GENETIC ENGINEERING OF BONE MARROW CELLS FOR THE
TREATMENT OF MYOCARDIAL INFARCTION

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DEDICATION

I dedicate this thesis to my family. My father Jaime, my sisters Jessica and Rebecca, my brother Thomas, and of course my lovely mother Laura. Your constant love and support has kept me on the right path, which is in the pursuit of happiness.
“If I can stop one heart from breaking.”

--Emily Dickinson
ABSTRACT OF THE THESIS

Genetic Engineering of Bone Marrow Cells for the Treatment of Myocardial Infarction
by
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Adoptive transfer of bone marrow stem cells (BMCs) for treatment of myocardial infarction has effectively improved cardiac function in several preliminary human clinical trials, but explanation of the mechanism is largely contested. In mouse models, BMCs have mediated cardiac repair after intramyocardial injection through direct and indirect reparations, but the large question remains is as to the survivability of injected cells. In this study, a veritable adult stem cell population, c-kit+ BMCs, were genetically engineered to over-express the cardioprotective kinase Pim-1 to enhance their survival in vitro as well as in vivo. Pim-1 modified BMCs (BMCeP) demonstrated increased proliferation and viability compared to un-modified BMCs (BMCe). Furthermore, BMCeP had increased resistance to apoptotic cell death with either hydrogen peroxide incubation or growth factor withdrawal as measured by flow cytometry. A comparative analysis was further performed in vivo, where after myocardial infarction, mice were intramyocardially injected with PBS, BMCe, or BMCeP and cardiac function was assessed for 12 weeks by echocardiography. At a three-week time point after injection, BMCe and BMCeP showed significant improvement in anterior wall dimensions compared to PBS and maintained this response up to 8 weeks. At a four-week time point after injection, BMCe and BMCeP had a significant improvement in fractional shortening and ejection fraction. Unfortunately, cell injected groups were not significantly different in hemodynamic parameters including heart weight body weight and function was never fully restored up to sham positive controls. Nonetheless, BMCe and BMCeP injected groups were statistically better than PBS negative controls by fractional shortening and ejection fraction analysis up to 12 weeks. Although, BMCeP did not recapitulate positive effects in vivo as previously shown in our laboratory, this is attributed to the low levels of Pim-1 protein initially expressed in BMCeP. Overall, these results allude to the protective effects of genetic engineering BMCs with Pim-1 to mediate damage after myocardial infarction.
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ABBREVIATIONS

7-AAD: 7-Aminoactinomycin D, marker of cell death
AV: annexin V, marker of early apoptosis
AWD: anterior wall dimension (thickness)
BAD: Bcl-2-associated death promoter, pro-apoptotic protein
Bcl-2: B-cell lymphoma 2, anti-apoptotic protein
Bcl-xL: member of the Bcl-2 family, anti-apoptotic protein
BMCe: bone marrow cells over-expressing eGFP (unmodified with Pim-1 BMCs)
BMCeP: bone marrow cells over-expressing eGFP and human Pim-1 isoform
BMCs: bone marrow cells; non-infected bone marrow cells
c-myb: proto-oncogene protein
c-myc: proto-oncogene protein
CAD: coronary artery disease
CAMK: calmodulin-dependent protein kinase-related group
CD34+ cells: human hematopoietic progenitor cells
Cdc25A: cell division cycle 25A protein (phosphatase regulating synthesis)
Cdc25C: cell division cycle 25C protein (phosphatase regulating mitosis)
CDKIs: cyclin dependent kinase inhibitors
CDKs: cyclin dependent kinases
CPCs: cardiac progenitor cells
EF: ejection fraction
eGFP: enhanced green fluorescent protein

EPCs: endothelial progenitor cells

ESCs: embryonic stem cells

FACS: flow activated cell sorting

FS: fractional shortening

G-CSF: granulocyte-colony stimulating factor

G_{1}/S: Gap1/Synthesis transition of the cell cycle

G_{2}/M: Gap1/Mitosis transition of the cell cycle

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

HGF: hepatocyte-growth factor

HSCs: hematopoietic stem cells

IGF: insulin-growth factor

IHC: immunohistochemistry

iPSCs: induced pluripotent stem cells

Lin-/c-kit+: Lineage negative and c-kit positive

MAPK: mitogen-activated protein kinase

MDM2: murine double minute 2 oncogene

MI: myocardial infarction (heart attack due to coronary occlusion)

MND: myeloproliferative sarcoma virus LTR-negative control region deleted

MSCs: mesenchymal stem cells

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1

NRCMs: neonatal rat cardiomyocytes

NuMa: nuclear mitotic apparatus protein

p21: cyclin dependent kinase inhibitor

p53: protein 53 or tumor protein 53

PI3K: Phosphatidylinositol 3-kinases

Pim-1: proviral insertion site for the moloney murine leukemia virus induced T cell lymphomas

Pim-KO: Pim-1, Pim-2, Pim-3 global knockout mouse

Pim-WT: Pim-1 over-expressing transgenic mouse

PKB: protein kinase B or Akt

Sca-1: stem-cell antigen 1

SCF: stem cell factor; steel factor

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

αMHC: alpha myosin heavy chain
INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in the United States, with over 80 million people suffering from one or more forms of this disease (1,2). CVD includes high blood pressure, coronary artery disease, cerebrovascular disease (stroke) and congenital heart disease (2). Coronary artery disease (CAD) is the leading cause of CVD, which is potentiated by a blockage of the normal supply of blood and oxygen to the heart by atherosclerotic plaques (3). The most common symptom of CAD is a myocardial infarction (heart attack) where sobering statistics present that approximately 800,000 people alive today have been affected by a myocardial infarction, angina pectoris, or both (2). The incidence of death from myocardial infarction is reported to be half a million cases per year in the U.S. and one million cases per year worldwide (4,5). Myocardial infarction leads to a progression of CAD, resulting in irreversible ischemic damage to the cardiomyocytes and fibrotic scarring, as well as the deterioration of the overall function and contractile capacity of the heart (6). Despite established pharmaceutical administration and medical procedures for treating surviving patients after a heart attack (7), these approaches do not help regenerate damaged myocardium necessary to improve long-term cardiac function.

THE HEART: A DYNAMIC ORGAN

The heart for a long time was believed to be a terminally differentiated organ, where compensation for extreme myocyte loss was restricted to endogenous mechanisms such as cardiomyocyte hypertrophy or inherent pro-survival signaling (8,9). This theory was initially challenged in zebrafish and newt heart models, where activation of endogenous cardiomyocyte regeneration by myocardial infarction (MI) increased cycling of differentiated cardiomyocytes (10). Furthermore, transplantation of adult cardiomyocytes into an infarcted heart model demonstrated modest improvements in cardiac function and improved physiological electrical coupling with the existing myocardium (10,11). Although, cardiomyocytes are suggested to have limited proliferation (12), adult myocytes have demonstrated the ability to de-differentiate, increase DNA synthesis and cycling, and subsequently have a hand in myocardial regeneration in non-mammal models (8).

