pro-proliferative proteins and influences their subcellular localization through phosphorylation and/or direct association. Examples include nuclear export and subsequent degradation of cyclin dependent kinase inhibitors p27 and p21 (72, 73), stabilization of c-myc (69), and mitotic complex assembly with NuMA(70). Additionally CPCs from Pim-1 transgenic mice show elevated levels of nucleostemin, a protein whose expression is associated with maintenance of proliferation in stem cells (74). Consistent with Pim-1's role in increased proliferation, our studies demonstrate injection of CPCeP promotes increased density of eGFP+ c-kit+ cells in the region of infarction (Figure 2.12B, 2.15B). As well, anti-apoptotic(121, 122) and pro-proliferative (66) actions of Pim-1 were observed with CPCs *in vitro* (Figure 2.2 and 2.3) and *in vivo* (Figure

2.13). CPCeP promote salutary effects relative to CPCe for up to 32-weeks (Figure 2.7, 2.10), presumably by conferring upon the limited number (100,000) of donated cells increased ability to survive and proliferate (Figure 2.13). CPCeP may also provide enhanced survival and increased cycling of the resident (GFP-) myocardium (Figure 2.13) through both paracrine effects and formation of de novo vasculature.

Stem cells derived from the heart, capable of generating myocytes and vasculature(4, 23, 67), may be "primed" for differentiation into cardiac lineages as evidenced by expression of c-kit in conjunction with MEF2C and NKX2.5 and increased expression of vWF (Figure 2.4, 2.9, 2.5A,C, and 2.6). Consistent with the role of Pim-1 in endothelial cell differentiation(102), CPCeP express vWF prior to differentiation (Figure 2.6A) and incorporate Ac-LDL (Figure 2.6B). CPCeP hearts also had significant increases in eGFP+ cells staining positive for vasculature markers vWF and SMA (Figure 2.9F). Pim-1 is also necessary for vascular smooth muscle cell (VSMC) proliferation, while knockdown inhibited capillary formation and reduced VSMC proliferation(67). These results infer that CPCeP may be predisposed toward vasculature lineages and suggests CPCeP-mediated angiogenesis may enhance new myocyte formation as well as retention of existing myocytes. Intramyocardial injection of CPCs has been shown to enhance cardiac function 4-weeks after injection(20, 21, 26, 112), but functionally relevant myocardial regeneration requires long-term repopulation of all three cardiac lineages: myocytes,

endothelial, and VSMC. Collectively, our results indicate CPCeP possess the inherent ability to provide such persistent multifaceted cellular repair.

Evidence of engraftment and differentiation of adoptively transferred stem cells remains a major focus of contention, but persistent presence of the donated CPCeP population is supported by immunostaining for Pim-1 (Figure 2.11B) as well as presence of SRY and eGFP DNA by PCR in cells and infarcted hearts (Figure 2.11C-D). Increased telomere length in small eGFP+ myocytes relative to small eGFP- myocytes in hearts receiving CPCeP (Figure 2.11A) are consistent with de novo myocyte formation from the CPCeP population.

Autologous stem cell therapy suffers from critical limitations in cellular survival and persistence that may be amenable to *ex vivo* genetic modification for regenerative therapy. However, lentiviral modification allowing chromosomal integration and long-term persistence also poses regulatory and safety concerns. Although oncogenic transformation has not been observed with CPCeP, safe and efficacious viral modification of CPCs may require inducible Pim-1 expression. Taken together our data suggest Pim-1 mediated CPC reprogramming provides profound improvements in cardiac structure and function that may prove to be superior to current treatment modalities. The long-term goal is to advance these findings toward translational therapeutic implementation of *ex vivo* gene therapy to enhance stem cell-based cardiac myogenesis.

Chapter 2, in full, is a reprint of the material as it appears in Circulation 2009. Fischer KM, Cottage CT, Wu W, Din S, Gude NA, Avitabile D, Quijada P,

Collins BL, Fransioli J, Sussman MA. 2009. The dissertation author was the primary investigator and author of this paper.

FIGURES

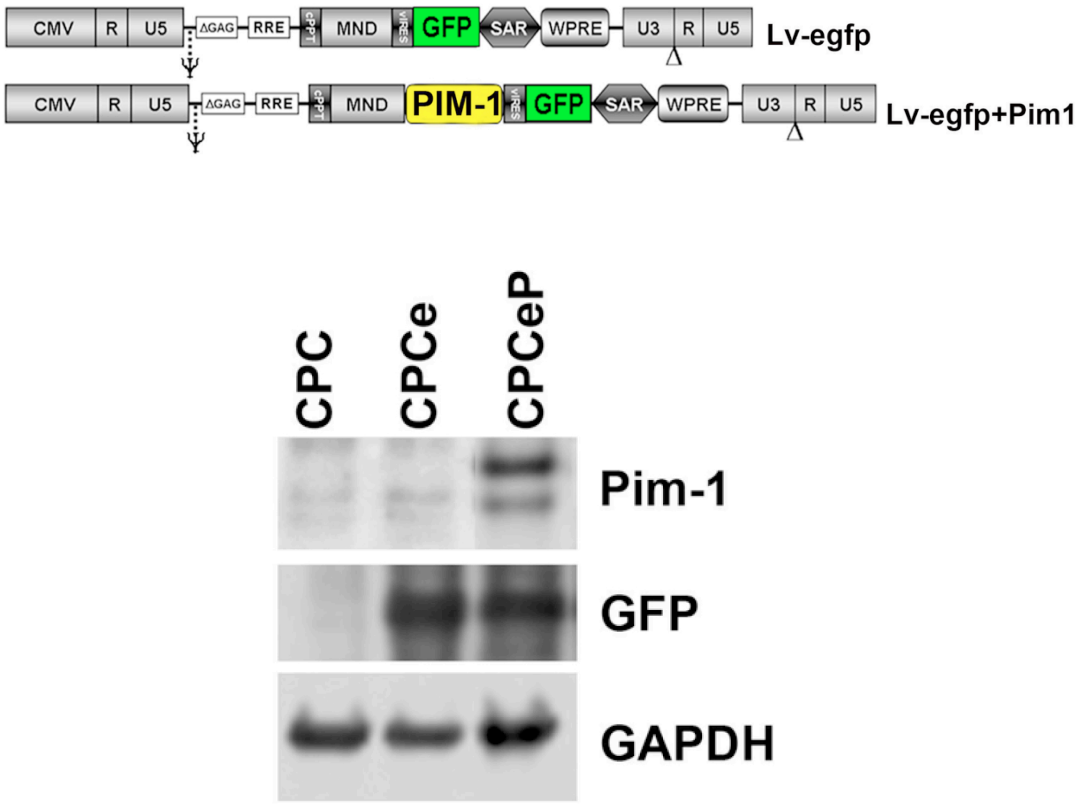
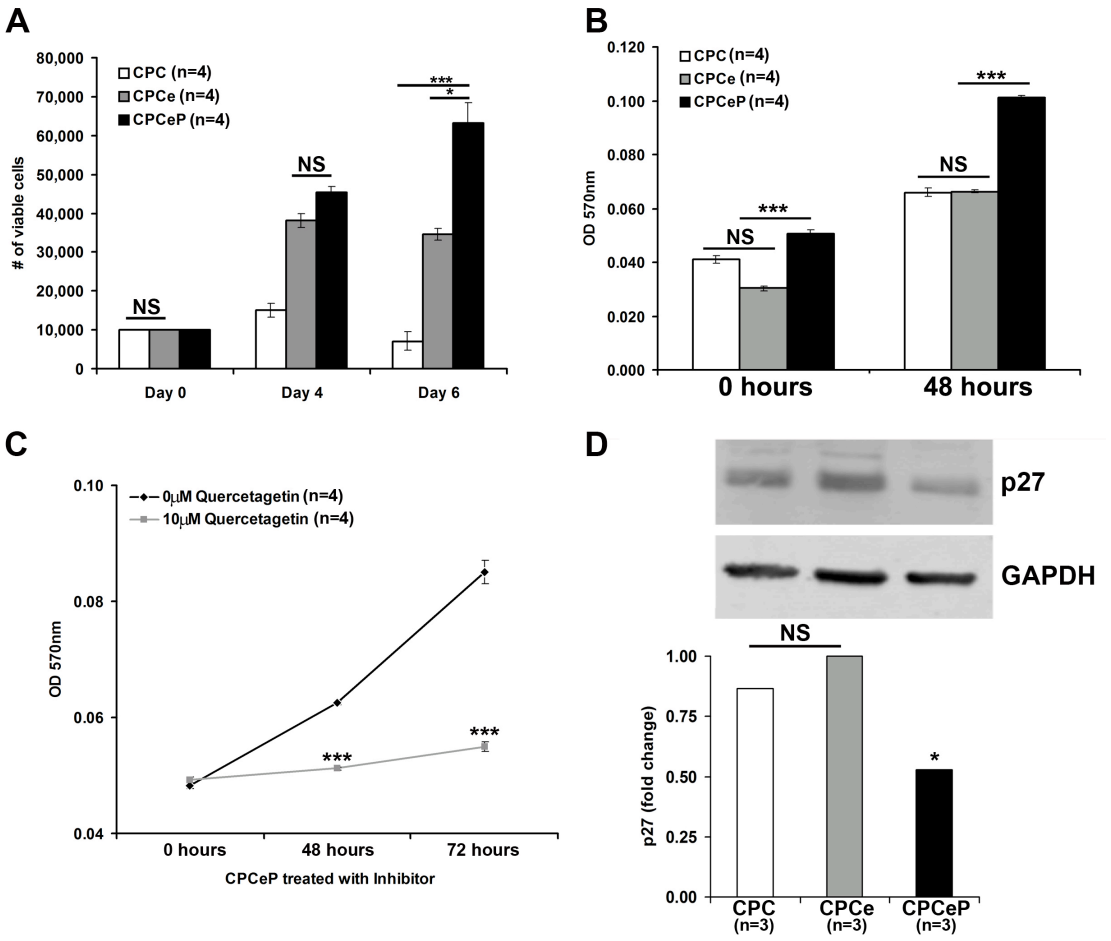


Figure 2.1: Lentiviral gene expression in c-kit+ CPCs

(**A**) Self-inactivating lentiviral vectors, termed Lv-egfp (GFP control) and Lv-egfp+Pim1. (**B**) Representative immunoblot of CPC (n=3), CPCe (n=3), and CPCeP (n=3), immunolabeled for Pim-1, GFP, and GAPDH.

