Cardiac Progenitor Cell Cycling Stimulated by Pim-1 Kinase

Christopher T. Cottage, Brandi Bailey, Kimberlee M. Fischer, Daniele Avitable, Brett Collins, Savilla Tuck, Pearl Quijada, Natalie Gude, Roberto Alvarez, John Muraski, Mark A. Sussman

Rationale: Cardioprotective effects of Pim-1 kinase have been previously reported but the underlying mechanistic basis may involve a combination of cellular and molecular mechanisms that remain unresolved. The elucidation of the mechanistic basis for Pim-1 mediated cardioprotection provides important insights for designing therapeutic interventional strategies to treat heart disease.

Objective: Effects of cardiac-specific Pim-1 kinase expression on the cardiac progenitor cell (CPC) population were examined to determine whether Pim-1 mediates beneficial effects through augmenting CPC activity.

Methods and Results: Transgenic mice created with cardiac-specific Pim-1 overexpression (Pim-wt) exhibit enhanced Pim-1 expression in both cardiomyocytes and CPCs, both of which show increased proliferative activity assessed using 5-bromodeoxyuridine (BrdU), Ki-67, and c-Myc relative to nontransgenic controls. However, the total number of CPCs was not increased in the Pim-wt hearts during normal postnatal growth or after infarction challenge. These results suggest that Pim-1 overexpression leads to asymmetric division resulting in maintenance of the CPC population. Localization and quantitation of cell fate determinants Numb and α-adaptin by confocal microscopy were used to assess frequency of asymmetric division in the CPC population. Polarization of Numb in mitotic phospho-histone positive cells demonstrates asymmetric division in 65% of the CPC population in hearts of Pim-wt mice versus 26% in nontransgenic hearts after infarction challenge. Similarly, Pim-wt hearts had fewer cells with uniform α-adaptin staining indicative of symmetrically dividing CPCs, with 36% of the CPCs versus 73% in nontransgenic sections.

Conclusions: These findings define a mechanistic basis for enhanced myocardial regeneration in transgenic mice overexpressing Pim-1 kinase. (Circ Res. 2010;106:891-901.)

Key Words: Pim-1 ■ Progenitor Cells ■ Asymmetric Division

Discovery of cycling progenitor cells residing in the myocardium has challenged the paradigm that the heart is a postmitotic organ. Instead, present research indicates that the heart is a self-renewing organ comprised primarily of terminally differentiated myocytes, vascular smooth muscle cells, endothelial cells together with cardiac progenitor cells (CPCs). These CPCs are c-kit+ cells, have the ability to self renew, and can differentiate into all 3 cardiac cell lineage. \(^2\) The stem cell antigen c-kit has been used to identify several types of adult stem cells including those residing in cardiac, hematopoietic, liver, brain, and pancreatic tissues. \(^3\) \(^6\) The primary characteristic of commitment to the cardiogenic lineage distinguishes c-kit+ CPCs from other stem cell types. \(^1\) \(^4\) CPCs reside within the myocardium in specialized niche structures where they self renew and produce daughter progeny that supply the heart with new myocytes and vessels, allowing for myocardial regeneration. \(^8\)

Increased generation of new cardiomyocytes in postnatal development can be stimulated by cardiac-specific expression of proliferative factors or signaling proteins leading to myocardial hyperplasia. \(^9\) \(^11\) Specifically, cardiac specific nuclear-targeted Akt expression leads to increased cycling and ultimately an increase in the CPC population, \(^11\) which may contribute to the cardioprotective effects seen when Akt is overexpressed. \(^12\) \(^15\) Akt is a nodal signaling kinase that influences multiple cellular processes including metabolism, cycling, cell growth and apoptosis. \(^16\) \(^20\) Akt exerts cardioprotective effects in concert with another serine/threonine kinase called Pim-1 that lies downstream of nuclear Akt accumulation. Pim-1 expression inhibits pathological damage and remodeling resulting from myocardial infarction (MI) and pressure overload-induced hypertrophy. \(^9\) Antia apoptotic effects of Pim-1 activity in the myocardium are linked to phosphorylation of Bad and inhibition of caspase cleavage. \(^21\)