The understanding of the heart as a dynamic post-mitotic organ is linked to its embryonic development from the mesoderm. At this important developmental stage, researchers discovered an increased number of c-kit positive (c-kit+) cells, a marker of stem
or progenitor cells, in the neonatal heart (13). Cells expressing green fluorescent protein (GFP) under control of the c-kit promoter were tracked through development and appeared to acquire lineages consistent with myocardial, endothelial and vascular lineages (13). Further studies have identified a resident adult c-kit+ progenitor pool, referred to as cardiac progenitor cells or CPCs (6,14,15). CPCs can be mobilized and stimulated to cycle with administration of cytokines such as hepatocyte-growth (HGF) factor or insulin-growth factor (IGF). Additionally, after MI, CPCs have demonstrated the ability to home in towards sites of injury, improving cardiac function and regeneration of ischemic regions (6,14,15). It has also been suggested that progression of heart disease in older individuals is attributed to the deteriorating function of aging CPCs, as seen in their decreased proliferation and senescent phenotype limiting their regenerative potential (14). Studies of the post-mitotic heart confirm it’s ability to regenerate cardiomyocytes and vascular structures after a myocardial injury, but this is often limited to the viable myocardium (16). These collective studies suggest that endogenous CPCs and cardiomyocytes do not sufficiently regenerate damaged tissue after injury, concluding that innate cellular repair mechanisms remain inadequate.

**ENDOGENOUS AND EXOGENOUS BONE MARROW DERIVED CELLS**

The arrival of cell-based therapies has become a novel approach in promoting recovery after ischemic damage. Modeled after the limited regenerative capacity of the heart, isolation and implantation of CPCs or bone marrow cells (BMCs) are actively being explored (8). Relevant to the clinical field, several types of stem cells are being used for clinical trials for the treatment of end stage heart failure. Most common is the use of bone marrow derived cells in the form of whole bone marrow or the bone marrow mononuclear layer (17). These isolated populations are considered to be a rich source of stem and progenitor cells such as hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs), which all have been shown to mediate cardiac function after injury (18-21). Preliminary use of whole bone marrow or specific progenitor cells from the bone marrow in human clinical trials appears to be promising (22). Several randomized studies have shown autologous BMC transplantation enhances cardiac function compared to placebo within one month after intravenous injection (8). More specifically, transfer of autologous human CD34+ hematopoietic progenitor cells promote re-vascularization in the heart improving overall cardiac function (23).
Induced mobilization of endogenous BMCs to the heart rather than intramyocardial injection of cells has also shown promising results. However, their presumed limit in cell plasticity remains to be their downfall in both cases (24,25). Researchers have discovered an endogenous subpopulation of stem cells within the bone marrow that migrates to sites of injury in the heart. One study demonstrated that hearts subjected to cytokine therapy, stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) increased the number of lineage negative and c-kit+ (Lin-/c-kit+) BMCs in the peripheral blood (16). Interestingly, after induction of a myocardial infarction, mice that were given cytokine therapy showed an increase in migration of Lin-/c-kit+ cells to the myocardium, reduced infarct size and improved left ventricular function (16). In a doxorubicin-induced cardiomyopathic model, new cardiomyocytes were attributed to mobilized BMCs from the peripheral blood (26). This progression of studies substantiates that c-kit+ cells not only mitigate damage by homing to sites of injury but also demonstrate BMCs potential to cross lineage paths through transdifferentiation (27,28).

Studies that involve injection of Lin-/c-kit+ BMCs show significant improvement in cardiac function mediated by transdifferentiation of cells into the cardiogenic, endothelial and vascular lineages void of cell fusion (29-31). Despite optimism of BMC derived cell therapy, cell engraftment is imperfect in these models (30,32), and there remains substantial controversy on the differentiation potential of bone marrow derived cells. It remains however, that transplantation of stem cells result only in modest recovery in cardiac structure and function (32). It is known that many of the transplanted stem cells die shortly after delivery, in part accounting for these modest benefits (17,33,34). This leads to the contention that only small improvements can become of bone marrow transplantation because these cells are not structurally or functionally fit to withstand the adversity of the heart environment after injury (33,34). Therefore, enhancing the ability of BMCs to survive and proliferate may substantially improve their ability to provide long-term structural benefits to the heart.

**GENETIC MODIFICATION OF STEM CELLS**

There is much contention as to what factors are ideal for injection experiments to repair damaged tissue. Those factors include: cell type and how the cells should be delivered to the myocardium (35). Cell type is the most common variable because of conflicting results on the ease of isolation and survival of stem cells after transplantation (35). As for the delivery of stem cells, it appears that it is much easier to intramyocardially
inject stem cells in small rodent models, but difficult to translate into human clinical trials which require myocardial intravenous injection. Researchers are continuously advancing their knowledge of the cell biology of stem cells in order to understand the modest improvements in myocardial repair. This has led to increased investigation in ways to enhance their potential for *in vivo* therapy through *ex vivo* gene modification. There are several ways to genetically modify stem cells, either through use of non-viral and viral vectors, which include simple transfections, or creation of adeno-, retro- or lentiviruses (36,37). There has been advanced interest in the use of lentiviruses because of their ability to integrate into the genome of dividing as well as quiescent cells (37). With use of a lentivirus, daughter progeny will inherit the transgene indefinitely, which ensures creation of stable cell lines and successful *ex vivo* modification (37,38). The downfall of this method is that it is difficult to determine the number of transgenes that will be incorporated into the genome and most importantly is as to where the transgene will insert (39). This could have tremendous consequences on the internal transcription of the cell as well as mutagenic or oncogenic detriments (39). In contrast, researchers are actively investigating the use of zinc-finger proteins (ZFP) in order to target incorporation of transgenes at specific DNA sites, random integration with current lentiviral constructs (40).

Genetic modification of cells for introduction into damaged tissue is a growing technique. Gene modification is particularly useful for tracking purposes such as through fluorescence tags (i.e. GFP) or small gene tags (i.e. myc or flag) in order to determine the cardiac potential of selected stem cells *in vivo* (10). Setting precedent for genetic modification of stem cells is important for advancing therapeutic intervention for the heart, but also for other organ types. Lentiviral transduction of bone marrow derived cells has been established in the past decade, but the efficiency is still very low (38,41). In recent studies, several researchers have successfully applied genetically modified MSCs over-expressing Akt, using a retrovirus, to the heart after MI (9,35,42-45). In the rat heart, Akt over-expressing MSCs (Akt-MSCs) had an increased propensity to reduce fibrotic scarring and restored cardiac function back to normal compared to MSCs alone (9). In another study by this group, Akt-MSCs created enhanced metabolic properties in the heart such as increased ATP reserves important for preserving cell integrity (45). Concurrent studies confirm that the improvement of these cellular functions was due to minimal cell fusion, but did not display apparent differentiation into the cardiogenic lineages (44). The researchers concluded that over-expression of Akt was an ideal source to generate enhanced MSCs (9,44) and sets
precedent for future studies that combine isolation of a progenitor population from a rich source such as the bone marrow and subsequent genetic enhancement to promote proliferation and survival in the heart after injury.