Figure 2.2: Pim-1 increases the proliferation rate of CPCs *in vitro*

(**A**) The number of viable cells as determined by trypan blue exclusion was determined for CPCeP, CPCe, and CPCs over a six day time course (mean ± SEM, n=4/group). (**B**) Metabolic rate of CPCeP, CPCe, and CPCs was measured by MTT assay over a 48 hour time course (mean ± SEM, n=4/group). (**C**) Metabolic rate evaluated by MTT assay in CPCeP treated with and without 10mM Quercetagetin over a 72 hour time course (mean ± SEM, n=4/group). (**D**) Immunoblot analysis of p27 protein in CPCeP, CPCe, and CPCs (n=3/group). *p<.05, **p<.01, ***p<.001.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NS
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B
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OD 570nm
CPC (n=4)
CPCe (n=4)
CPCeP (n=4)
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CPCeP
(n=3)

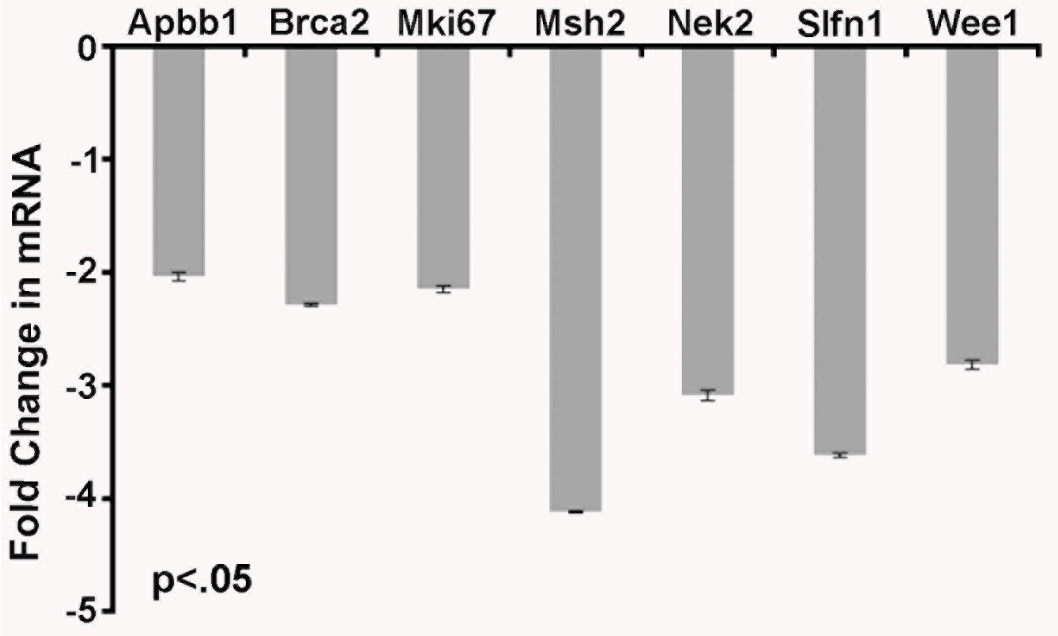


Figure 2.3: Pim-1 transduced CPCs mediate expression of genes involved in proliferation

Total mRNA was extracted from CPCe (n=3) and CPCeP (n=3) cells and run on the cell cycle RT^2 Profiler Cell Proliferation Array from SuperArray in triplicate. CPCeP and CPCe were normalized to GAPDH. Samples analyzed had ≥2-fold difference from control, with $p<0.05$, (mean ± SEM, n=3).

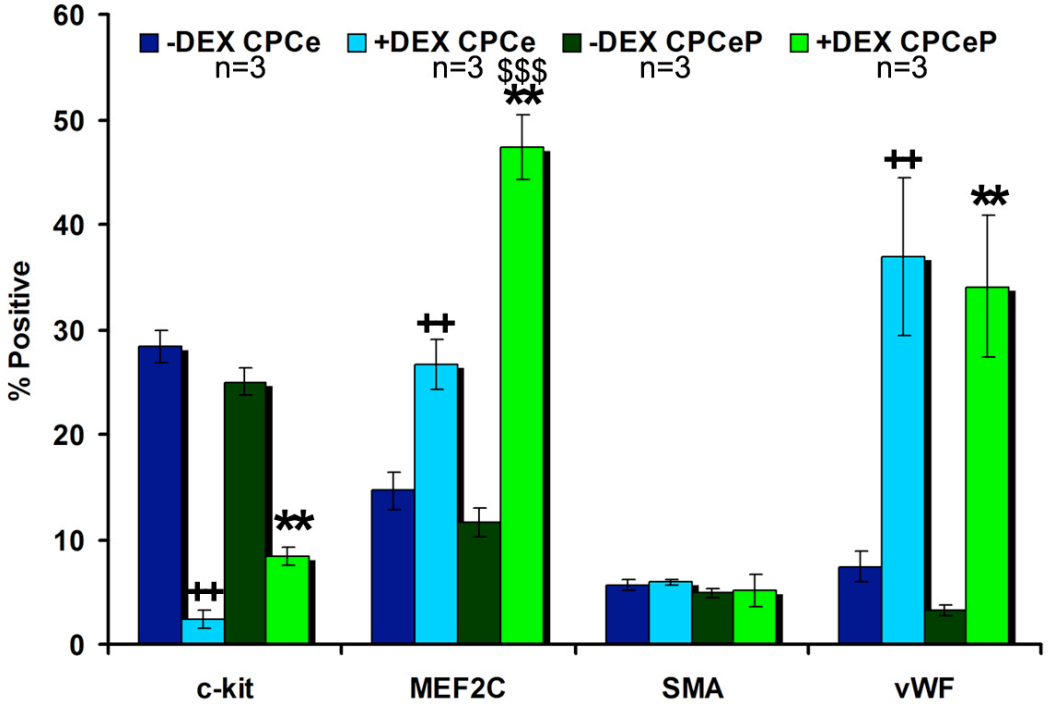


Figure 2.4: Flow cytometric analysis of CPCe and CPCeP

Analysis of cardiogenic lineages of CPCe and CPCeP before (dark blue and dark green) and after (light blue and light green) dexamethasone treatment (mean ± SEM, n=3/group). ++p<.01 compared to CPCe –Dex. **p<.01 compared to CPCeP -DEX, $$$p<.001 compared to CPCe +Dex.

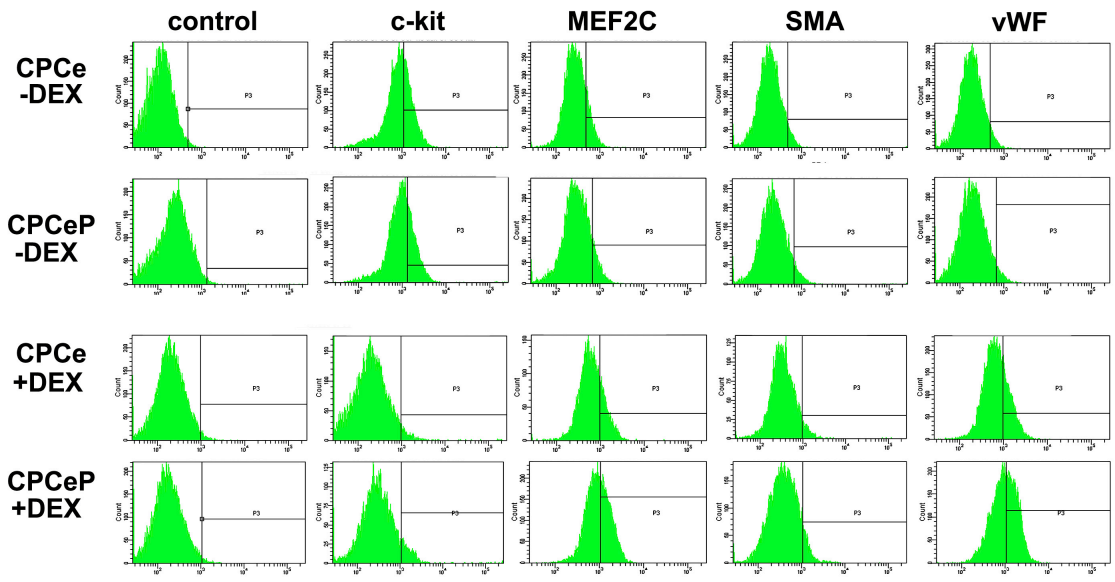


Figure 2.5: Phenotypic FACS characterization of CPCe and CPCeP *in vitro*

Flow cytometric analysis of CPCe (n=3) and CPCeP (n=3) treated with and without dexamethasone for seven days stained with specific antibodies and analyzed.

Figure 2.6: Phenotypic characterization of CPCe and CPCeP *in vitro*

(**A**) Immunolabeling of CPCe and CPCeP for cardiac (Mef2C), endothelial (VonWillebrands Factor), and smooth muscle (Gata6). White arrowheads indicate cells of interest. In each case eGFP is represented in green, lineage marker in red, and Topro-3-iodide (nuclei) in blue. (**B**) Ac-LDL-DiI uptake assay in CPCe and CPCeP using HUVEC positive control. (**C**) Immunolabeling of CPCe (n=3) and CPCeP (n=3) ran in triplicate after exposure for 7 days to dexamethasone for cardiac (Mef2C), endothelial (VonWillebrands Factor), and smooth muscle (Gata6). White arrowheads indicate cells of interest.

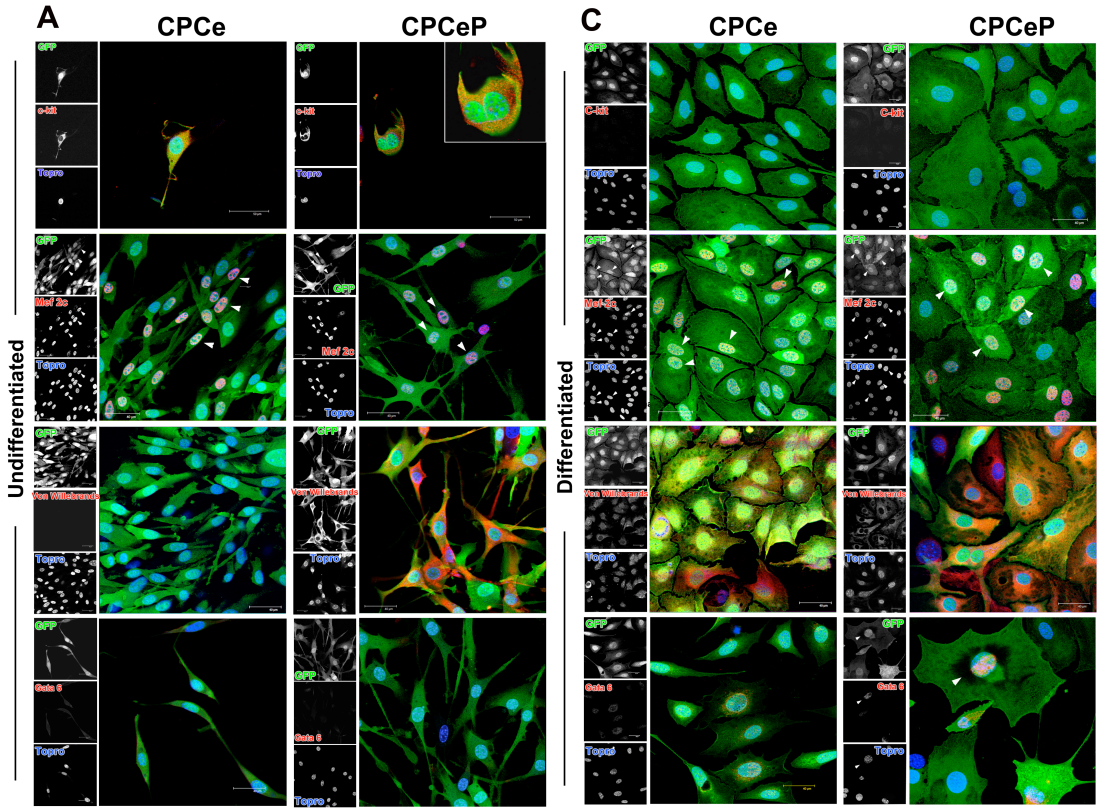

A
CPCe
CPCeP
Undifferentiated
C
CPCe
CPCeP
Differentiated
B
huvec
CPCe
CPCeP

Figure 2.7: Intramyocardial injection of CPCeP improves cardiac function 12-weeks post infarction

(**A-C**) Electrocardiographic assessment of AWD (**A**), EF (**B**), and FS (**C**), in sham (■, orange, n=8), vehicle (●, black, n=9), CPCe (▲, blue, n=8), and CPCeP (♦, green, n=9), 12-weeks post-infarction (mean ± SEM). (**D-F**) Cardiac function of sham (orange, n=5), vehicle (black, n=5), CPCe (blue, n=5), and CPCeP (green, n=5) were evaluated using *in vivo* hemodynamic measurements of LVDP (**D**), LVEDP (**E**), and dP/dT (**F**) 12-weeks post-intramyocardial injection (mean ± SEM). ϕp<.05, ϕϕp<.01, ϕϕϕp<.001 compared to sham; #p<.05, ##p<.01, ###p<.001 compared to vehicle, * p<.05, **p<.01, ***p<.001 compared to CPCe. Echocardiography significant (p<.05) by two-way repeated measures ANOVA.