Transgenic cardiac-specific overexpression of Pim-1, like nuclear-targeted Akt, produces postnatal myocardial hyperplasia consistent with increased cardiomyocyte or CPC cycling. In neoplastic cell types, Pim-1 activity is associated...
with enhanced cellular proliferation. Similarly, Pim-1 induction leads to enhanced proliferation of hematopoietic stem/progenitor cells downstream of STAT5 activation.22 Pim-1 exerts proliferative effects through phosphorylation of p21 on Thr145,23 stabilizing c-Myc24 and increasing MDM2-mediated degradation of p53 via the proteasome.25 Subsequent loss of p53 leads to hyperproliferative phenotypes in several cancer cell lines.26–28 Collectively these studies point toward an important influence of Pim-1 expression to increase cell cycling, but the role of Pim-1 on CPC proliferation has yet to be elucidated. To determine whether Pim-1 enhances CPC cycling, control nontransgenic mice (NTG) were compared to 3 genetically engineered mouse lines with altered Pim-1 activity: cardiac-specific overexpression of Pim-1 (Pim-wt), a kinase dead form of Pim-1 (Pim-DN),9 and Pim-1–null mice (Pim-KO). Results indicate that Pim-1 overexpression leads to substantial increases in CPC cycling during development and after MI without an increase in overall myocardial CPC population number. Reconciliation of this apparent paradox rests in the observation of increased asymmetric division in cycling CPCs found in hearts overexpressing Pim-1. Thus we find that Pim-1 overexpression leads to increased CPC cycling, which ultimately leads to a preservation of the stem cell pool.

Methods

Construction of Pim-KO and Pim Transgenic Animals and Animal Use
Creation and characterization of Pim-1 transgenic lines has been described previously9,21 with further details in the Online Data Supplement (available at http://circres.ahajournals.org). Murine surgical procedures were performed as previously described.29 All animal studies were approved by the Institutional Animal Use and Care Committee.

Immunohistochemistry, Confocal Microscopy, and Immunoblot Analysis
Formalin fixed, paraffin embedded hearts were used for immunohistochemistry as previously described11,30 with details provided in the Online Data Supplement. Immunoblot methods with antibody information are detailed in the Online Data Supplement.

Adult Cardiac Progenitor Cell Isolation, Trypan Blue Exclusion Assay, CyQuant Proliferation Assay, and MTT Assay
Adult cardiac progenitor cells were isolated from nontransgenic hearts between the ages of 8 and 12 weeks as described previously.2

Trypan blue exclusion assay used a 50% trypan blue solution with hemocytometer determination. CyQuant proliferation assay (Invitrogen no. C35007) was performed as per manufacturer instructions. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as previously described,31 with additional details in the Online Data Supplement.

Statistical Analysis
All data are expressed as means±SEM. Statistical analysis was performed using Student’s t test and ANOVA with Tukey’s post hoc as appropriate. Probability values of <0.05 were considered significant.

Results

Pim-1 Is Expressed in CPCs
Consistent with reports of Pim-1 activity in stem cells,32–34 confocal microscopy performed on myocardial sections of neonatal (2 day) nontransgenic (NTG) hearts shows Pim-1 colocalization with the progenitor cell marker c-kit (Figure 1A). Consequences of increased myocardial Pim-1 expression on the CPC pool was assessed by confocal microscopy of tissue sections from transgenic mice overexpressing Pim-1 and green fluorescent protein (GFP) downstream of the α-myosin heavy chain promoter (Pim-wt).21 Immunolabeling for c-kit and GFP in myocardial sections from Pim-wt hearts (Figure 1B) show colocalization of c-kit and GFP in both 2-day-old (61% colocalization) and 2-week-old (51% colocalization) hearts consistent with α-myosin heavy chain promoter activity in CPCs.35 Pim-1 expression in CPCs is corroborated by immunoblots of CPCs isolated from NTG and Pim-wt hearts, which are immunoreactive for the transgenically encoded 34 kDa Pim-1 protein (Figure 1C). CPC lysates are negative for sarcomeric desmin, indicating no cardiomyocyte contamination. Collectively, these results indicate that c-kit+ cells of cardiac origin express Pim-1 and that expression of Pim-1 as a transgenic protein can be detected in CPCs isolated from murine lines engineered with cardiac-specific overexpression of Pim-1 protein.