**THE MANY ROLES OF PIM-1**

Akt is a survival kinase that regulates important cardioprotective pathways such as programmed cell death and proliferation (46,47). Akt exerts these protective effects when it is localized in the nucleus, where in cardiomyocytes, nuclear Akt, effects cell proliferation and prevents progression of cardiac hypertrophy (46,47). Akt acts upstream of another serine/threonine kinase Pim-1 (48), which is also described as an important cardioprotective molecule because of its role in cell survival, cell growth, apoptosis and differentiation in the heart (48-50). These processes are not only limited to myocytes, but Pim-1 regulates these essential processes in non-myocyte populations such as hematopoietic cells, smooth muscle cells and endothelial cells (50-54). Pim-1 was first discovered as a proviral insertion site for the moloney murine leukemia virus induced T-cell lymphomas (51,55). Additionally, Pim kinases belong to the calcium calmodulin family, the calmodulin-dependent protein kinase-related group or CAMK (56,57). The *pim-1* gene encodes for two proteins: one at 44kDa and the other 33-34kDa (51) and are constitutively active kinases once translated (58). The Pim family is a series of proto-oncogenes that include Pim-1, Pim-2 and Pim-3, which are crucial for normal development and maintenance of the hematopoietic system (54,55,59). Complete knockout of Pim-1, Pim-2 and Pim-3 (Pim-KO) in a mouse model created a significant reduction in body mass, symptoms that continued until the mice reached adulthood (48,55). Interestingly, Pim-KO mice had considerably less hematopoietic cells than their wild type controls, implying to the physiological necessity of Pim-1 for normal development (55). With these preliminary findings, our laboratory showed that that Pim-KO mice had impaired cell survival signaling after myocardial infarction and pressure-overload induced hypertrophy as assessed by measurement of proliferative and apoptotic markers *in vitro* and *in vivo* (48). By contrast, Pim-1 over-expression (Pim-WT) mice driven by a heart specific promoter (α-MHC) had increased resistance to pathological stress such as myocardial infarction or trans-aortic constriction (48,49). Additionally, isolated cardiomyocytes from Pim-WT mice showed enhanced cell survival properties and increased calcium transients as a consequence of Pim-1 over-expression (48).
Pim-1 has dynamic regulation of the cell with its dual compartmentalization in the nucleus and cytoplasm to promote proliferation and cell survival. Pim-1 interacts and stabilizes a variety of transcription factors such as NFATc, c-Myb and c-Myc, which suggests a role for Pim-1 in differentiation and proliferation (51,60,61). In reference to the cell cycle, Pim-1 was first described to regulate G1/S transition by phosphorylation of Cdc25A phosphatase indirectly enhancing the action of cyclin dependent kinases (CDKs) promoting progression through the cell cycle and DNA synthesis (51). In contrast, Pim-1 has a direct effect on inhibition of cyclin dependent kinase inhibitors (CKIs) such as p21\textsuperscript{G1p1/WAF1} by phosphorylation on the threonine 145 (51,58,62). Additionally, Pim-1 regulates mitotic proteins such as the Cdc25C phosphatase and NuMa, required for G2/M progression and proper mitotic spindle formation, respectively (51,63-65). MDM2, an E3 ubiquitin ligase, when phosphorylated by Pim-1, allows for p53 degradation, creating a hyperproliferative status of the cell (66,67). Overall, these studies allude to the pro-proliferative roles of Pim-1 that were initially discovered in cancer cell lines, but are actively being implemented as therapeutic tool for expansion of adult stem cells to enhance cellular regeneration in the heart.

One of the mechanisms of cell survival depends upon preservation of mitochondrial integrity. Protection of mitochondria after pathological stress relies on inhibition of apoptotic cell death and preservation of regulatory mechanisms in the heart involving survival kinase signaling. Pim-1 plays such role in repressing pathological stress by antagonizing the mitochondria’s pro-apoptotic cascades. Most commonly in the hematopoietic system, Pim-1 over-expression prevents adverse apoptotic processes caused by growth factor deletion in the media and treatment with pro-apoptotic agents such as doxorubicin (68,69). One mechanism where Pim-1 is known to induce its pro-survival effects is by phosphorylation and inactivation of BAD protein on the serine residue 112 in hematopoietic cells as well as in cardiomyocytes (48,68). Inactivation of the BAD protein causes it to leave the mitochondria by way of the protein 14-3-3 permitting anti-apoptotic proteins such as Bcl-xL and Bcl-2 to exert their effects (57,68,70). Additionally, Pim-1 has been show to translocate to the mitochondria after ischemic reperfusion injury allowing Pim-1 to be on site to inhibit pro-apoptotic proteins and preserve cellular integrity via the mitochondria (71).

Pim-1, from these studies, is demonstrated to be involved in several essential cellular processes including promoting proliferation, inhibiting apoptosis, and regulating transcription and signal transduction by targeting different compartments of the cell (51).
From these initial analyses, our laboratory, has shown Pim-1 not only mitigates pathological injury but is a compelling choice for genetic modification of isolated stem/progenitor cells. In our laboratory, we have isolated c-kit+ CPCs from mouse hearts and genetically modified them with Pim-1 kinase (72). Transfer of these cells into infarcted myocardium not only showed functional improvement but also enhanced engraftment and differentiation of implanted Pim-1 CPCs compared to control cells (72). In addition, analysis of CPCs from Pim-WT mice displayed increased cycling \textit{in vitro} as well as \textit{in vivo} compared to controls (73). Stem cell therapy to enhance cardiac regeneration is still a novel approach to treat ischemic injury. Our laboratory is interested in CPC transplantation, however translation of this cell type in the clinical setting may be problematic because of invasive procedures to obtain progenitor cells and the relatively limited number of cells (6,8,14). This has led to an approach that combines isolation of a stem cell population from a familiar source such as the bone marrow and subsequent over-expression of Pim-1 to promote survival and growth of transplanted BMCs in the heart after injury. Additionally, ease of isolation and injection of bone marrow cells has made it an established procedure for treatment of a myocardial infarction in clinical trials with positive although modest results (8,17); therefore, genetically modified BMCs should have a positive effect on improving recovery after pathological damage.

**RATIONALE**

This study is addressed to show enhanced myocardial repair by delivery of genetically modified BMCs with Pim-1 kinase through preservation of the existing myocardium and improving overall cardiac function. Description of Pim-1 as a pro-survival molecule even in the instance of myocardial damage promotes the notion that genetic modification with this kinase will not only show pro-survival effects \textit{in vitro} but also \textit{in vivo}. Taken together, thus I hypothesize that adoptive transfer of BMCs over-expressing cardioprotective Pim-1 kinase in the heart will enhance cardiac function after infarction compared to unmodified BMCs. The long-term goal of this study is to identify a novel approach to enhance cellular therapy for the treatment of heart failure.
MATERIALS AND METHODS

LENTIVIRUS PRODUCTION

BMCs were lentivirally transduced using a bicistronic construct that either over-expressed the human Pim-1 gene under control of a myeloproliferative sarcoma virus LTR-negative control region deleted (MND) promoter and enhanced green fluorescence protein (eGFP) driven off a vIRES noted as BMCeP or the control lentivirus that drives expression of eGFP alone noted as BMCe. Untransduced cells are referred to as BMCe.