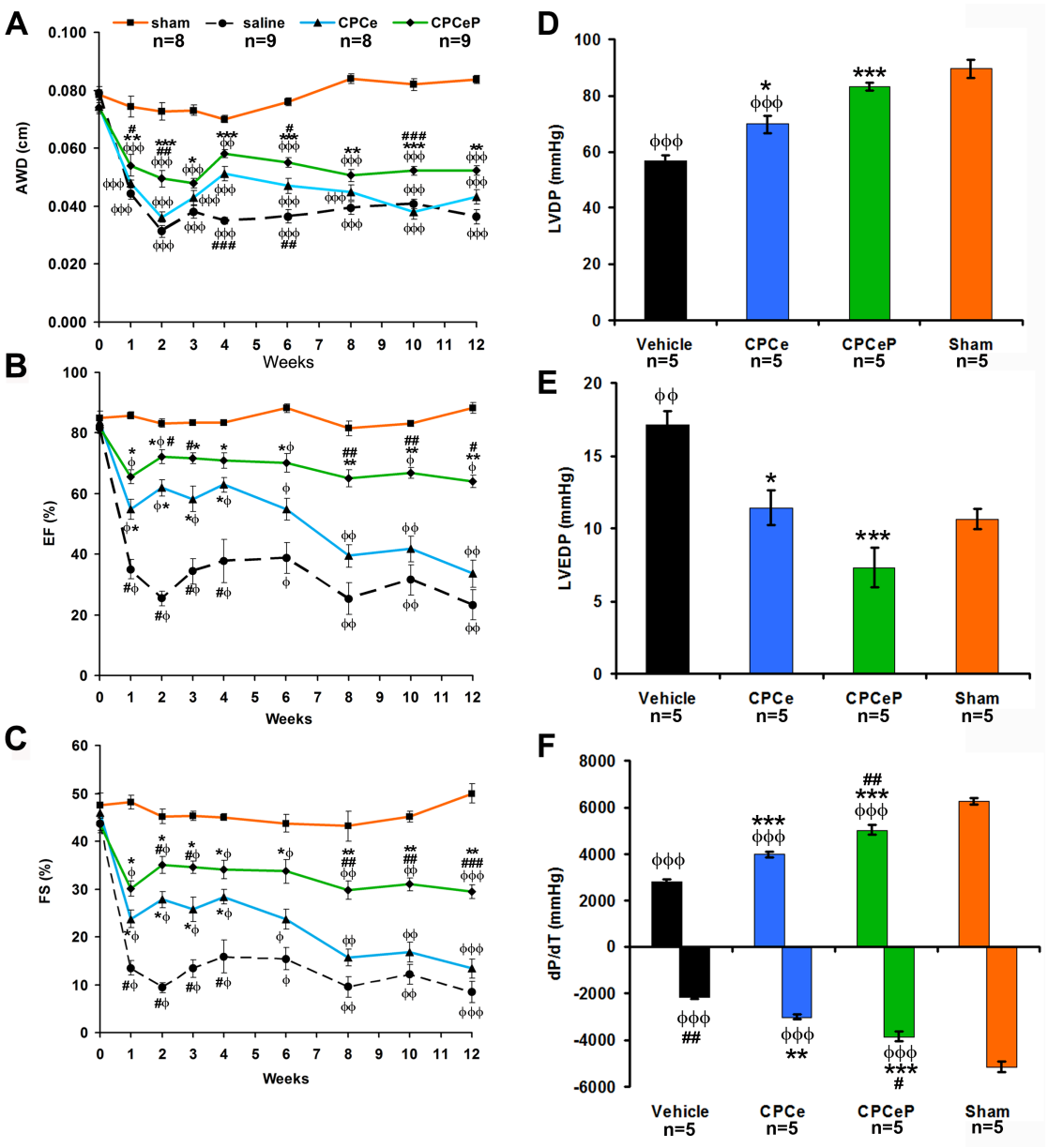

A
sham n=8
saline n=9
CPCe n=8
CPCeP n=9
AWD (cm)
Weeks
B
EF (%)
Weeks
C
FS (%)
Weeks
D
LVDP (mmHg)
Vehicle n=5
CPCe n=5
CPCeP n=5
Sham n=5
E
LVEDP (mmHg)
Vehicle n=5
CPCe n=5
CPCeP n=5
Sham n=5
F
dP/dT (mmHg)
Vehicle n=5
CPCe n=5
CPCeP n=5
Sham n=5

Figure 2.8: Improved cardiac function in CPCe and CPCeP treated animals

(**A-C**) In-vivo hemodynamic measurements of left ventricular developed pressure (DP) (**A**), left ventricular end diastolic pressure (Ped) (**B**), and dP/dT maximum and minimum (**C**) assessing cardiac function of sham (n=4), vehicle (n=4), CPCe (n=4), and CPCeP (n=4) injected animals 4 weeks post-intramyocardial injection (mean ± SEM). $\phi p<.05$, $\phi\phi p<.01$ compared to Sham and $*p<.05$ compared to CPCe.

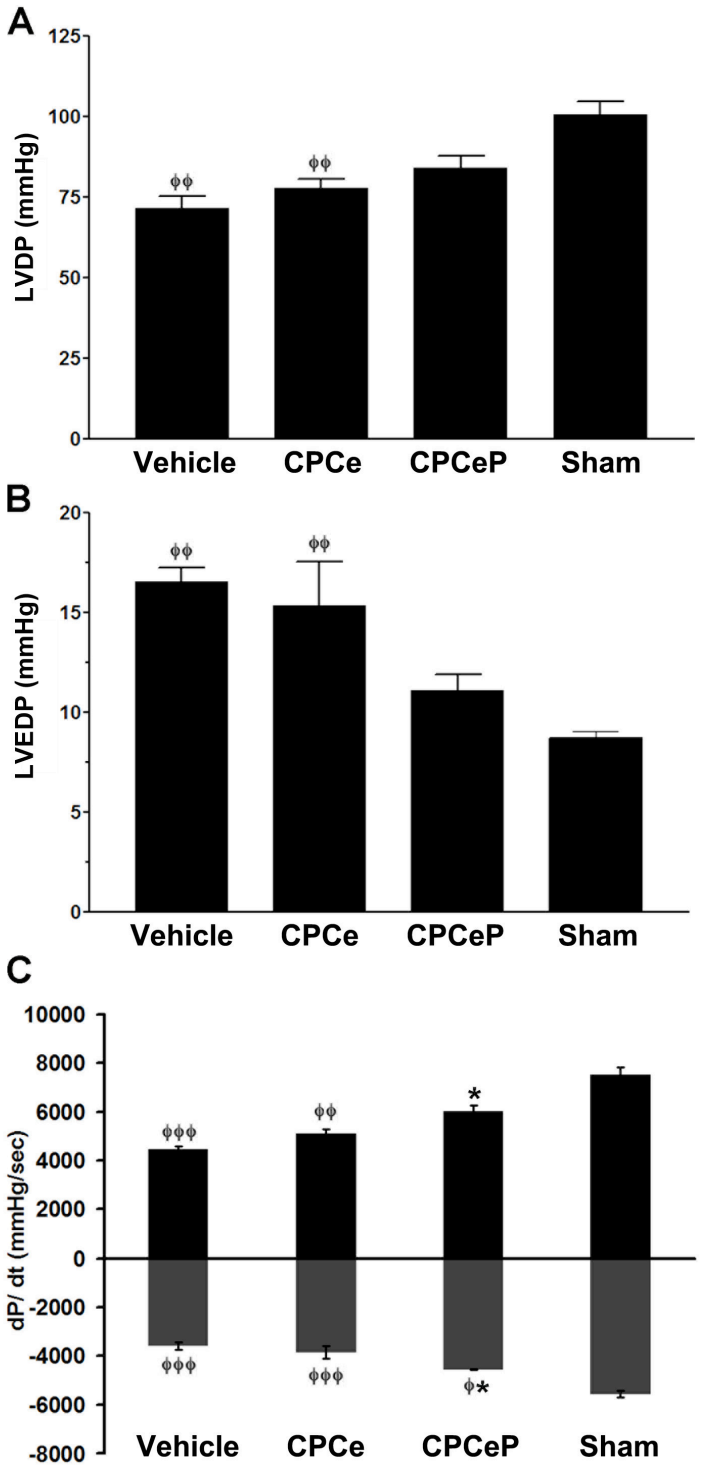
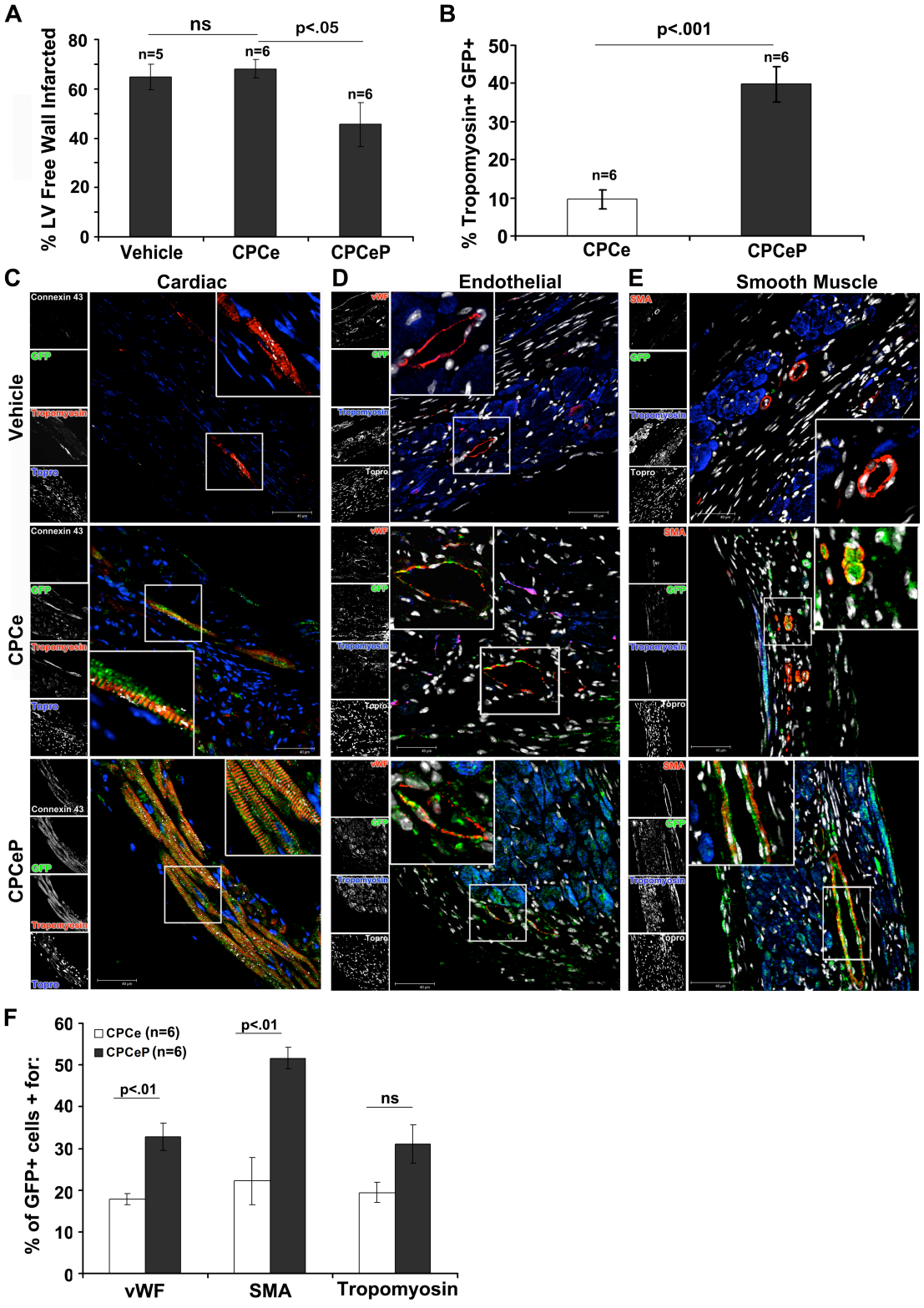

A
LVDP (mmHg)
125
100
75
50
25
0
ϕϕ
ϕϕ
Vehicle
CPCe
CPCeP
Sham
B
LVEDP (mmHg)
20
15
10
5
0
ϕϕ
ϕϕ
Vehicle
CPCe
CPCeP
Sham
C
dP/ dt (mmHg/sec)
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
ϕϕϕ
ϕϕ
*
ϕϕϕ
ϕϕϕ
ϕ*
Vehicle
CPCe
CPCeP
Sham

Figure 2.9: De novo myocyte formation and neovascularization results in reduction of infarct size in CPCeP treated animals

(**A**) Quantitation of infarction area in vehicle (n=5), CPCe (n=6), and CPCeP (n=6) treated hearts 12-weeks post injection (mean ± SEM). (**B**) Quantitation of Tropomyosin+ eGFP+ cells in CPCe (n=6) and CPCeP (n=6) animals (mean ± SEM). (**C-E**) Representative immunostaining for colocalization of eGFP (green) and cardiac myocytes (tropomyosin, red) and connexin-43 (white) (**C**), endothelial cells (vWF, red) (**D**), and smooth muscle cells (SMA, red) (**E**) in vehicle, CPCe, and CPCeP treated hearts 12-weeks post-intramyocardial injection. Enlarged areas are represented with indicated boxes. Scale bars represent 40mm. (**F**) Quantitation of eGFP+ population in CPCe (n=6) and CPCeP (n=6) for cardiac lineage markers.