Pim-1 Stimulates Cardiac Progenitor Cell Cycling In Vitro and In Vivo
Consequences of Pim-1 overexpression on DNA content, CPC viability, and metabolic activity were measured in vitro using CyQuant DNA content assay, trypan blue exclusion assay, and tetrazolium salt (MTT) colorimetric reduction assay with cultures derived from NTG or Pim-wt hearts. Pim-wt CPCs show significantly increased DNA content following culture for 1 or 5 days by CyQuant assay (4.3- and 2-fold, respectively; Figure 2A). CPC viability was assessed in cultures seeded with 20,000 cells from NTG or Pim-wt hearts followed by trypan blue exclusion assay, which reveals that the number of Pim-wt CPCs increase at a significantly greater rate than NTG (3.8-fold at day 2 and 4.4-fold at day 4; Figure 2B). Involvement of Pim-1 activity in cell division and metabolic activity of CPC cultures was confirmed by subsequent treatment with the Pim-specific inhibitor quercetin (10 μmol/L) leading to significantly less DNA incorporation (Figure 2C). Pim-wt CPCs also show significantly higher metabolic activity compared to NTG CPCs at 3 days (1.7-fold) after plating, and lower MTT conversion following Quercetin exposure (Figure 2D) indicative of enhanced
proliferation. Levels of c-Myc were increased 3-fold in Pim-wt CPCs and reduced by 63% with treatment of Quercetin (Figure 2E), consistent with previous studies linking c-Myc stabilization and increased cycling of neoplastic cells with overexpression of Pim-1.24 Increases in phosphorylation of p21 at Thr145, a direct target of Pim-1, and increased levels of Cyclin E relative to NTG CPCs are findings consistent with enhanced proliferative signaling in Pim-wt CPCs (Online Figure I). During mitosis, nuclear mitotic apparatus protein (NuMA) organizes and tethers microtubules to the spindle poles and has been shown to interact with Pim-1.36 Therefore, the localization of NuMA was investigated; enhanced proliferative signaling in Pim-wt CPCs does not correlate to changes in NuMA localization (Online Figure II).

Cell cycling in vivo was examined in NTG and Pim-wt hearts with Ki-67, PCNA, and c-kit immunolabeling of myocardial sections. Coincident Ki67+/c-kit+ labeling is significantly increased in postnatal Pim-wt hearts at 2 days (58.1% versus 41.5%) and 2 weeks (52.4% versus 26.8%) after birth compared to NTG. (Figure 3A; *P<0.05). In comparison, cycling CPC numbers were significantly decreased in myocardial sections from Pim-DN hearts at both 2 days (64.7%) and 2 weeks (55.3%) relative to NTG samples. Significant increases in PCNA+/c-kit+ cells at 2 days and 2 weeks of age (1.7- and 1.8-fold, respectively) confirms enhanced cell cycling in Pim-wt CPCs (Figure 3B). These effects on the proportion of cycling CPCs are consistent with proproliferative effects of Pim-1 in the postnatal myocardium.

To further assess physiological CPC dynamics within the developing myocardium the number of apoptotic CPCs was determined. Figure 3C demonstrates that NTG hearts have significantly more apoptotic CPCs than Pim-wt at 2 days and 2 weeks (9.75- and 5-fold respectively). Expansion of the CPC population resulting from altered Pim-1 expression was assessed by quantitation of c-kit+ cells in the left ventricles at 2 days, 2 weeks, and 3 months of age. Indeed, the number of CPCs was significantly decreased in the Pim-KO (2.1-fold less at 2 days and 3-fold less at 2 weeks), indicating that loss of Pim-1 by genetic deletion impairs CPC production. Of note however is a significant increase in PCNA+/GATA4+ cells at 2 days in the Pim-KO hearts (2.6-fold, Online Figure III) possibly in an attempt to retain homeostasis caused by the lack of CPC cycling. Curiously, an increase in total number of c-kit+ cells versus the NTG controls was not observed in Pim-wt hearts despite evidence of increased CPC cycling (Figure 3). Thus, Pim-1 expression increases the frequency of cycling CPCs (Ki67+/c-kit+) without increasing the population of CPCs (c-kit+). The increased number of CPCs observed in the Pim-DN heart relative to NTG controls (1.7-fold; Figure 3D) may reflect increased recruitment of the CPC pool in response to cardiomyopathic effects of the Pim-DN construct.9 Clearly, these results imply Pim-1-mediated effects on the CPC pool, but straightforward interpretation of these findings is challenging because of the multifaceted nature of Pim-1 mediated effects that necessitated further experiments to unravel.