BONE MARROW ISOLATION: CULTURE AND TRANSDUCTION

BMCs were isolated from 8-15 week old FVB mice by flushing the femur and tibia with 5% FBS/PBS. Mononuclear layer from the bone marrow was obtained by a Histopaque gradient (Sigma). Cells were then labeled with anti-CD117 magnetic antibodies (Miltenyi Biotec) and sorted for CD117 using MACS columns (Miltenyi Biotec). Cell were plated in round bottom 96-well plates at 1.5 X 10^5 cells per well in Stemspan media (Stem Cell Technologies) supplemented with cytokines IL-3, IL-6, TPO at 20ng/mL and Flt-3 ligand and H-SCF at 50 ng/mL (Peprotech) considered to be complete media. Transduction of c-kit+ enriched BMCs by the bicistronic lentivirus Lv-egfp or Lv-eGFP+Pim-1 was performed with a multiplicity of infection (MOI) of 50 in the presence of Polybrene (4 μg/ml) to facilitate lentiviral transduction. Cells were allowed 48 hours to express the eGFP reporter gene and human Pim-1, and then infected again with a MOI of 25. Western blot analysis, immunohistochemistry and FACS analysis were utilized to assess over-expression of lentiviral vectors in BMCe and BMCeP populations.

IMMUNOBLOT ANALYSIS

Western blot analysis with BMC protein samples were separated on a 4%–12% Bis-Tris mini-gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour with 8% milk in TBST (1%Tris-buffered saline/0.1% Tween) and then probed with primary antibody overnight in milk. Next day blots were washed with TBST buffer and incubated in secondary antibodies in milk for 1.5 hours. Membranes were washed with TBST and imaged with a Typhoon 9410 scanner. Primary antibodies: Pim-1 anti-goat (Santa Cruz cocktail E-16, N-16, C-20 clone 1:200), GFP anti-chicken (Invitrogen 1:1000), CDC25A anti-rabbit (Cell Signaling Technologies 1:500), Bcl-2α
anti-moue (Invitrogen 1:1000), GAPDH anti-mouse (Chemicon 1:3000).

**IMMUNOFUORESCENCE MICROSCOPY: FIXED CELL STAINING**

BMCs were placed in minimal volume on glass slides and allowed to dry for two hours. Cells were then fixed in 4% paraformaldehyde for 30 minutes then washed in phosphate buffered saline. Cells were blocked in 10% horse serum/PBS for one hour and then incubated with primary antibodies overnight. Next day, cells were washed in PBS and incubated with corresponding secondary antibodies for 1.5 hours. Cells were washed in PBS followed by a final wash containing TO-PRO-3 iodide for 10 minutes to stain for nuclei. Primary antibodies used: CD117 (c-kit) anti-goat (R&D systems 1:50), GFP anti-rabbit (Invitrogen 1:50).

**FACS STAINING AND ANALYSIS**

Cells in suspension were counted and placed in samples of 1.5-2.0 x 10^5 per tube. Cells were washed two times in 1% FBS/PBS before incubation with conjugated antibodies for 15 minutes at room temperature. After incubation cells were washed two times with 1% FBS/PBS and resuspended in minimal amount of buffer for analysis.

**MTT ASSAY**

BMCe and BMCeP were subjected to a MTT colorimetric reducing assay which analyzes (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole being reduced to a purple formazan in living cells. This assay was used in order to determine relative rates of metabolic activity between cell lines. In this assay 5,000 cells were plated per well in a 96-well flat bottom plate and given 10µL of 5mg/mL solution of MTT reagent for four hours and then incubated with 100µL (diluted HCL for lysis) of stopping reagent overnight. MTT assay was performed 1,3,5 and 7 days after plating and absorbance was read at 595nm in order to determine relative concentrations of cells at given days. MTT assay was repeated with use of Pim-1 kinase inhibitor Quercetengentin (Calbiochem) at 50 µM on BMCe and BMCeP at day 0 and evaluated for metabolic activity at Day 1 and Day 3.

**CELL VIABILITY AND TRYPAN BLUE EXCLUSION ASSAY**

BMCs were evaluated for cell viability by plating 10,000 cells per well in a 96-well round bottom plates in quadruplicate in complete media. The number of viable cells were
counted at days 1, 3, 5 and 7 after plating with use of hemocytometer and trypan blue in order to exclude non-viable cells from viable (visibly bright) cells.

**APOPTOSIS/CELL DEATH ASSAY**

Cells in suspension were counted and plated at equal densities in 48-well plates in complete media. Cells were treated with hydrogen peroxide either 18 hours at 25μM or 100μM from 30 minutes the next day. At the end of the incubation period, all cells were collected and washed once with PBS then for the second time with Annexin-V binding buffer. Cells were incubated with Annexin-V conjugated to allophycocyanin (APC), 7-Aminoactinomycin D (7-AAD) or both for at least 15 minutes at room temperature. Cells were then analyzed for frequency of Annexin-V positive cells and Annevin-V/7-AAD positive cells compared to control (cells with complete media and no treatment with hydrogen peroxide). All flow cytometric analysis was done with FlowJo 8.7 software.

**MYOCARDIAL INFARCTION AND INJECTION**

Ten-week old female FVB mice that received myocardial infarctions were carried out under isoflurane anesthesia and by tying off the left anterior descending artery (LAD) that supplies blood to the anterior left ventricle. After infarction injury, injections with either PBS (5μL per injection 5 injections total per mouse) or genetically enhanced cells, BMCe or BMCeP cells (20,000 cells per 5 μL injection, 5 injections making a total of 100,000 cells injected per mouse) were introduced to the pre-ischemic border along the infarcted region. The sham operations were performed by opening and closing the chest.

**ANIMAL MODELS**

All experimental models were designed in accordance with NIH guidelines and approved by the San Diego State University Medical Center Institutional Animal Care and Use Committee.

**ECHOCARDIOGRAPHY**

Echocardiography was used to evaluate cardiac function after a MI and injections. Infarction size was standardized by echocardiography performed on animals imaged along a parasternal short-axis view by M-mode recorded at two days post infarction/injection and successively for 4 weeks, then every other week up to 12 weeks. Functional recovery was determined by measurement of left ventricle (LV) anterior wall diameter (AWD) in
centimeters (cm) for wall thinning, the end diastolic diameter (EDD) and end systolic
diameter (ESD) in order to calculate LV fractional shortening (FS) and LV ejection fraction
(EF). Hemodynamic performance assessed by echocardiography two days post-infarction
was not statistically different between infarcted/injected groups (PBS, CPCe, and CPCeP).
FS a measure of contractile function, is measured by EDD minus ESD divided by EDD * 
100. The proportion of blood ejected out of the left ventricle during each heartbeat is
referred to as the EF and calculated as EDD^3-ESD^3/EDD^3 *100.