A
% LV Free Wall Infarcted
ns
p<.05
n=5
n=6
n=6
Vehicle
CPCe
CPCeP
B
% Tropomyosin+ GFP+
p<.001
n=6
n=6
CPCe
CPCeP
C
Cardiac
D
Endothelial
E
Smooth Muscle
Vehicle
CPCe
CPCeP
Connexin 43
GFP
Tropomyosin
Topro
vWF
SMA
F
% of GFP+ cells + for:
CPCe (n=6)
CPCeP (n=6)
p<.01
p<.01
ns
vWF
SMA
Tropomyosin

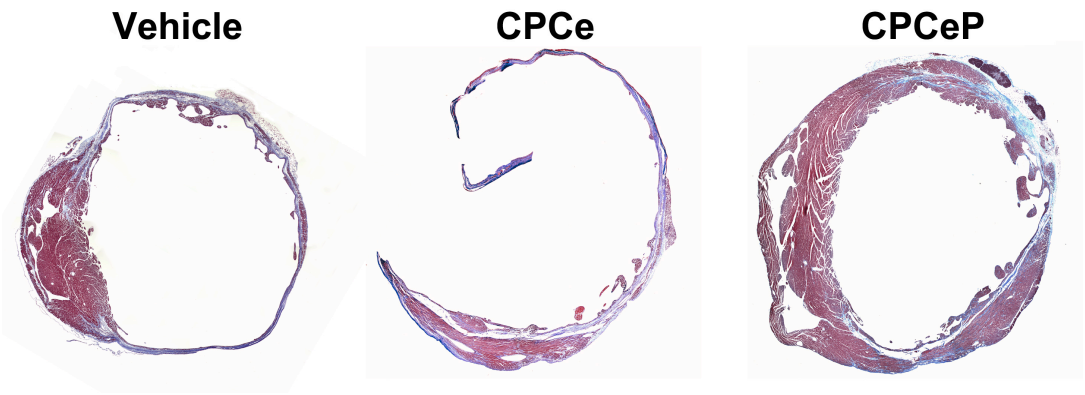


Figure 2.10: Decreased fibrosis in heart of CPCeP treated animals

Representative images of Masson's Trichrome staining in hearts from CPC, CPCe, and CPCeP injected mice.

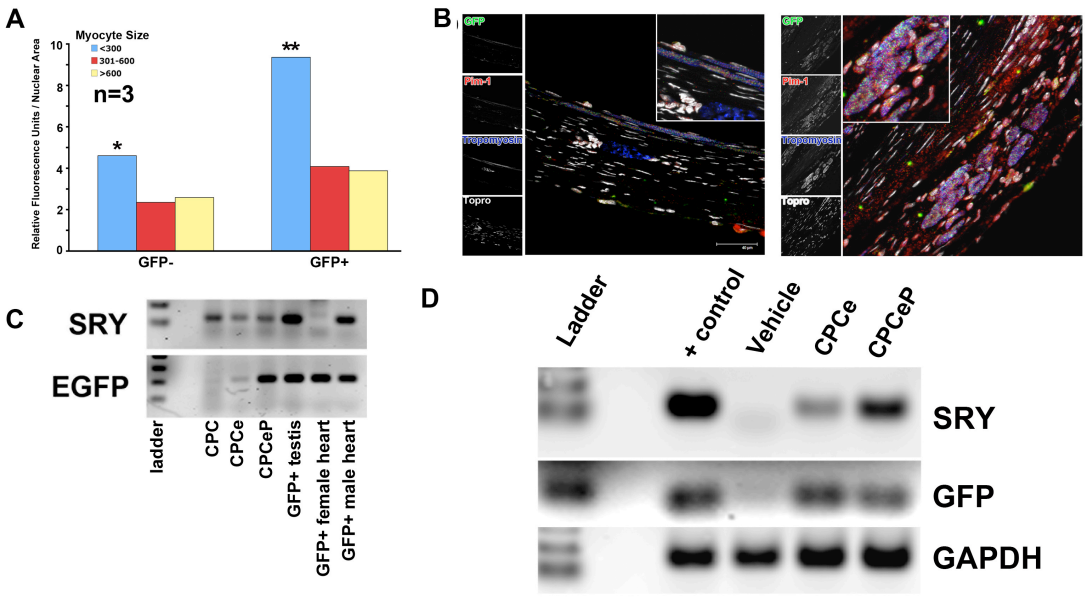


Figure 2.11: Preserved telomere length in CPCeP and detection of male SRY gene

(**A**) Quantitation of telomeric length in small to large border zone myocytes in the hearts of CPCeP injected mice 12-weeks post infarction (mean ± SEM, n=3). (**B**) Myocytes (tropomyosin, blue) immunolabeled for Pim-1 (4), eGFP (green), and nuclei (white) in CPCe and CPCeP treated hearts 12-weeks post infarction. (*p<.05, **p<.01). (**C**) PCR of genomic DNA of lentivirally infected cells, CPCe and CPCeP, to confirm male origin. (**D**) PCR of genomic DNA detecting SRY and eGFP from excised infarct region in CPCe and CPCeP injected hearts 12-weeks post infarction. Scale bars represent 40mm.

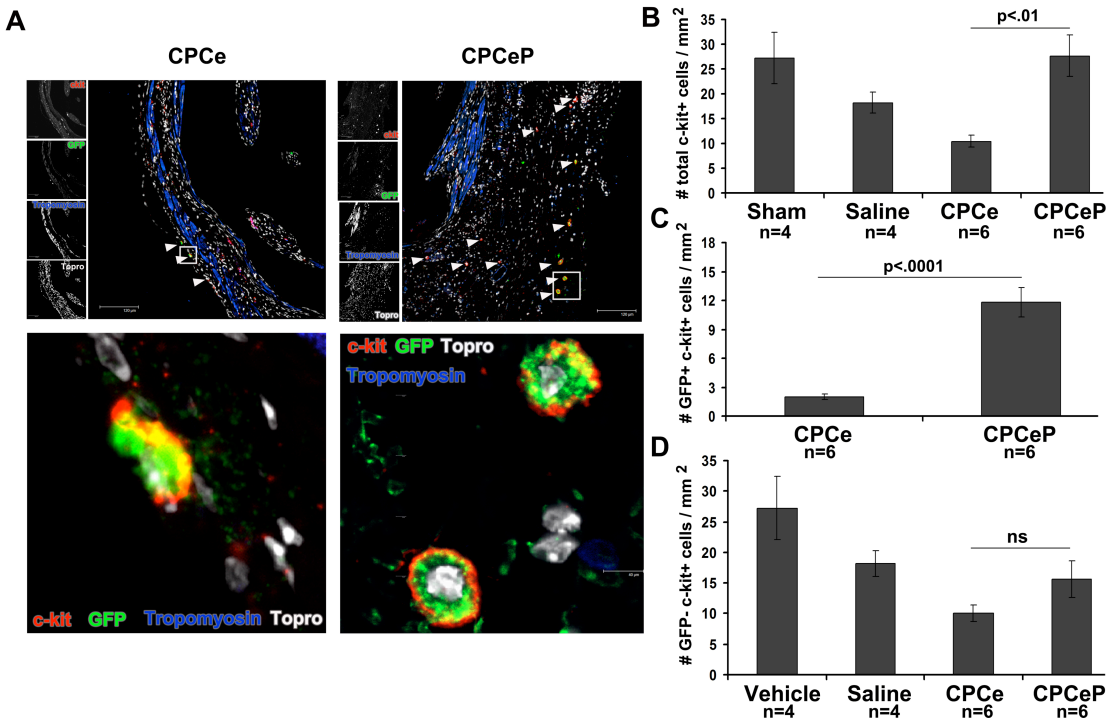


Figure 2.12: CPCeP treated hearts have increased numbers of c-kit+ cells 12-weeks post intramyocardial injection

(**A**) Immunostaining for c-kit and eGFP (green) in heart sections from mice treated with CPCe (n=6) or CPCeP (n=6) 12-weeks post-intramyocardial injection. Enlarged areas are represented with indicated boxes. (**B**) Quantitation of the number of total, (**C**) eGFP+, and (**D**) eGFP- c-kit+ cells in hearts of mice injected with CPCe (n=6) or CPCeP (n=6) (mean ± SEM). Scale bars represent 120mm in top panels.

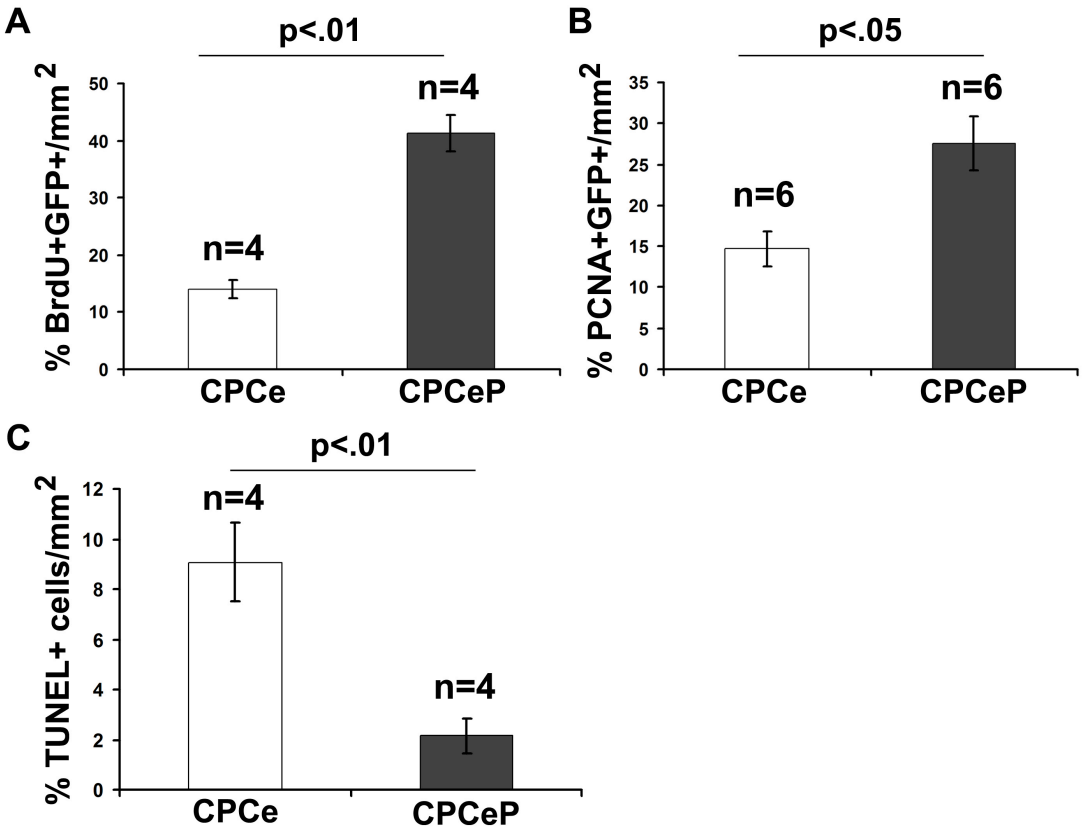


Figure 2.13: Increased proliferation of CPCeP *in-vivo*

(**A**) Quantitation of BrdU+ GFP+ colocalization in CPCe (n=4) and CPCeP (n=4) injected animals at 5 weeks post infarction and CPC injection. (**B**) Quantitation of PCNA and GFP colocalization in CPCe (n=6) and CPCeP (n=6) injected animals 12 weeks post infarction and CPC injection. (**C**) Quantitation of TUNEL+ GFP+ cells in CPCe (n=4) and CPCeP (n=4) injected animals at 5 weeks post infarction and CPC injection.