**Pim-1 Colocalizes With but Does Not Increase the Number of CPCs Following MI**

Previous studies showed elevated levels of Pim-1 in cardiomyocytes following pathological challenge.9 Consistent
with the accumulation of c-kit+ CPCs following infarction2. Pim-1 colocalizes with c-kit+ cells in myocardial sections of NTG as well as Pim-wt mice at 7, 10, and 21 days postinfarction (Figure 4A through 4C). The impact of Pim-1 expression on accumulation of CPCs in the border zone surrounding MI was assessed at 7, 10 and 21 days following challenge. Despite severe damage from coronary ligation assessed by infarct size (Online Figure IV, A) the number of accumulated CPCs in the border zone region is comparable between heart samples of Pim-wt versus NTG at all 3 time points examined (Figure 4D). In comparison, myocardial sections from Pim-KO mice subjected to infarction show significantly fewer accumulated CPCs at the one week time point (2-fold); samples at later stages were unavailable because Pim-KO mice were unable to survive for more than one week postinfarction. Analysis of apoptotic CPCs by TUNEL staining revealed fewer TUNEL+ CPCs in Pim-wt hearts 10 days and 3 weeks after infarction (Online Figure IV, B). Thus, although loss of Pim-1 may impair the CPC response to infarction, the cardiac-specific overexpression of Pim-1 does not provide an increased benefit through increasing total CPCs generated in response to infarction.

**Pim-1 Stimulates CPC Cycling After MI**

Because the primary effect of cardiac-specific Pim-1 overexpression is to expand the number of cycling CPCs in the transgenic heart (Figure 3), myocardial sections from Pim-wt hearts were immunolabeled to assess the quantity of cycling CPCs following infarction challenge. CPCs with coincident labeling for Ki-67+/c-kit+ (Online Figure IV, yellow arrows) and PCNA+/c-kit+ cells in the border zone surrounding the infarct region were quantitated at 7, 10, and 21 days after infarction. Indeed, Pim-wt hearts had a significant increase in the percent of Ki-67+/c-kit+ (2.1-, 2.24-, and 1.80-fold, respectively) and PCNA+/c-kit+ (1.98-, 2.05-, and 1.59-fold, respectively) CPCs versus NTG controls at all 3 time points (Figure 5A, B).
through 5F). In contrast, Pim-KO mice show a drastic decrease in cycling c-kit$^+$ cells seven days after infarction versus NTG controls (1.7-fold). In addition to increased CPC cycling Pim-wt hearts exhibit increased myocyte cycling measured by Ki-67$^/$c-kit$^+$ and PCNA$^/$c-kit$^+$ myocytes (Online Figure VI, A [yellow arrow]; Online Figure VI, B through E). In addition, Pim-wt hearts contain small myocytes with longer telomeres (2.3-fold longer), indicative of new myocytes of CPC origin (Online Figure VI, F). These findings are consistent with earlier results (Figures 2 and 3) that point toward a role for Pim-1 in expansion of the cycling CPC population.

Pim-1 Stimulates 5-Bromodeoxyuridine Incorporation in CPCs After MI

CPCs undergoing DNA synthesis as defined by coincidence of 5-bromodeoxyuridine (BrdU)$^/$c-kit$^+$ immunolabeling (Figure 5G) were quantitated in myocardial sections from sham operated versus infarcted hearts. Consistent with findings of increased Ki-67$^+$ and PCNA$^+$ CPCs in Pim-wt hearts following infarction challenge Pim-wt hearts possess significantly more BrdU$^+$/c-kit$^+$ CPCs (1.7-fold; Figure 5H). Pim-wt hearts also show a significant increase of BrdU$^+$ nuclei (8.3-fold increase overall versus NTG) including BrdU$^+$/tropomyosin$^+$ labeled cardiomyocytes (4.4-fold increase; Online Figure VII).