**HEART WEIGHT, BODY WEIGHT RATIO (HW/BW)**

Mice were sacrificed under chloral hydrate sedation at 12 weeks. Before the
procedure of removing the hearts, mice were weighed in milligrams. Thereafter, hearts were
arrested in diastole by catheterizing the abdominal aorta and flushing the heart with a high-
potassium/cadmium solution. Phosphate buffered formalin fixative was perfused into the
coronary arteries at systolic pressure while the left ventricle was filled with formalin at
diastolic pressure. Retroperfused hearts were then removed from the chest cavity and
placed in formalin overnight. Next day hearts were removed from formalin and weighed in
milligrams, then placed in 70% ethanol for paraffin embedding and tissue processing.

**IMMUNOFLOURESCENCE MICROSCOPY: PARAFFIN
SECTIONS**

Retroperfused hearts were removed from the chest cavity and placed in formalin
overnight, followed by 70% ethanol, followed by processing for paraffin embedding using an
automated tissue processor. Heart sections were deparaffinized, and antigen was retrieved
in 1 mmol/L citrate (pH 6.0), followed by a one-hour block in TNB. Primary antibodies were
incubated overnight at 4°C at appropriate dilutions. Slides were washed in 1% Tris/NaC1
(TN buffer) followed by secondary antibody incubation for two hours at room temperature.
Subsequent tyramide amplification was performed as necessary. Cells were washed after
secondary antibodies in TN followed by a final wash containing TO-PRO-3 iodide for 10
minutes to stain for nuclei. Primary antibodies used: CD117 (c-kit) anti-goat (R&D Systems
1:40), GFP anti-rabbit (Invitrogen 1:500), tropomyosin anti-mouse (Sigma 1:100). CD117
and GFP required tyramide amplification.
**STATISTICAL ANALYSES**

Statistical analysis was carried out using student’s t-test when comparing two groups. More than two group comparisons were analyzed using one-way ANOVA with Tukey’s post-test. P-values of less than 0.05 were considered statistically significant. Error bars represent standard error of the mean (SEM).
RESULTS

GENETIC MODIFICATION OF C-kit+ BMCS WITH Pim-1

Bone marrow is a rich source of stem and progenitor cells including HSCs, EPCs, and MSCs that ultimately lead to an array of progenitors that can reconstitute the entire blood system in irradiated mice (74). In this study we isolated c-kit+ BMCS and analyzed the cells for lineage and stem cell markers in order to establish the phenotype of the cell populations involved in long-term culture and transduction. Lentiviral vector constructs expressing GFP or GFP in combination with human Pim-1 (Figure 1A, pg.15) were created to efficiently deliver genes to BMCS denoted as BMCe and BMCeP, respectively. Immunoblot analysis and use of a specific antibody was used to confirm and recognize over-expression of the human Pim-1 at 33-34 kDa, but not the endogenous mouse Pim-1 in BMCeP cell line and GFP over-expression in BMCe and BMCeP (Figure 1B, pg. 15). Subsequently, FACS analysis demonstrated BMCe and BMCeP expressed GFP and c-kit + populations, and further confirmed high levels of GFP and c-kit expression after long-term culture and passaging. Results demonstrated GFP expression for BMCe at 89.2% and BMCeP at 90.9% while c-kit expression in BMCe at 94.5%, BMCeP at 98.2% and BMC at 87.9% (Figure 1C, pg. 16). Additionally, immunohistochemistry confirmed the presence of the stem cell marker c-kit and GFP tag in engineered cell lines (Figure 1D, pg.16). These results demonstrate the ability to over-express Pim-1 in BMCS and the opportunity to demonstrate pro-survival effects in our system by Pim-1 over-expression.

CHARACTERIZATION OF C-kit+ BMCS OVER-EXPRESSING Pim-1

Whole bone marrow is a natural heterogeneous cell population. In order to understand the benefit and regenerative capacity of enhanced BMCS when applied to an in vivo setting, progenitor cell markers were evaluated by flow cytometry. Preliminary results suggest that BMCeP have reduced expression of lineage positive markers such as CD4 and CD8 (T-cells), B220/CD45R (B-cells), Mac-1 (monocytes/macrophages), and Gr-1 (granulocytes) compared to BMCe (Figure 2B, pg. 18). Additionally, both BMCe and BMCeP
Figure 1. Genetic modification of BMCs.
A. Bicistronic lentiviral vectors for introduction of cDNA into c-kit+ BMCs. Top, MND promoter driving GFP expression. Bottom, MND promoter driving both Pim-1 and GFP expression. B. Immunoblot for GFP expression in BMCe and BMCeP and Pim-1 overexpression in BMCeP. BMCs were loaded as a negative control. GAPDH was imaged as a loading control. C. FACS analysis of GFP expression and c-kit expression. D. Immunostaining showing expression of c-kit and GFP in BMCe and BMCeP as shown in single channel scans on the left and merged on larger image (right).
A. 

Lv-egfp = BMCe

Lv-egfp + Pim-1 = BMCeP

B. 

BMC  BMCe  BMCeP

Pim-1

GFP

GAPDH

C. 

BMC

BMCe

BMCeP

GFP +

c-kit +

87.9%

89.2%

90.9%
D.

BMC

GFP

c-kit

nuclei

BMCe

GFP

c-kit

nuclei

BMCeP

GFP

c-kit

nuclei
demonstrated low expression levels of Ter119 (erythrocyte) (Figure 2B, pg.18). In reference to the “stemness” of the populations, BMCs expressed high levels of sca-1 and low levels of CD34 at equal levels (Figure 2A-2B, pg. 18). In studies, acquisition of the CD34 surface marker ensures a more differentiated state indicative of progression down the hematopoietic lineage. BMC populations were also positive for the ubiquitous hematopoietic marker CD45 and the endothelial marker CD31 (Figure 2A, pg.18). Our detailed analysis produces a comprehensive picture of characteristics possessed by Pim-1 engineered cells versus control cells prior to adoptive transfer into the myocardium. Phenotypic characterization of genetically modified BMCs does reveal variable expression of markers for HSCs, EPCs and lineage positive cells. In conclusion, BMCeP revealed relatively few lineage positive cells that included B cells, T cells, granulocytes, and macrophages compared to BMCe, which could be attributed to the cells hyper-proliferative status compared to unmodified BMC and BMCe populations.