Figure 2.14: Long-term persistent cardiac functional recovery in animals treated with CPCeP

(**A-C**) Electrocardiographic assessment of FS (**A**), EF (**B**), and AWD (**C**), in sham (■, orange, n=6), vehicle (●, black, n=9), CPCe (▲, blue, n=9), and CPCeP (♦, green, n=6), 32-weeks post-infarction (mean ± SEM). Statistics for each time point are provided in Table S1. (**D-F**) Cardiac function of sham (n=5), vehicle (n=5), CPCe (n=5), and CPCeP (n=5) were evaluated using *in vivo* hemodynamic measurements of LVEDP (**D**), LVDP (**E**), and dP/dT (**F**), 32-weeks post-intramyocardial injection (mean ± SEM). (**G**) Heart weight: body weight ratios. Statistically significant p<.05 (ANOVA). (**H**) Wall stress assessment comparing ratio of left ventricular diameter (r) to wall thickness (h) from sham (n=6), vehicle (n=9), CPCe (n=9), and CPCeP (n=6) treated animals (mean ± SEM). ϕp<.05, ϕϕp<.01, ϕϕϕp<.001 compared to sham; #p<.05, ##p<.01, ###p<.001 compared to vehicle, * p<.05, **p<.01, ***p<.001 compared to CPCe. Echocardiography significant (p<.05) by two-way repeated measures ANOVA.

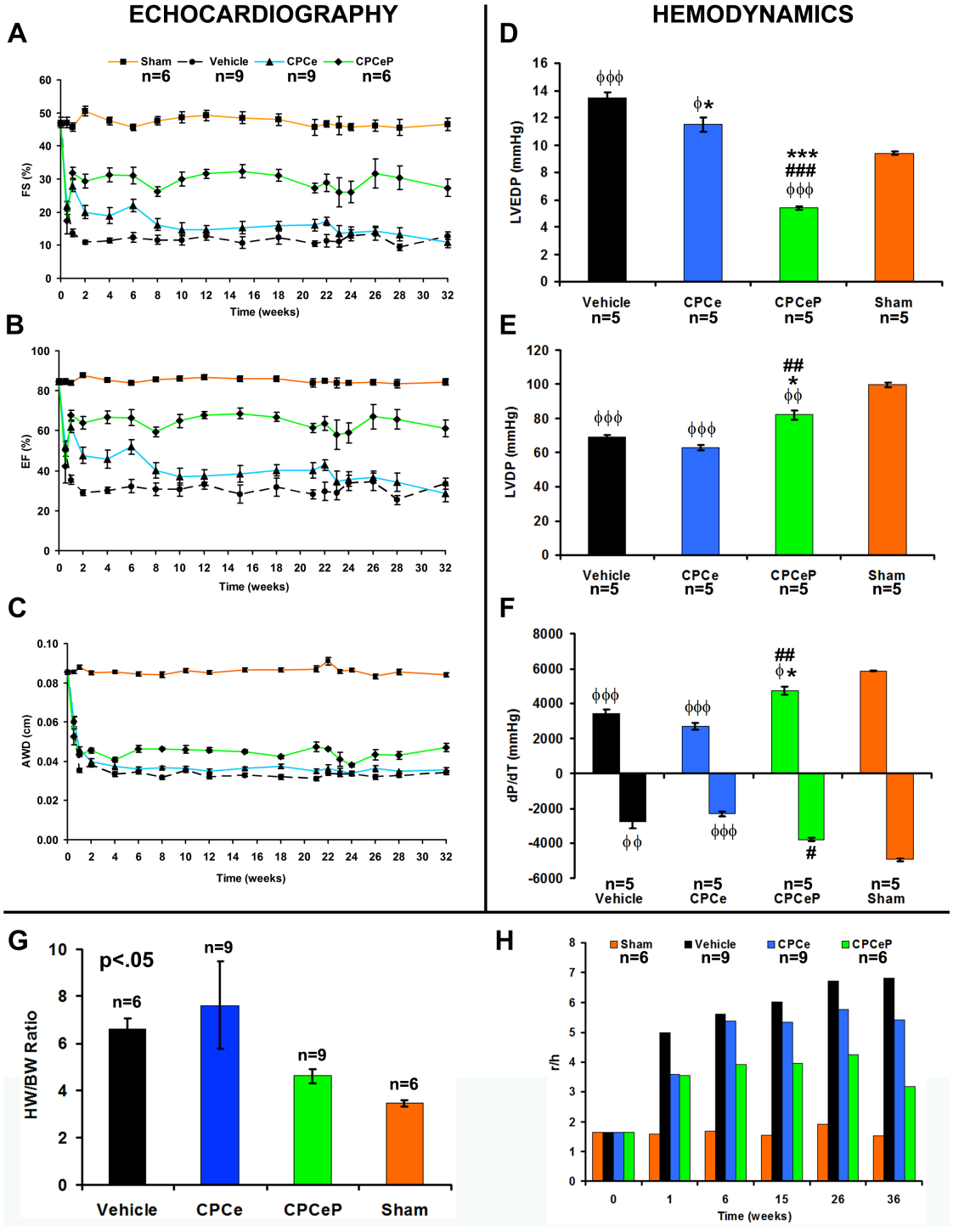

ECHOCARDIOGRAPHY
HEMODYNAMICS
A
Sham n=6
Vehicle n=9
CPCe n=9
CPCeP n=6
FS (%)
Time (weeks)
B
EF (%)
Time (weeks)
C
AWD (cm)
Time (weeks)
D
LVEDP (mmHg)
Vehicle n=5
CPCe n=5
CPCeP n=5
Sham n=5
E
LVDP (mmHg)
Vehicle n=5
CPCe n=5
CPCeP n=5
Sham n=5
F
dP/dT (mmHg)
n=5 Vehicle
n=5 CPCe
n=5 CPCeP
n=5 Sham
G
p<.05
n=6
n=9
n=9
n=6
HW/BW Ratio
Vehicle
CPCe
CPCeP
Sham
H
Sham n=6
Vehicle n=9
CPCe n=9
CPCeP n=6
r/h
Time (weeks)

Figure 2.15: Persistent engraftment and differentiation of Pim-1 expressing CPCs 32-weeks post intramyocardial injection

(**A**) Infarct size measurement, (**B**) quantitation of Tropomyosin+ eGFP+ cells, (**C**) and confocal micrographs of hearts injected with CPCe (n=3) or CPCeP (n=3) 32-weeks post-infarction. (mean ± SEM). Sections were stained for eGFP (green), Tropomyosin (4), c-kit (white), and nuclear stain Topro-3-iodide (blue). Quantitation of total (**D**), eGFP+ (**E**), and eGFP- (**F**) c-kit+ cells in heart sections from mice treated with CPCe (n=3) or CPCeP (n=3) 32-weeks post-infarction. (**G**) Immunolabeling with eGFP (green), c-kit (4), tropomyosin (blue), and nuclear stain Topro-3-iodide (white). (**H**) Quantitation of total number of vessels, (**I**) small vessels and (**J**) immunolabeling for vasculature in hearts from mice treated with CPCe (n=3) or CPCeP (n=3) 32-weeks post-infarction. Sections were stained with eGFP (green), smooth muscle actin (4), tropomyosin (blue), and nuclear stain Topro-3-iodide (white). Mean ± SEM, n=3, * $p<.05$, ** $p<.01$, *** $p<.001$. Scale bars represent 50mm.

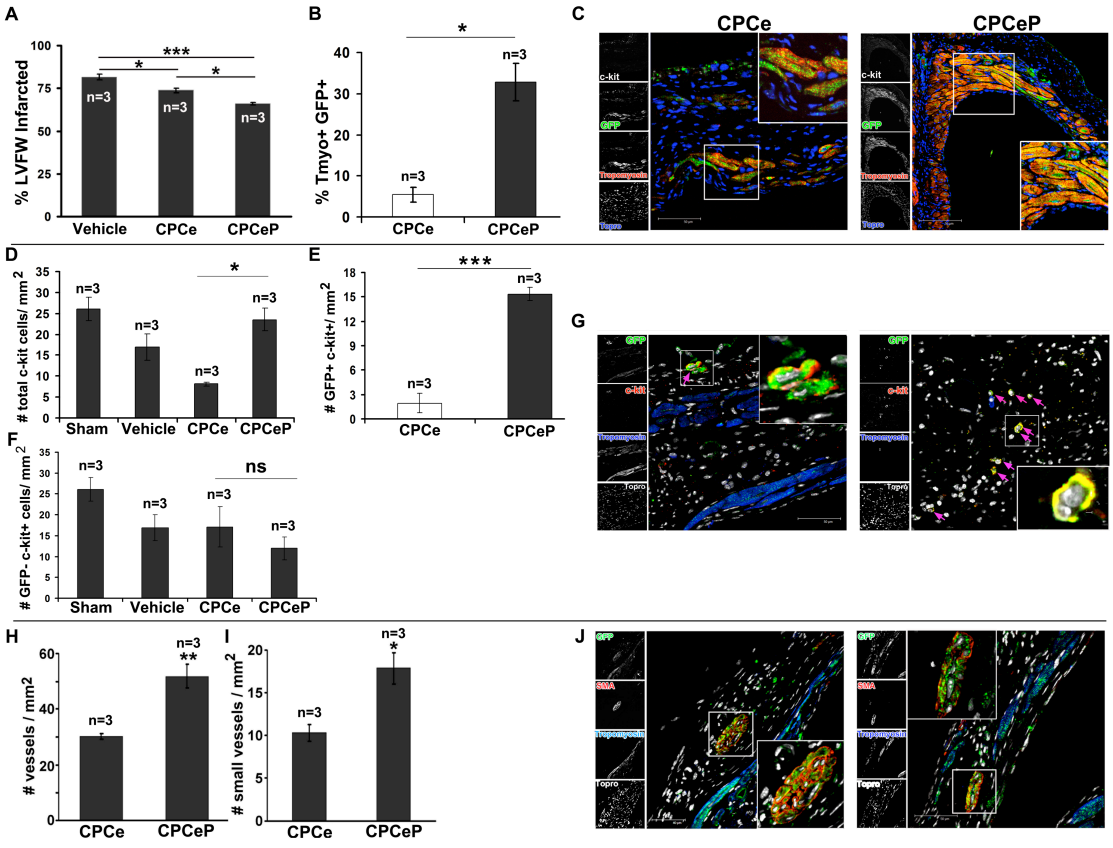

A
% LVFW Infarcted
Vehicle
CPCe
CPCeP
B
% Tmyo+ GFP+
C
CPCe
CPCeP
c-kit
GFP
Troponyosin
Topro
D
total c-kit cells/ mm2
Sham
E
GFP+ c-kit+/ mm2
F
GFP- c-kit+ cells/ mm2
ns
G
SMA
H
vessels / mm2
I
small vessels / mm2
J
n=3

Table 2.1: Immunohistochemisty Antibody Table

Primary Ab	Species	Dilution	Amplification	Manufacturer
c-kit	Goat	1:40	Yes	R&D Systems
Pim-1	Rabbit	1:500	Yes	CST
Tropomyosin	Mouse	1:75	No	Sigma
GFP	Rabbit	1:500	Yes	Molecular Probes
vWF	Rabbit	1:100	No	Sigma
Mef2C	Rabbit	1:100	No	Invitrogen
Connexin 43	Rabbit	1:100	Yes	Sigma
SMA	Rabbit	1:300	No	Inviva