Pim-1 Expression Promotes Asymmetric Division of CPCs Following Infarction

The conundrum of increased cycling CPCs without expansion of the CPC population could be reconciled by increased frequency of asymmetric division in the presence of Pim-1 overexpression. Frequency of asymmetric cell division in CPCs was assessed using cell determinant markers α-adaptin and Numb (Figure 6A and 6B). Asymmetric division is characterized by sequestration of Numb or α-adaptin immunolabeling lateralized to one side of the mitotic cell, whereas symmetric division of CPCs is characterized by uniform α-adaptin and Numb distribution.7 Cycling CPCs are identified by coincident phospho-histone$^/$c-kit$^+$ immunolabeling and colocalized with the cell determinant markers. With respect to asymmetric division, Numb sequestration is significantly greater in cycling CPCs from Pim-wt mice relative to NTG samples (65% versus 26%, respectively; Figure 6C). Alternatively, symmetric division evidenced by uniform α-adaptin labeling is higher in NTG CPC relative to Pim-wt (73% versus 36%, respectively; Figure 6D). Collectively, these results indicate that cardiac-specific Pim-1 overexpression leads to increased asymmetric division of the CPC population in response to infarction challenge.

Discussion

Collectively, this report delineates a previously unrecognized mechanistic cellular basis for the enhanced resistance to cardiomyopathic injury observed in mice created with cardiac-specific overexpression of Pim-1 kinase.21 In combination with the previously detailed prosurvival properties of Pim-1,9,21,33 the multifaceted consequences of Pim-1 actions seem well suited to the task of augmenting CPC regenerative
potential. The capacity of Pim-1 to influence the CPC population through increased cycling and asymmetric division would be a valuable molecular interventional approach to potentiate CPC-based regeneration following myocardial injury by preserving the CPC pool as well as providing cardiogenic daughter cells. With the advent of regenerative medicine new possibilities are being explored to mediate myocardial repair and cellular replacement, but our understanding of CPC biology lags far behind the comparatively rapid implementation of adoptive transfer studies in experi-

Figure 4. Colocalization of Pim-1 with CPCs in pathologically challenged myocardium. Hearts subjected to infarction challenge and processed for immunohistochemistry at time points of 7 (A), 10 (B), and 21 (C) days postinfarction. Samples for NTG (left column) immunolabeled for c-kit (red), Pim-1 (green), tropomyosin (blue), and Topro (white). Samples for Pim-wt (right column) immunolabeled for c-kit (red), GFP (green), tropomyosin (blue), and Topro (white). Coincidence of Pim-1 or GFP with c-kit is shown for each image (inset in yellow box). D, c-kit+/mm² of left ventricles (n=4). *P<0.05 vs NTG by Student’s t test.
Figure 5. Pim-1 stimulates CPC cycling after MI. 

A through C, Percentage of CPCs with coincident immunolabeling for Ki-67/c-kit at 7, 10, and 21 days postinfarction from NTG, Pim-wt, and Pim-KO as indicated. 

D through F, Percentage of CPCs with coincident immunolabeling for PCNA/c-kit at 7, 10, and 21 days postinfarction from NTG, Pim-wt, and Pim-KO as indicated. *P<0.05 vs NTG, **P<0.01 vs NTG by Student’s t test (n=4 per group; error bars are SEM).

G, Representative confocal images of CPCs from sections of infarct zones within a NTG or Pim-wt heart. Enlarged images of yellow-boxed areas depict typical CPCs positive for BrdU in the infarct zone. Sections were immunolabeled with c-kit (red), BrdU (green), Tropomyosin (blue), and Topro (white). Cells representing BrdU-/c-kit- (arrows) and BrdU-/c-kit+ (arrowheads) are indicated. 

H, Percentage of BrdU-/c-kit- cells quantitated from left ventricular samples of NTG or Pim-WT hearts 7 days postinfarction. *P<0.05 vs Pim-wt by Student’s t test (n=3; error bars are SEM).
mental animal models and the clinical setting.\textsuperscript{37,38} Although not surprising that such studies are being pursued, it is similarly predictable that the underlying mechanistic basis for salutary effects observed remains in debate.\textsuperscript{1,39–41} Virtually all studies show modest engraftment, persistence, proliferation, and survival of CPCs on adoptive transfer into infarcted myocardium. Manipulation of cellular signaling to expand the CPC pool, enhance survival, and promote repopulation of damaged tissue is an attractive approach to augment the limited regenerative potential that currently hampers cellular-based intervention strategies for myocardial repair.