**BMCeP have enhanced proliferation**

In order to assess the growth advantage of Pim-1 over-expression in BMCs, basic biochemical and growth assays were performed. BMCeP showed an increased number of viable cells at day 5 (p<0.02) and day 7 (p<0.05) relative to BMCe by trypan blue exclusion assay, which allows for quantification of viable cells (Figure 3A, pg. 20). Additionally BMCeP had increased metabolic activity assessed by a colorimetric MTT assay compared to BMCe at day 5 (p< 0.0007) and day 7 (p<0.008) (Figure 3B, pg. 20). The MTT assay was repeated in the presence of a Pim-1 kinase inhibitor quercetagetin in order to corroborate the increased growth rates associated with over-expression of Pim-1. Here, BMCeP metabolic activity was decreased substantially after 24 hours and 4 days (p<0.0002) (Figure 3C, pg. 21). Interestingly, BMCe showed also a significant decrease in metabolic activity at 24 hours and 4 days with addition of the Pim-1 inhibitor. This result is attributed to the fact that BMCs express a significant amount of endogenous Pim-1 to regulate hematopoietic growth and cell survival (54). Additionally, to confirm Pim-1 kinase activity, cell lines were subjected to immunoblot analyses for total CDC25A protein levels. Results demonstrate significantly more CDC25A in BMCeP cells compared to BMCe and BMCs at basal levels (Figure 3D, pg. 21). CDC25A is a phosphatase that regulates G1/S progression by interaction with various CDKs and its up-regulation is a direct consequence of Pim-1 over-expression in BMCs (51,75).
Figure 2. BMC characterization.
A. Flow cytometric analysis, showing histograms of BMC populations for hematopoietic lineage markers sca-1, CD34, CD45 and CD31. B. Analysis of GFP populations from BMCe and BMCeP for lineage markers represented as percent of total GFP population.
Figure 3. Pim-1 over-expressing BMCs have enhanced proliferation.
A. Cell viability assay, showing increasing number of viable cells in BMCeP vs BMCe at day 5 (*p<0.02) and day 7 (**p<0.05). B. MTT assay, showing increased metabolic activity in BMCeP vs BMCe at day 5 (***p<0.0007) and day 7 (#p<0.008). C. MTT assay in the presence of Pim-1 inhibitor. Day 0 represents BMCeP vs BMCe without the Pim-1 inhibitor (Φp<0.001). Day 1 BMCe vs inhibitor (πp<0.0001) BMCeP vs. inhibitor (πp<0.0001). Day 4 BMCe vs inhibitor (πp<0.0001) BMCeP vs inhibitor (θp<0.0002). D. Immunoblot for CDC25A in BMC, BMCe and BMCeP, quantification (right) was normalized to GAPDH as a loading control and represented as relative fluorescence units (rfu) n=1.
C.

![Graph with bar charts showing OD 595nm for different groups over days 0, 1, and 4.](graph.png)

D.

- **CDC25A**
  - BMC
  - BMCe
  - BMCeP

- **GAPDH**

![Western blot images for CDC25A and GAPDH](blot_images.png)

![Histogram showing rfu for BMC, BMCe, and BMCeP](histogram.png)
BMCeP are Resistant to Apoptotic Stimuli

Previously, our laboratory has shown that Pim-1 over-expression decreases incidence of apoptotic cell death in neonatal rat cardiomyocytes (NRCMs) even at basal levels by measurement using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (48). To assess for apoptotic associated cell death in BMCs, flow cytometric analysis was used in order to determine the extent of early apoptosis, positive for Annexin V (AV) alone or cell death AV and 7-Amino-actinomycin D (7-AAD) double staining (AV/7AAD). Induction of cell death in BMCs was measured with complete media or complete media with the addition of hydrogen peroxide (H$_2$O$_2$) at 25µM overnight, or 100µM H$_2$O$_2$ for 30 minutes. An oxidative stress stimuli, such as H$_2$O$_2$ was used in order simulate the environment genetically modified BMCs would be subjected to in the infarct region after injection, either at an early time point (30 minutes) or longer (18 hours). In vitro analysis, demonstrated BMCeP had only a 1.13-fold increase in AV+/7AAD+ cells after incubation with 100µM H$_2$O$_2$ for 30 minutes where as BMCe had a 2.04 fold increase in AV+/7AAD (Figure 4A, pg. 23). Furthermore, after longer treatment of 25µM H$_2$O$_2$, BMCe had a detrimental 9.5 fold change increase in cell death, whereas BMCeP had only a 4.1 fold increase in cell death (Figure 4A, pg. 23). To confirm the pro-survival role of Pim-1 over-expression in BMCs, cells were subjected to growth factor withdrawal. Removal of IL-3, IL-6, FLT-3 and SCF from the media was performed for 72 hours and subsequent immunoblot analysis demonstrated up-regulation of the anti-apoptotic protein Bcl-2α in BMCeP compared to BMCe (p<0.0003) and BMC (p<0.002) (Figure 4B, pg. 23). Bcl-2α has been previously described as an indirect substrate of Pim-1, which supports mitochondrial integrity in the myocardium (71,76).

Injection of C-kit+ BMCs Improve Cardiac Function

A longitudinal assessment of cardiac function by echocardiography was performed after MI and injection of BMCe or BMCeP. Hemodynamic analysis was measured to understand the impact of MI upon the left ventricle and the compensatory response from injected BMCs. Results from the in vivo experiments demonstrate that injection of BMCe and BMCeP had a significant improvement in AWD at 3 weeks BMCeP (p<0.01) and BMCe (p<0.05) compared to PBS (Figure 5A, pg. 26), FS at 4 weeks BMCeP (p<0.05) and BMCe (p<0.01) (Figure 5B, pg. 26) and EF at 4 weeks BMCeP (p<0.05) and BMCe (p<0.01) (Figure 5C, pg. 27) compared to PBS control. Cardiac function of BMCe and BMCeP injected hearts
Figure 4. Pim-1 over-expression in BMCs protects from apoptotic cell death. 
A. Flow cytometric analysis of BMCe and BMCeP after incubation with apoptotic stimuli represented as fold change from cells with media alone. B. Immunoblot analysis of Bcl-2α in BMCs. BMCeP vs BMCe (*p<0.0003) and vs BMC (ϕp<0.002). Protein levels were normalized to GAPDH loading controls and represented as relative fluorescence units (RFU).
Figure 5. Longitudinal assessment of cardiac function after injection of BMCs. A. AWD in cm. 3 weeks Sham vs BMCe (*p<0.05) and BMCeP (**p<0.01). 8 weeks PBS vs BMCe (**p<0.0001) and BMCeP (***p<0.0001). B. %FS. 4 weeks PBS vs BMCe (**p<0.01) and BMCeP (*p<0.05). 8 weeks PBS vs BMCe (*p<0.05) and BMCeP (*p<0.05). 12 weeks PBS vs BMCe (*p<0.05) and BMCeP (*p<0.05). C. %EF post myocardial infarction and injection of cells up to 12 weeks. 4 weeks PBS vs BMCe (**p<0.01) and BMCeP (*p<0.05). 8 weeks PBS vs BMCe (*p<0.05) and BMCeP (*p<0.05). 12 weeks PBS vs BMCe (*p<0.05) and BMCeP (**p<0.01). D. Heart weight, body weight at 12 weeks. PBS vs Sham (**p<0.0006), PBS vs BMCe (**p<0.01) and BMCeP (*p<0.05). Shams are represented as positive controls.
C.

![Graph showing EF (%) vs Time (weeks) for different treatment groups.]

D.

![Graph showing HW/BW ratio (g/mg) for different treatment groups.]