Table 2.2: Thirty-two week echocardiography statistical analysis

Fractional Shortening	3 days	7 days	2 weeks	4 weeks	6 week	8 week	10 week	12 week	15 week	18 week	21 week	22 week	23 week	24 week	26 week	28 week	32 week
Sham vs Vehicle	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCe	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCeP	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P < 0.001
Vehicle vs CPCe	P > 0.05	P < 0.001	P < 0.001	P < 0.05	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Vehicle vs CPCeP	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P < 0.001	P < 0.001	P < 0.01
CPCe vs CPCeP	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.05	P < 0.01	P < 0.001	P < 0.001	P < 0.001
Ejection Fraction																	
Sham vs Vehicle	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCe	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCeP	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P < 0.01	P < 0.01	P < 0.05	P < 0.05	P < 0.01	P < 0.05	P > 0.05	P < 0.001
Vehicle vs CPCe	P > 0.05	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Vehicle vs CPCeP	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P < 0.001	P < 0.001	P < 0.001
CPCe vs CPCeP	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.05	P < 0.01	P < 0.001	P < 0.001	P < 0.001
Anterior Wall Dimension																	
Sham vs Vehicle	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCe	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Sham vs CPCeP	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Vehicle vs CPCe	P > 0.05	P < 0.001	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P > 0.05	P > 0.05	P < 0.05	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Vehicle vs CPCeP	P > 0.05	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P < 0.05	P < 0.01	P < 0.001	P < 0.001
CPCe vs CPCeP	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.01	P > 0.05	P < 0.05	P > 0.05	P < 0.01	P < 0.001

CHAPTER III:

Pim-1 Localization Influences Cellular Survival and Proliferation

INTRODUCTION

The serine threonine kinase Pim-1 is well known as a potent oncogene (66, 101, 123-126), stimulating pro-proliferative and anti-apoptotic effects upon a variety of cell populations (66, 68, 71, 72, 101, 122, 127). Similarly, overexpression of Pim-1 in the heart is also pro-proliferative and anti-apoptotic and promotes cardiac progenitor cell cycling (95, 127), expression of anti-apoptotic proteins (64, 65), and protects the heart from pathological damage (64, 65, 95). It remains unknown however, how Pim-1 governs these two distinct aspects of cardioprotection.

Previous studies by our group have demonstrated Pim-1 protein is highly expressed in the neonatal mouse heart, declining as the mice progress into adulthood (65). Additionally, Pim-1 protein expression in the heart is reactivated after pathological injury such as myocardial infarction (MI) or pressure overload. Interestingly, the localization of Pim-1 expression during these periods of activation is strikingly different (65). During periods of cardiac growth, Pim-1 expression is primarily nuclear in myocytes, potentially regulating cell cycle proteins previously demonstrated to interact with Pim-1, including p27 (73), p21 (71, 72), c-myc (69), and NUMA (70). In contrast, after pathological injury, Pim-1 expression is largely cytoplasmic, where it may be preventing cell death through interactions with Bcl2, BAD (65, 122), and/or Bcl-xl (128). Recently is has been demonstrated cardiac specific overexpression of Pim-1 kinase provided protective benefits to the heart by inhibiting oxidative stress induced cell death by preventing mitochondrial depolarization and

release of cytochrome c (109). These initial studies and observations led to the hypothesis that different stimuli may effect the localization of Pim-1, prompting the cell to proliferate or to stimulate survival signaling in order to abrogate cell death. Taken together, we believe that nuclear localization of Pim-1 kinase promotes proliferation while mitochondrial localization of Pim-1 inhibits cell death.

Herein, this chapter focuses on the design of viral vectors in order to investigate the proliferative and protective effects garnered by constructs delivering Pim-1 kinase to the nuclear or mitochondrial compartment of the cell. Understanding the mechanisms of how Pim-1 specifically influences cell survival and proliferation will provide strategies to safely improve upon stem cell mediated cardiac regeneration.

METHODS

Adenoviral vectors and generation of adenovirus

Adenoviral constructs were designed beginning from specific constructs to deliver the gene of interest to the nucleus, pCMV/myc/nuc (Invitrogen, catalog #V82120), or the mitochondria, pCMV/myc/mito (Invitrogen, catalog #V82220). Human Pim-1 cDNA was cloned into specific targeting vectors, after which the construct was removed through restriction digest and cloned into the pShuttle adenoviral backbone (Stratagene, catalog #240010). Subsequent plasmids were treated as per the AdEasy XL Adenoviral Vector System protocol to generate adenovirus. Adenovirus was amplified, purified by cesium chloride gradient, and titer determined by plaque assay.

Transfections

Cardiac progenitor cells (CPCs) or 293Ts were plated in six well dishes at 50,000 cells per well. The next day Fugene Reagent (Roche) was used to deliver 1ug of DNA to cells. Cells were harvested for western blot 48 hours later.

Cardiac Progenitor Cell Isolation, Cell culture, and Adenoviral Infection

CPCs were isolated from 10-12 week old male FVB mice and cultured in cardiac stem cell (CSC) media: DMEM F-12, 10% FBS, 1% PSG, 1X ITS (Lonza, 17-838Z), .4mg/ml EGF (Sigma, E9644), .02ng/ml bFGF (Peprotech, 100-18B), and 1000U/ml LIF (Chemicon #ESG1107). CPCs were plated in 6-well bottom plates at 50,000 cells per well and transduced with adenovirus (NLS or mito

constructs) at an MOI of 50 for 2 hours. CPCs were washed 18 hours later and fresh CSC media was added. Cells were expanded and analyzed by flow cytometry to determine the percentage of GFP+ cells.

Neonatal Rat Ventricular Cardiomyocyte Cell culture and Adenoviral Infection

NRCMs were plated on permanox coated slides at 70,000 per well in M199 media containing 10% FBS and 1% PSG. Cells were washed twice with PBS and infected with adenovirus (NLS or mitochondrial constructs) at an MOI of 50 for 2 hours, washed and replaced with fresh media. Forty-eight hours later cells were analyzed by flow cytometry to determine percentage of GFP+ cells.

Apoptotic treatments, Annexin V/ 7AAD analysis and TUNEL Assay

NRVCM or CPC cultures were infected with NLS or mitochondrial adenoviral constructs and treated 36 hours later with 250nM Staurosporin for 40 hours to induce cell death. Samples were then analyzed by flow cytometry after being stained with Annexin V and 7AAD (BD Pharmingen) to determine the percentage of early and late apoptotic cells. TUNEL assay was performed on cells treated with and without 250nM Staurosporin as per manufacturer's protocol (TMR Red TUNEL assay, Roche) to determine percentage of TUNEL positive cells.

CyQuant and MTT Assay

GFP-NLS, Pim1-NLS, mito-GFP, and mito-Pim1 infected CPCs were plated in quadruplicate (3500 cells/well) in 96 well flat bottom plates. CyQuant reagent

was added to the media at day 1 and day 3 as per manufactures protocol. Reagent was incubated for 45 minutes and read on a spectrophotometer at 530nm on day one, three, and five. For MTT assay, infected cells were plated the same as for CyQuant assay, however MTT reagent was added (1.2mM final concentration), incubated for 4 hours, after which stopping reagent was added (10% SDS +.015M HCl). Plates were read on a spectrophotometer at 595nM on day one, three and five.

Confocal Microscopy

NRVCMs were plated at 70,000 cells per well and CPCs plated at 25,000 cells per well on permanox coated plastic slides. Cells were fixed post-treatment in 4% PFA, washed twice in 1X PBS and blocked for one hour in 10% horse serum. Primary antibodies were incubated overnight at 4°C at appropriate dilutions. Slides were washed in 1X PBS followed by secondary antibody incubation, 2 hours at room temperature.

Statistics

Statistics were calculated using SPSS software. One-way ANOVA and two-way repeated measures ANOVA for echocardiography with Tukey's post-hoc test were calculated. Student's T test was used where appropriate. Values with $p<.05$ were considered statistically significant.

RESULTS

Design of Nuclear and Mitochondrial Pim-1 Constructs

Nuclear and mitochondrial targeted adenoviral constructs were designed to deliver GFP or GFP fused to Pim-1 kinase, to neonatal rat ventricular cardiomyocytes (NRVCMs) or cardiac progenitor cells (CPCs) (Figure 3.1A). All constructs included a fused myc-tag to facilitate identification in future studies. Transient transfections were performed on CPCs to demonstrate protein expression of fused products of nuclear-targeted GFP (GFP-NLS), nuclear-targeted Pim-1 (Pim1-NLS), mitochondrial-targeted GFP (mito-GFP), and mitochondrial-targeted Pim-1 (mito-Pim1) constructs. GFP-NLS and Pim1-NLS CPCs expressed myc-tag and GFP proteins, while only Pim1-NLS CPCs overexpressesed Pim-1 protein (Figure 3.1B, left). Similar results were obtained with mito-GFP and mito-Pim1 CPCs, whereby both constructs expressed myc-tag and GFP proteins, while Pim-1 protein was only overexpressed in mito-Pim1 transfected CPCs (Figure 3.1B). Further confirmation of proper protein expression of fused gene products was demonstrated by fluorescence microscopy analysis of transfected CPCs. GFP expression in NLS-GFP and NLS-Pim1CPCs was nuclear (Figure 3.1C, middle panel) while mito-GFP and mito-Pim1 expressing CPCs expressed GFP in the cytosol (Figure 3.1C, right panel). Wild type GFP and wild type Pim-1 were provided as a comparison (Figure 3.1C, left panel).

Expression and Localization of Mitochondrial Pim-1 and Nuclear Pim-1

Proper localization of mito-Pim1 and Pim1-NLS in adenoviral constructs was determined by immunocytofluorescence (ICF). Mito-GFP and mito-Pim1 infected NRVCMs and CPCs demonstrated precise co-localization of GFP and the mitochondrial specific protein Tom20 (Figure 3.2A and B, top panels). Similarly, GFP-NLS and Pim1-NLS infected NRVCMs and CPCs demonstrated co-localization of GFP protein with the nuclear marker Topro-3-iodide (Figure 3.2A and B, bottom panels).

Mitochondrial Pim-1 attenuates STS induced cell death in CPCs

Mito-Pim1 expressing CPCs attenuate staurosporine (STS) induced cell death when compared to STS treated Pim-NLS CPCs and non-infected control CPCs (Figure 3.3). While mito-Pim1 expressing CPCs abrogate cell death to a greater extent, both mito-Pim1 and Pim1-NLS expressing CPCs exhibit decreased expression of Annexin V and 7AAD (Figure 3.3A) compared to uninfected controls treated with STS.

Nuclear localized Pim-1 Influences Cell Proliferation

Nuclear localized Pim-1 was expressed in CPCs and had significantly reduced metabolic activity compared to control and mitochondrial Pim-1 infected CPCs at day three (Figure 3.4). Interestingly, mitochondrial localized Pim-1 increased the rate of CPC metabolic activity compared to Pim1-NLS and Pim control at day three (Figure 3.4).

DISCUSSION

Over the last ten years regenerative medicine has offered up new hope for the treatment of heart failure. At the inception of regenerative medicine for the heart, it appeared only modest improvements could be gained by delivering stem cells after pathological damage. However, consistent observations began to emerge, highlighting fundamental problems that had to be surmounted in order to provide therapeutically relevant improvement in cardiac structure and function. One primary goal focused on improving the proliferation and survival of the adoptively transferred stem cell pool within the damaged myocardium. Addressing this concern led the field to explore genetic modification of stem cells with pro-survival genes. A substantial number of studies now document improved cardiac performance after delivery of stem cells modified with cardioprotective genes compared to unmodified cells (30, 46, 49, 58, 60, 62, 95, 129). Protective benefits provided by genetically enhanced stem cells arise from protective paracrine signaling (55, 57, 59, 130) as well differentiation and integration of the genetically modified stem cells to into the surviving myocardium (4, 63, 95). Enhancing the ability of progenitor cells to survive and proliferate often requires overexpressing genes that may interfere with mechanisms governing normal cell cycle and survival. Thus a precise understanding of how cardioprotective genes, such as Pim-1, increase survival and proliferation of progenitor cells is critical to the safety of regenerative therapies.