Pim-1 influences cell proliferation in cancer and hematopoietic stem cells. Pim-1 phosphorylates heterochromatin protein-1 and NuMA, which are crucial in spindle fiber assembly and subsequent cell division during HeLa cell mitosis.\textsuperscript{36} Phosphorylation of NuMA leads to its translocation to the spindle pole where it anchors microtubule (\(\alpha\)) ends as a critical part of the chromosomal segregation process.\textsuperscript{42} Pim-1 cooperates with other cell cycle proteins such as c-Myc, which is stabilized by Pim-1 activity thereby promoting enhanced cell cycling.\textsuperscript{24} In stem cells, Pim-1 is implicated in the antiapoptotic and hyperproliferative phenotypes of hematopoietic progenitor cells.\textsuperscript{22} Hematopoietic progenitor cells overexpressing STAT5 induce Pim-1 protein expression, resulting in significantly enhanced proliferation.\textsuperscript{23} Proliferation is also enhanced by Pim-1 mediated phosphorylation of the cell cycle inhibitor p21,\textsuperscript{43} leading to cytoplasmic sequestration of p21 and inability to interact with cyclinE/cdk2 in the nucleus.\textsuperscript{44} The proliferative phenotype of transformed cell lines such chronic myelogenous leukemia cells K562 and BV173 is associated with increased Pim-1 expression in G1/S phase that is maintained at high levels through S phase until the end of M phase.\textsuperscript{45} Collectively, these observations indicate that Pim-1 activity enhances proliferative activity when present in cycling cells, as appears to be the case in the study where Pim-1 is regulated by the \(\alpha\)-myosin heavy chain promoter in CPCs.

The \(\alpha\)-myosin heavy chain promoter has been exploited to overexpress several signaling molecules in the heart of transgenic mice.\textsuperscript{46–51} Cardiac overexpression of proliferative and antiapoptotic molecules such as IGF-1, cyclin D2, Bcl-2, and nuclear Akt lead to a hyperplastic phenotype and are cardioprotective.\textsuperscript{10,11,46,51} Cardiac-specific transgenic overexpression of nuclear Akt was found to increase cell cycling, thereby expanding CPCs, indicating this population is influenced by activation of the \(\alpha\)-myosin heavy chain promoter (demonstrated elsewhere\textsuperscript{11,12,35}). However, because Akt is a nodal kinase with several biological functions including survival, proliferation, gene transcription, protein translation, metabolism, and differentiation,\textsuperscript{16} beneficial effects of Akt overexpression can be accompanied by deleterious consequences.\textsuperscript{52–54} Thus, Pim-1 with a relatively narrow spectrum of biological effects limited to cell proliferation and survival would seem a much more suitable cardioprotective molecular target for therapeutic interventional strategies, including expansion of the CPC population to promote repair and regeneration in the wake of pathologic injury.

CPCs in the adult mouse heart favor asymmetric division in an attempt to maintain cardiac homeostasis and replenish myocardial cells rather than constant self-renewal.\textsuperscript{7} Similarly, asymmetric cell division creates cell type diversity during early mammalian development as observed in neuroblasts and embryonic stem cells.\textsuperscript{55–57} Cancer stem cells use normal stem cell

![Figure 6. Pim-1 expression correlates with increased asymmetric division of CPCs.](image-url)
characteristics, including asymmetric cell division to evade chemotherapy and promote growth. However, in the context of the myocardium that is notoriously resistant to oncogenic transformation, amplifying the inherent ability of CPCs to divide asymmetrically may prove a useful tool in regenerative medicine.

Taking our findings with myocardial Pim-1 expression together in the context of the literature, a hypothetical model is proposed wherein overexpression of Pim-1 leads to increased CPC cycling during hyperplastic growth occurring during pre/postnatal development and after infarction induced stress (Figure 7). Increased cycling in Pim-wt CPCs correlates with elevated levels of c-Myc in the CPCs (Figure 2E) and previous reports of c-Myc levels decline in the adult heart, c-Myc levels are relatively low and CPCs exhibit basal proliferative rates to replace cells lost because of normal aging. However, postnatal growth or cardiomyopathic injury characteristics, including asymmetric cell division to evade chemotherapy and promote growth. However, in the context of the myocardium that is notoriously resistant to oncogenic transformation, amplifying the inherent ability of CPCs to divide asymmetrically may prove a useful tool in regenerative medicine.