- **PBS**
- **BMCe**
- **BMCeP**
- **Sham**
was never fully restored to sham controls in all physiological parameters by 12 weeks, although both BMCe and BMCeP were statistically better than PBS in FS and EF, but not AWD, at the end of 12 weeks. Additionally, surviving mice from the experiment were subjected to heart weight, body weight (HW/BW) analysis. This phenotypic characterization is based on the hypertrophic compensatory response to myocardial damage. As the heart sustains damage it will begin to enlarge and the ratio between the animals body mass will become larger (77). In our experiment, PBS negative controls had a high HW/BW ratio compared to BMCeP (p<0.05), BMCe (p<0.01) and sham controls (p<0.0001) (Figure 5D, pg. 27). This physiological response (FS, EF and HW/BW) was sustained up to twelve weeks after MI and injection, where the patterns between BMCeP and BMCe were not considered to be statistically different, although statistically improved over PBS negative controls.

C-KIT+ BMCs HAVE LIMITED SUSTAINABILITY AFTER INJECTION INTO THE MYOCARDIUM

Studies that involve injection of Lin-/c-kit+ BMCs show significant improvement in cardiac function (16,17), although adoptively transferred cells are rarely observed within the infarct after delivery. Extent of persistence and engraftment was measured by immunohistochemical (IHC) analysis using confocal microscopy with mouse hearts receiving PBS injections, BMCe or BMCeP injections after 12 weeks. Presence of donated cell populations in the myocardium was determined by staining of sections with GFP and c-kit antibodies. In PBS controls, c-kit positive cells with no GFP were observed after myocardial infarction (Figure 6A, pg. 29) within the border zone of the infarct consistent with previous published findings (15). By contrast, BMCe (Figure 6B, pg. 29) and BMCeP (Figure 6C, pg. 30) were found after 12 weeks by co-expression of c-kit and GFP. Cells were observed to be within the infarct region of the left ventricle and retained the stem cell marker c-kit without apparent engraftment or differentiation into the myocardium. Although relatively small numbers of cells were observed in the infarct region, mice who received intramyocardial injections of BMCe or BMCeP sustained a positive response in cardiac function over the time course of 12 weeks. This observation alludes to the ability of injected BMCs to mediate pathological injury, but most likely at very early time point through a paracrine response.
Figure 6. *In vivo* assessment of cell engraftment after 12 weeks of MI. A. Representative micrographs of the mouse myocardium after PBS (vehicle) injection. B. The mouse myocardium after BMCe injection. C. Mouse myocardium after BMCeP injection 12 weeks post infarction. Cells are represented with c-kit (PBS, BMCe and BMCeP), GFP tag (BMCe and BMCeP). Myocardium is labeled with tropomyosin and nuclei with TO-PRO staining. White boxes depict enlarged region of c-kit+ cells, co-expressing GFP as the case for BMCe and BMCeP.
A. PBS injected

B. BMCe injected
C. BMCeP injected
DISCUSSION

This present study demonstrates that adult stem cells should not be underestimated in their ability to repair or mediate damaged tissue, in our case, the myocardium. Although there are several studies that insist that the positive potential of BMCs is through their transdifferentiation into the cardiogenic lineages (29,31), these studies are continually being refuted. Arguments come from several contrasting studies; one suggests transdifferentiation of BMCs is customary cell fusion within the existing myocardium while the other argues BMCs mediate cardiac function based solely on a paracrine effect in the infarcted heart (22). Furthermore, BMCs are suggested to have difficulty surviving after injection into the hostile infarcted region (34). The last point concerning the viability of injected cells leads our laboratory’s interest in enhancing adult BMCs to overcome the adversity of the ischemic heart, by improving their proliferative potential and survival. In order to appropriately enhance BMCs, we genetically engineered them to over-express Pim-1 kinase using a lentivirus (Figure 1). BMCs were isolated from mice by the marker c-kit and characterized for several hematopoietic lineage and stem cell markers (Figure 2) in order to understand how to expand and transduce these cells for in vitro analysis and in vivo experiments. Cells that were enhanced with Pim-1 supported a hyper-proliferative status by MTT assay and viability quantitation where data was supported with use of Pim-1 specific inhibitor (Figure 3). Additionally, Pim-1 over-expressing cells had an increased resistance to apoptotic related cell death (Figure 4), which correlates with previous studies in cardiomyocytes and CPCs in our laboratory (48,72). Injection of BMCe and BMCeP had a significant effect on improving cardiac function and maintaining a healthier phenotype in mice after myocardial infarction (Figure 5). Lastly, after 12 weeks of injection, cell populations were observed, albeit at low number, and still expressed c-kit with no noticeable engraftment or differentiation into the remote or infarcted myocardium (Figure 6).

Our study aimed at rescuing myocardial function addressed and raised interesting questions on the proliferative and survival capacity of BMCs. C-kit is an ideal marker for cell sorting because it is ubiquitously expressed on stem and progenitor cells from the bone marrow. Furthermore, BMCs ex vivo maintained this homogenous population in culture after several passages in the presence of SCF (Figure 1C). Interestingly, activation of the c-kit receptor results is a variety of downstream cascades such as Mitogen-activated protein
kinases (MAPK) and phosphoinositide-3 kinase (PI3K) regulating cell proliferation and survival (78). The PI3K pathway in turn activates Akt, which regulates these essential cellular functions upstream of Pim-1 (78). Although progression of tumors is often linked to aberrant activation of tyrosine kinases (79), over-expression of c-kit is a positive enhancer of expansion of adult stem cells. One study has shown that differentiation of c-kit+ BMCs into the cardiogenic lineage is dependent on activation of the receptor by SCF in vitro, where in contrast, BMCs from c-kit null mice failed to turn into beating cardiac clusters in similar media conditions (27). In addition, the characterization of BMCeP compared to BMCe revealed an enriched c-kit expression after several days in culture, but the differences in expression of lineage positive markers has yet to be fully confirmed such as with Gr-1, Mac-1 and CD90.1 expression (Figure 2B). Our laboratory believes that BMCeP hyperproliferative status limits commitment to differentiation in culture attributing to lower expression of lineage positive markers compared to BMCe. This observation leads to intriguing possibilities of using Pim-1 kinase as a cardioprotective agent to supplement the expansion, survival and differentiation of adult stem cells from the bone marrow.