Previous studies demonstrate Pim-1 has distinct localization patterns within the heart depending on age or pathological status. Nuclear localization of Pim-1 was observed in young hearts, while perinuclear and cytosolic localization was observed after TAC banding or infarction. In support of this last point, a recent publication demonstrated transgenic animals overexpressing cardiac specific Pim-1 protected the heart by attenuating mitochondrial depolarization and release of cytochrome c. Taken together these data support the idea that Pim-1 enhances proliferation when nuclear and is inhibits cell death when localized to the mitochondria. To this end, the current chapter provides a system that allows for the separate investigation of survival and proliferation pathways influenced by Pim-1.

Adenoviral constructs were used to successfully deliver Pim-1 to the nucleus or mitochondria of both CPCs and NRVCMs (Figure 3.1 and 3.2). Preliminary in-vitro data suggest mitochondrial Pim-1 transduced CPCs inhibit STS induced cell death compared to Pim-1-NLS and non-infected controls (Figure 3.3A).

Cell proliferation was evaluated using an MTT assay to assess metabolic activity. These results were at odds with our hypothesis in that Pim1-NLS CPCs had significantly lower MTT activity compared to mito-Pim1 CPCs. Although MTT is often used as an assay to assess cell number, the assay measures mitochondrial reductase enzyme and therefore may not accurately reflect cell proliferation. Further experiments are planned to more accurately test cell proliferation. Interestingly, we also observed Pim1-NLS, at low MOI, appears to

be localized primarily in the nucleolar compartment. These results support previous studies by our group indicating that nucleostemin, a nucleolar protein involved in promoting proliferation, is significantly elevated in Pim-1 transgenic mice. Further studies are planned to investigate the interaction and significance of Pim-1 at the nucleolar compartment.

Our preliminary data also demonstrate that adenoviral infection efficiencies are relatively low, resulting in approximately 30% GFP positive population as indicated by FACS analysis (data not shown). Given the low transduction efficiency our results may underestimate the effects of nuclear or mitochondrial targeted Pim-1. Future studies include the design of lentiviral constructs to deliver Pim-1 to the nucleus or mitochondria in order to make stable lines. Stable lines facilitate future experiments allowing for the selection of a more pure population. Additionally these new vectors will incorporate the use of a bicistronic vector in order to more accurately reflect protein conformation *in-vivo*. The current adenoviral vectors are fusion proteins of Pim-1 with GFP as well as a myc-tag. The size of this fusion protein may inhibit proper expression of the protein, proper folding, and/or activity. Although future experiments are planned to confirm downstream targets are properly phosphorylated by Pim-1 fusion constructs, it is likely that vectors more closely representing endogenous Pim-1 conformation will produce more accurate results.

Taken together, the results presented here provide a platform on which to base future experiments assessing the effect of Pim-1 localization on

proliferation and cell survival. Preliminary data suggest Pim-1 influences these pathways but mechanistically remains to be elucidated.

FIGURES

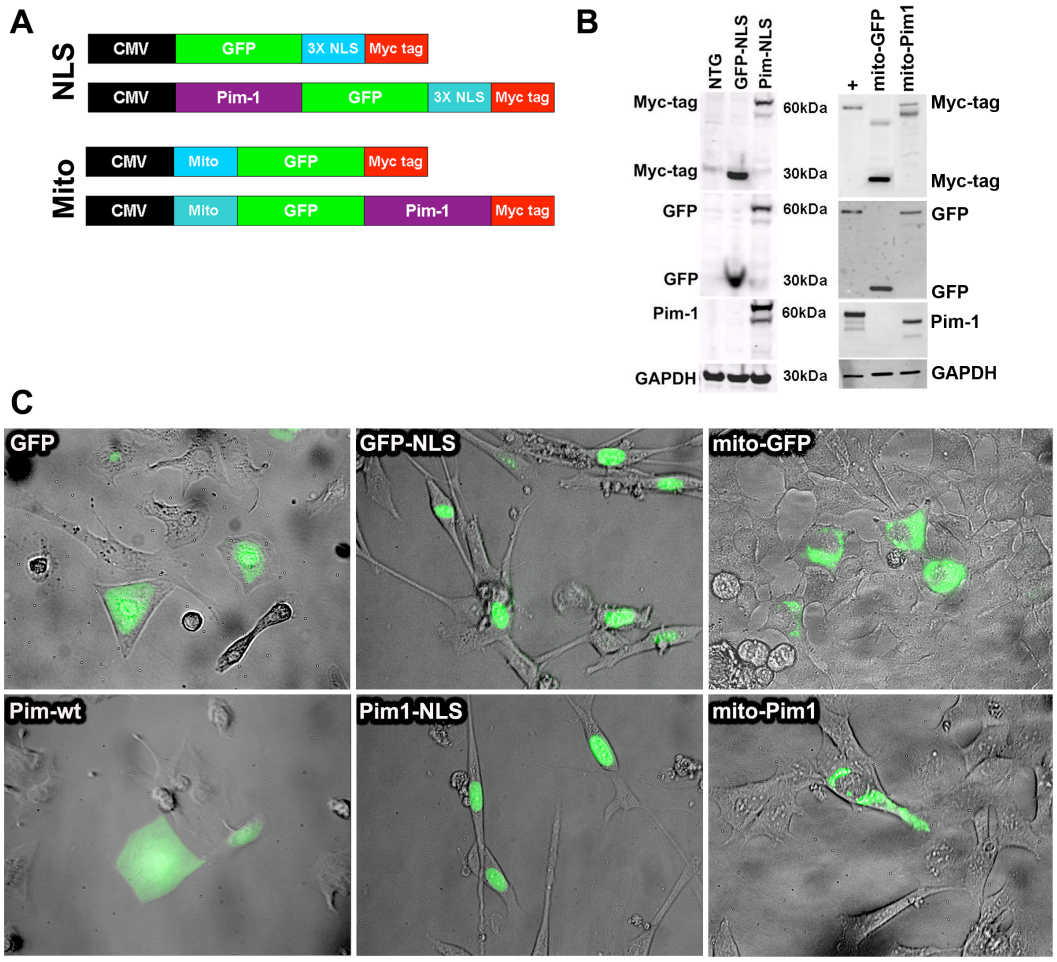
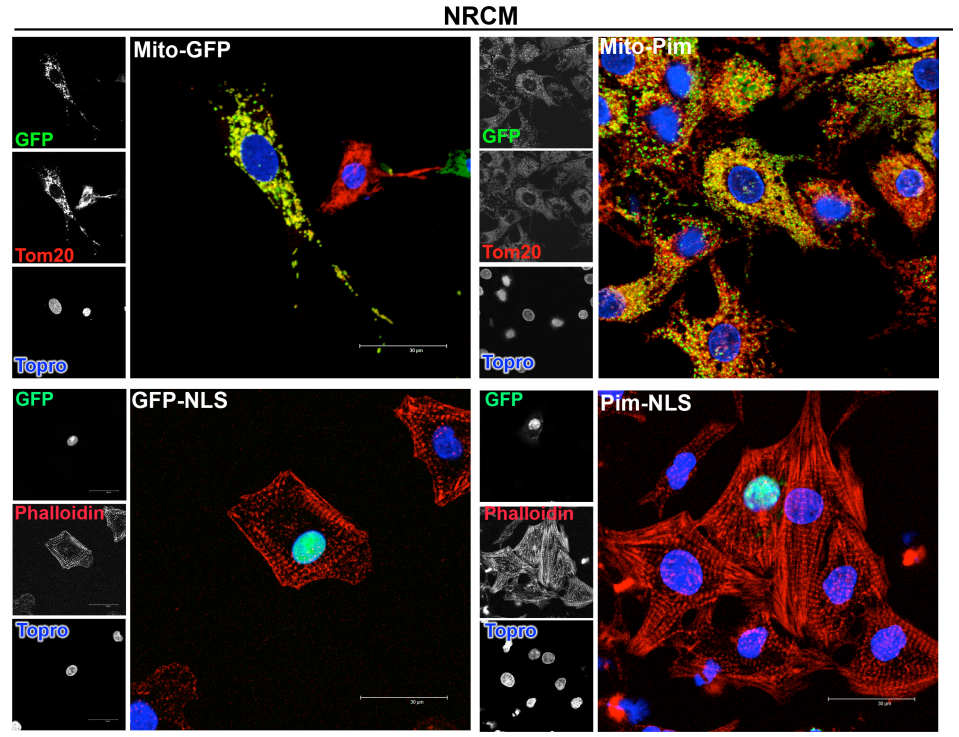


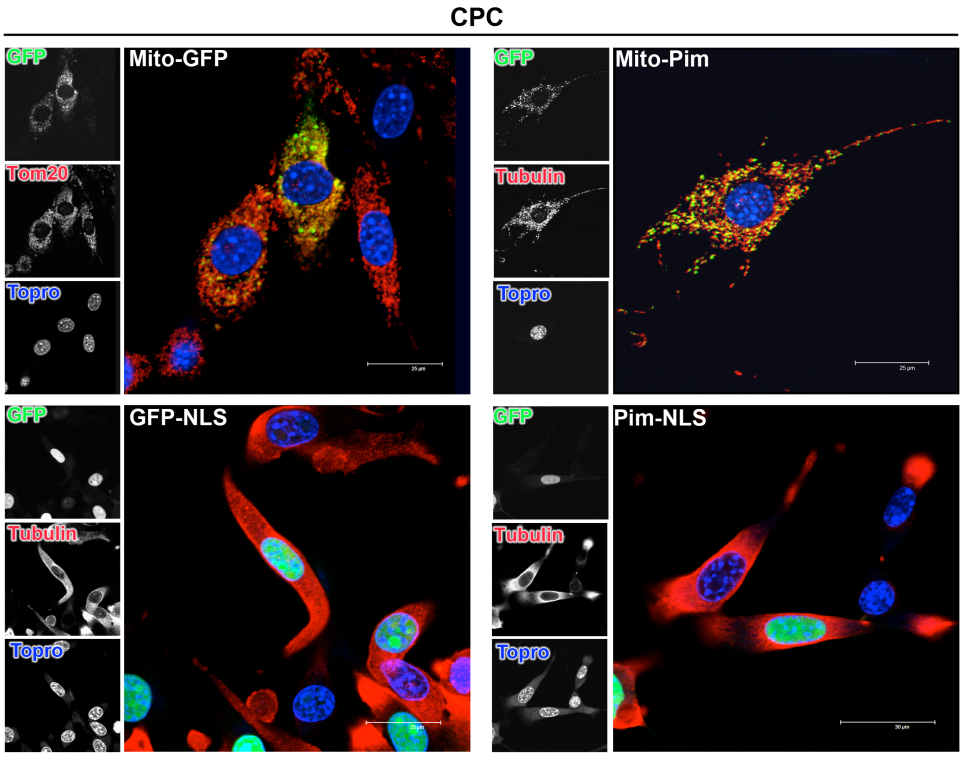
Figure 3.1: Nuclear and Mitochondrial Targeted Pim-1 Constructs

(**A**) Schematic of nuclear-targeted GFP (GFP-NLS) and Nuclear Pim-1 (Pim1-NLS) as well as mitochondrial-targeted GFP (mito-GFP) and mitochondrial Pim-1 (mito-Pim1). (**B**) Immunoblot analysis of CPCs transfected with GFP-NLS, Pim1-NLS, or mito-GFP and mito-Pim1 constructs. (**C**) Immunocytofluorescence demonstrating localization of fused constructs in wild type GFP and Pim-1 (left panels), GFP-NLS and Pim1-NLS (middle panels), or mito-GFP and mito-Pim1 (right panels).