Although the majority of the studies of Pim-1 highlight its involvement in cell survival through hyper-proliferation and inhibition of cell death, Pim-1 plays an influential role in differentiation of hematopoietic cells (54) and preservation of vasculogenesis in different organ types (52,53). Additionally, our laboratory has shown that injection of Pim-1 CPCs potentiated neovascularization in vivo after 12 weeks. These results mediated a more than modest improvement in cardiac function and as a consequence reduced infarct size and fibrotic scarring (72). From these preliminary studies our laboratory hypothesizes that BMCs, which have been modified with Pim-1, will have enhanced ability to survive and differentiate within the infarct. Another study from our laboratory has proposed a mechanism that targets Pim-1 as a mediator of the endogenous regenerative response after myocardial injury by activation of the nucleolar protein nucleostemin. After infarction, nucleostemin was up regulated in resident CPCs and mature cardiomyocytes in the border zone of the infarct, which increased DNA synthesis in both cell types (80). CPC proliferation and survival is, in part, attributed to activation of nucleostemin, which preserves the “stemness” and proliferation of c-kit+ cells in the heart (81). In contrast, down-regulation of nucleostemin induces differentiation of CPCs in vitro, suggesting that this important protein in involved in regulating endogenous stem cells and their differentiation into cardiomyocytes downstream of Pim-1 (80,81).
Adult stem cell trials for treatment of ischemic heart diseases have led to promising results, but the incidence of engraftment and differentiation is minimal. Consequently, researchers have attributed paracrine effects of donated adult stem cells as the reason behind their protective benefits. Adult stem cells have been known to secrete a plethora of cytokines such as VEGF, bFGF and IGF which stimulate resident cells in the myocardium to mediate cardiac repair on site (22). Additionally, the enhanced secretion of growth factors such as IGF-1 and HGF-1 from injected cells or resident myocardium potentiates survival-signaling mechanisms through activation of PI3K/Akt, JAK/STAT and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathways (46). Additionally, paracrine secretion from either direct cytokine injection or stem cell transplantation mediates homing and migration of endogenous stem cells to the site of injury. BMCs are one such type of cells that have been suggested to arrive at sites of damage not only as inflammatory cells (82), but as mediators of cardiac repair by increasing cytokine secretion and possible transdifferentiation into cardiac lineages (22,34). Pim-1 has been suggested as an important kinase for survival after system damage, where bone marrow from Pim-1 global knockout mice failed to reconstitute the hematopoietic system in lethally irradiated mice (79). This result was attributed to Pim-1 not only being important in development of the animal model but for homing and migration of injected HSCs (79). Although this study focused on hematopoiesis, we believe that from this study Pim-1 may facilitate recruitment of endogenous stem cells, cardiac or bone marrow derived, through secretion of CXCL12 and activation of CXCR4 receptor (79). In future studies it would be useful to understand the impact of delivering BMCs over-expressing Pim-1 kinase to not only mediate the survival of the donated population, but to characterize the positive physiological responses that occur after injection. To this effect, delivery of Pim-1 may mediate cardiac function after injury by salvaging the myocardium though induction of homing and migration of endogenous BMC and CPC stem cell populations through a novel paracrine mechanism.

The heart after ischemic injury is a hodgepodge of inflammation and endogenous repair (82). Pim-1 activity is greatly influenced by cytokines, and in turn, Pim-1 has been shown to influence cytokine secretion by promoting localization of NFκB and subsequent production of interleukin 6 (IL-6) (83). Interestingly our lab subjected Pim-WT hearts to a qRT-PCR cytokine array comparative to non-transgenic hearts (NTG). Notably, Pim-WT hearts had increased expression of interleukin 20 (IL-20) and a significant down-regulation of pro-apoptotic cytokines such as Fas death domain (CD95) compared to NTG hearts.
(John Muraski Dissertation, 2007). It is important to address that Pim-WT hearts have limited over-expression in cardiomyocytes and CPC populations through the αMHC promoter and at basal levels, which contributed to 90% identical cytokine profiles between Pim-WT and NTG hearts (84). Since Pim-1 and its protective effects are induced by pathological injury (48), it would be interesting to assess cytokine profiles in the heart after adoptive transfer of CPCs or BMCs over-expressing Pim-1 after MI. This type of study would be important in creating a deviated analysis of the potential paracrine effects that arise from genetically modified stem cells in a disease model compared to unmodified cells.

The use of Pim-1 in order to promote proliferation and inhibit cell death in adult stem cells raises questions about the tumorigenic potential of these cells if they were to migrate systemically. Pim-1 transgenic mouse models were observed to have a great delay in the incidence of tumors and at a very low percentage (50). The role of Pim-1 as a proto-oncogene is limited by its expression, but ultimately creates a synergism with transcription factors such as c-myc (50), which further increases the rate and incidence of tumors in mouse models if these proto-oncogenes were to be deregulated. Alone, Pim-1 over-expression rarely results in tumors, although has been suggested to create a predisposed phenotype in Pim-WT mice (50). In reference to our 12-week in-vivo study, our mice, which received injection of BMCeP or BMCe, did not form tumors. Similarly, injected Pim-1 over-expressing CPCs up to 32 weeks did not show a tumorigenic phenotype (72), suggesting that Pim-1 over-expression in adult stem cells is safe for short-term use.

This study has promoted intriguing leads to the study of adult stem cells for the use of mitigating myocardial injury. Unfortunately, the in vivo injection of BMCeP did not accurately recapitulate results as previously described (48,49,72). This may be attributed to the fact that at the time of injection, BMCeP expressed significantly lower levels of Pim-1 protein compared to CPCs (72). To this end, the expression of Pim-1 in hematopoietic cells was optimized and enhanced after the in vivo study (Figure 5-6), where functional assays reveal increased positive effects of Pim-1 on BMCs (Figure1-3). Nonetheless, results demonstrated a small improvement in cardiac function with BMCe and BMCeP intramyocardial injections compared to PBS. Hemodynamic analysis was consistent with previous published results, which further supports the salutary effects of using BMCs to ameliorate myocardial damage. Unfortunately because of small group sizes and high death rates after MI, it was difficult to retroperfuse and fix hearts at earlier time points (4 weeks) in order to assess for potential mechanisms attributing improvement of cardiac function up to
12 weeks by the injection of BMCe and BMCeP into the infarcted myocardium. Overall, hemodynamic analysis was consistent with previous published results, which further supports the salutary effects of using BMCs to ameliorate myocardial damage.

The future directions of this study are to highlight the pro-proliferative and anti-apoptotic role of Pim-1 in BMCs as well as optimizing the over-expression of our protein. In order to understand the protective functional benefits of Pim-1 in BMCs, alternative strategies for genetic modification of stem cells is actively being researched in our laboratory. In this study, a viral promoter was used to express our gene of interest, but we believe that over-expression of Pim-1 could be improved using a more efficient mammalian promoter. In addition, lentiviral constructs that incorporate a selectable marker (i.e. puromycin, neomycin) will assist in sorting for infected cells in vitro, creating a pure population of Pim-1 over-expressing cell for in vivo analysis. We believe that increased Pim-1 protein levels, as well as pure population, will result in improved cardioprotective benefits after infarction compared to unmodified control cells. In addition, conclusion of the first injection experiment, has clarified the need for increased number of mice in the in vivo experimental groups in order to delineate the protective effect of Pim-1 engineered BMCs. BMCs are a reliable source of delivery of cardioprotective genes, and because Pim-1 implies a plethora of cellular responses, it is important to allude to a synergistic effect of using BMCs in combination with an over-expression system. In order to assess for this positive response of delivering BMCeP, a variety of methodologies will be employed, such as co-staining GFP for cardiac lineage markers, measuring infarct size, and extent of fibrosis by Massons Trichome. This could have tremendous implications of the regenerative response in the heart after delivery of BMCs after an infarct, through direct differentiation of injected c-kit+/GFP+ or endogenous stem cells (c-kit+/GFP-) into the cardiac, endothelial or vascular lineages. It is imperative for our laboratory to not only to continue the quest to further our knowledge of adult stem cells, both in and out of the heart, but also to take advantage of Pim-1 and it’s cardioprotective role in a disease model.
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