Figure 3.2: NRVCMs and CPCs Infected with Mitochondrial Targeted or Nuclear Targeted Pim-1 Adenoviral Constructs

(**A**) NRVCMs and (**B**) CPCs were infected with adenoviral constructs expressing mito-GFP or mito-Pim1 and GFP-NLS or GFP-Pim1. GFP (green) expression was co-localized with either the mitochondrial specific marker Tom20 (red) or the nuclear DNA stain Topro-3-iodide (blue).


NRCM
GFP
Tom20
Topro
Mito-GFP
GFP
Tom20
Topro
Mito-Pim
GFP
Phalloidin
Topro
GFP-NLS
GFP
Phalloidin
Topro
Pim-NLS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Tom20
Topro
Mito-GFP
GFP
Tubulin
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Tubulin
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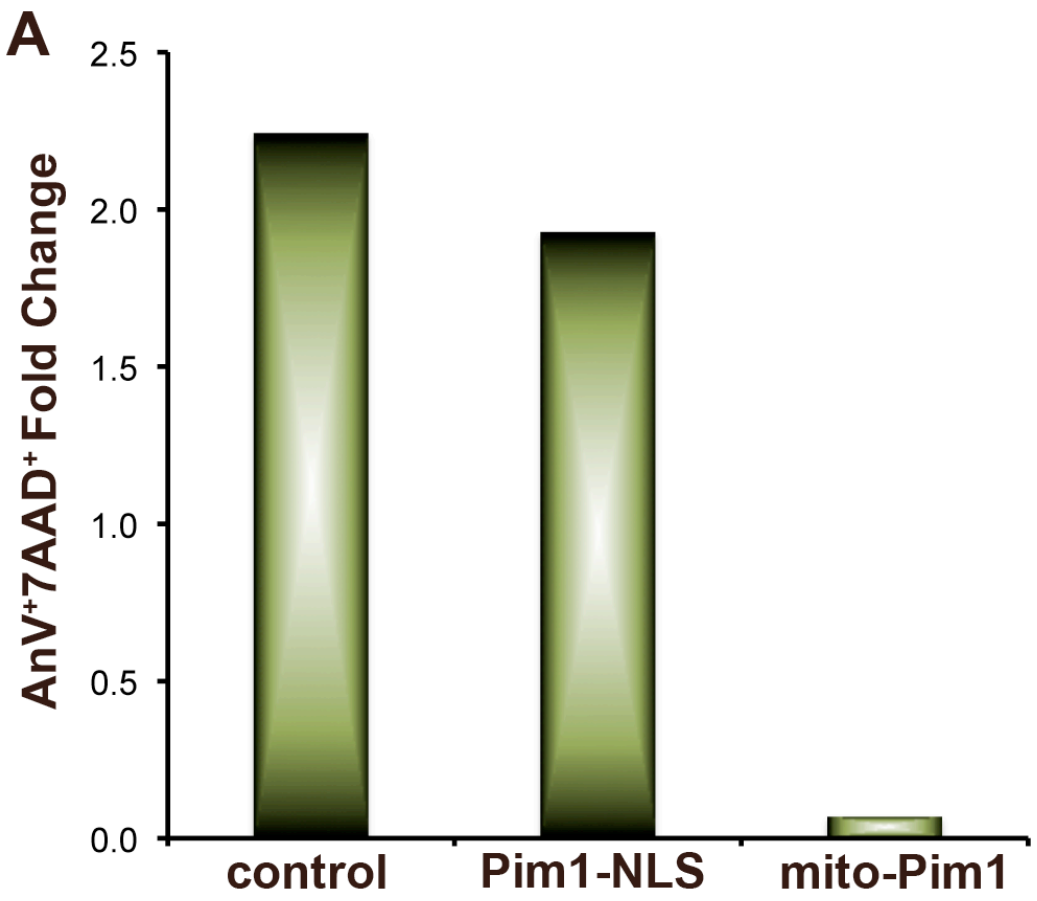

Figure 3.3: Mitochondrial Pim-1 attenuates STS induced cell death in CPCs

(**A**) Control, Pim1-NLS, and mito-Pim1 CPCs were treated with 250nM of STS for 40 hours and analyzed by flow cytometry to determine the number of Annexin V+, 7AAD+ cells. Data are expressed as fold change compared to respective controls treated with STS (n=1).

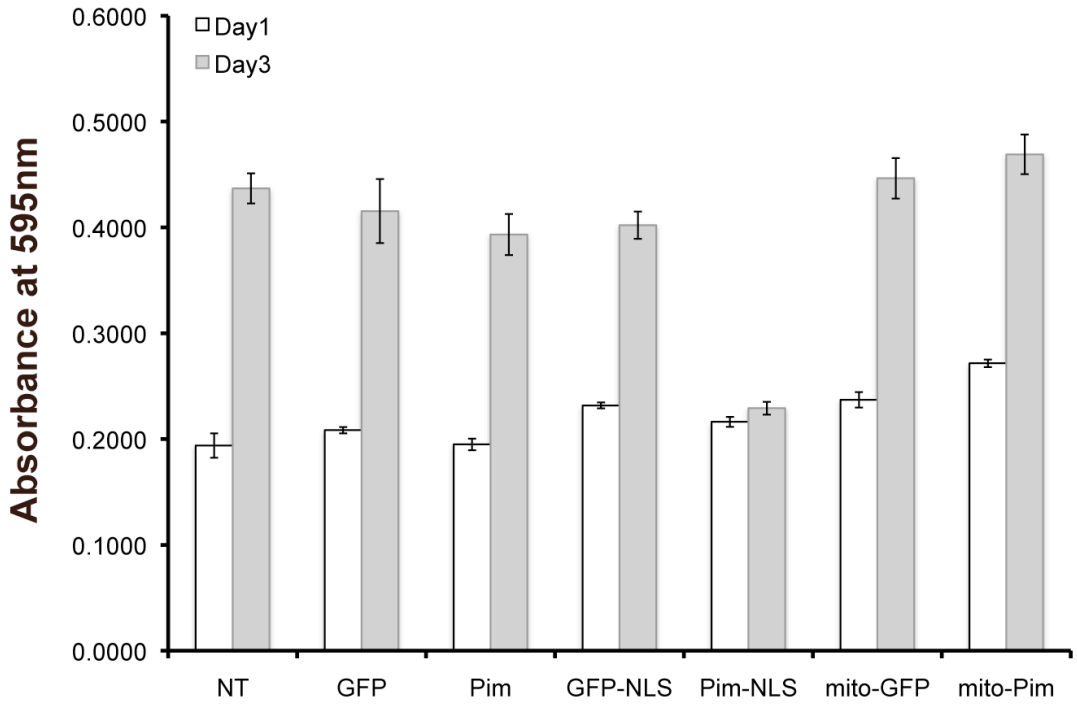

Figure 3.4: CPCs expressing Nuclear Pim-1 abrogate cell proliferation

CPCs expressing nuclear Pim-1 and mitochondrial Pim-1 were plated and incubated with MTT reagent for 4 hours at Day 1 and Day 3. Samples were compared to appropriate controls (nuclear GFP, mitochondrial GFP, GFP and Pim-1).

CONCLUSION OF THE DISSERTATION

A revolution in the way we currently treat heart disease has begun, spurred on by the unmet need for cardiac repair and regeneration after pathological insult. While initial results from adoptively transferred stem cells are hopeful, optimism is stymied by the modest improvements routinely seen in experimental studies. Although these findings offer hope for a novel modality of treatment, the benefits garnered through stem cell delivery still fall short of the improvements needed for complete and successful long-term cardiac functional recovery. Fortunately, the recurrent observation that the vast majority of adoptively transferred stem cells die or vanish shortly after delivery offers insight into how researchers may be able to improve current stem cell delivery strategies (Table 1). If indeed a minority of injected stem cells survive in pathological tissue after delivery, then conceivably modification of stem cell populations with cardioprotective genes would allow for increased survival, proliferation, and regeneration leading to dramatic increases in the efficacy of repair (Figure 1 and Chapter 2).

A majority of studies thus far seem to focus on a "one or the other", paracrine vs. engraftment, explanation for cardiac improvement. However, it seems more likely that improvements are mediated by a combination of these effects. It is feasible that early survival of injected stem cells relies on paracrine factors, while long-term improvements come from engraftment and differentiation of the adoptively transferred stem cell population.

Modification of CPCs with nuclear Akt or Pim-1 resulted in enhanced progenitor cell proliferation. These results are consistent with previous studies demonstrating the pro-proliferative effects of Akt and Pim-1 kinase. However, the success of treating damaged hearts with these genetically modified CPCs was strikingly different. Our data demonstrated CPCs modified to overexpress nuclear Akt resulted in enhanced proliferation, however abrogated lineage commitment *in vitro* and *in vivo*. Formation of de novo myocardium was not observed in hearts of animals that received CPCeA. *In vivo*, lack of lineage commitment inhibited the formation of both myocyte and vascular lineages needed to restore cardiac structure and function. Further evidence supporting overexpression of nuclear Akt repressed lineage commitment was demonstrated using an Akt inhibitor. When CPCeA were incubated with an Akt inhibitor and induced to differentiate, statistically significant increases in cardiac TnT transcript were observed. These results indicate CPC differentiation is sensitive to the amount of Akt expressed within the cellular system and that precise regulation of Akt may dictate whether the cell should proliferate or differentiate. Although these results may seem at odds with studies demonstrating nuclear Akt is cardioprotective, the majority of these studies investigate the cardioprotective effect within the myocyte population and not within the stem cell population. Overexpression of nuclear Akt in a non-proliferating cell type within the adult myocardium may impart only protective effects, as pro-proliferative effects may not be stimulated in a non-cycling population.

Interestingly, our data demonstrate CPCs modified with Pim-1 kinase, a downstream target of Akt, were able to mitigate cardiac damage. Long-term, 32-week improvements, in both myocardial structure and function, were observed when compared to unmodified cells. Hearts of animals that received Pim-1 modified CPCs had increased numbers of cycling CPCs, indicating enhanced proliferation *in vivo*. However the most striking difference between animals that received CPCeA and CPCeP was observed in the ability of CPCeP to terminally differentiate *in vivo*. Quantitative results demonstrated CPCeP were able to regenerate de novo myocardium, with a tendency towards forming vascular lineages over myocytes. Taken together these results demonstrate improving cardiac regeneration relies on enhancing survival and proliferation without inhibiting differentiation of the progenitor cells pool. Additionally, our results give supporting evidence that in order to effectively repair damaged myocardium, myocytes as well as vascular lineages must be generated.

The data provided in this thesis provides direct evidence demonstrating lentiviral gene delivery can enhance regenerative cellular therapy long term. Safety concerns regarding lentiviral vectors have arisen due to integration of the desired gene into areas of the genome by which a proto-oncogene can become abnormally regulated. To address this problem, targeted integration is being explored as an effective and safe way to deliver genes where integration will not result in aberrant activation of endogenous proto-oncogenic genes. Although safety regarding the use of genetically modified

stem cells currently remains a major issue of contention, successful treatment of a handful of diseases has been achieved through the delivery of genetically corrected stem cells. Some of the most promising studies have shown that delivery of a functional ADA gene can cure patients who suffer from severe combined immunodeficiency (SCID) (99, 129, 131-134). These results, albeit not in the cardiovascular system, provide hope for a novel treatment using genetic modification to deliver cardioprotective genes to stem cells, providing cells with an increased capacity to proliferate and survive in the damaged myocardium. Ultimately, appropriate viral vector selection largely relies on the target cells as well as the length of time of desired gene expression.

Many unanswered questions remain in the search for an effective stem cell therapy to treat cardiovascular disease. However, the data provided in this thesis advances the concept of *ex vivo* stem cell gene therapy to enhance cardiogenesis and demonstrates CPC commitment is essential to the regenerative response. In conclusion, our results provide direct evidence that enhancing stem cell survival without inhibiting lineage commitment allows for dramatic improvements in tissue regeneration. Our results provide a platform for future studies investigating pathways regulating differentiation of progenitor cells that may be able to further advance the concept of safe and effective *ex vivo* gene therapy.

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