References


Novelty and Significance

What Is Known?

- Pim-1 is a cardioprotective kinase that inhibits cell death and cardiomyocyte hypertrophy induced by pathological injury.
- Myocardial regeneration is enhanced using cardiac stem cells genetically engineered to overexpress Pim-1.

What New Information Does This Article Contribute?

- Pim-1 overexpression in the heart increases proliferation of cardiac progenitor cells during postnatal growth or after myocardial infarction.
- Pim-1–mediated increases in cardiac progenitor cell proliferation are mirrored by an elevated rate of asymmetric cell division that produces more cardiogenic cells to populate the myocardium.

The regulatory mechanisms governing cardiac progenitor cell growth and lineage commitment are poorly understood but are critically important issues in developing therapeutic strategies for enhancing regenerative and reparative processes in the damaged heart. The cardioprotective properties of Pim-1 kinase activity render the heart resistant to injury, but the involvement of cardiac progenitor cells remains undetermined. In this study, we demonstrate that Pim-1 enhances cardiac progenitor cell cycling without increasing the progenitor cell population during physiological growth and after myocardial infarction. This apparently paradoxical outcome is reconciled by concomitant increases in progenitor cell asymmetric division in hearts overexpressing Pim-1. Higher rates of asymmetric division coupled with proliferation maintain the progenitor cell population while generating de novo cardiogenic differentiated daughter cells, which likely accounts in part for enhanced resistance to pathological injury in Pim-1 overexpressing transgenic mice. These findings, in conjunction with the ability of Pim-1–engineered cardiac progenitor cells to mediate enhanced regeneration in the damaged heart, suggest that Pim-1 kinase is an important target for therapeutic strategies aimed at augmenting the limited reparative potential of cell-based approaches in damaged myocardium.
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In the *Circulation Research* article by Cottage et al (Cardiac Progenitor Cell Cycling Stimulated by Pim-1 Kinase. *Circ Res*. 2010:106:891–901), a correction was needed.

The fourth author’s name was spelled incorrectly. The name should read “Daniele Avitabile”.

The authors apologize for the error, and the correction appears in the online version of the article, which is available at http://circres.ahajournals.org/content/106/5/891.full
Supplemental Methods

Transgenic Animals
Litter-mates for the non-transgenic (NTG) strain were cross-bred to maintain the FVB colony (FVBN/J, Jackson Labs, Stock # 001800). Both Pim-WT and Pim-DN transgenic mice were created in the FVBN/J background. Embryos at 0.5 days post coitus (dpc) were extracted from FVBN/J females (Jackson Labs) crossed with FVBN/J males (Jackson Labs), injected with Pim-WT or Pim-DN transgenes at a concentration of 2ng/uL, and transferred into ICR pseudo-pregnant females. Pups were screened for the presence of the transgene, weaned at three weeks of age and labeled as founders. Founders were backcrossed into FVBN/J males or females (Jackson Labs), the F1 generation was used to check for vertical transmission and protein expression. The positive littermates from the F1 generation were crossed for ten generations to stabilize the lines used for experiments. Pim-wt mice were constructed to overexpress the human 34 kd Pim-1 and GFP in a bicistronic format downstream of the αMHC promoter.

Immunohistochemistry
Animals were sacrificed at time points described in the text; the abdominal aorta was cannulated and perfused with phosphate buffered heparin. Following perfusion the heart was arrested in diastole by the injection of CdCl2 through the aortic cannula. Hearts were then flushed with formalin for 15 minutes, excised and fixed in formalin for 24 hours at room temperature. For paraffin processing, heart samples were placed in an automated tissue processor. Samples were then embedded and sectioned at a thickness of 4µm. Slides were deparaffinized and rehydrated using xylene and decreasing amounts of alcohol ending with a wash in deionized water. Antigen retrieval was performed using 10mM citrate, pH 6.0, for 15 minutes at 50% power in an 1100 W microwave. Slides were allowed to cool to room temperature and washed several times in distilled water. Next endogenous peroxidase activity was quenched using 3% H2O2 in DI water for 20 minutes at room temperature. Slides were then washed three times with DI water, twice with 1X TN (150mM NaCl, 100mM Tris, pH 7.5) and blocked with TNB (1X TN and blocking reagent supplied by Perkin Elmer) for 1 hour. Primary antibodies were applied overnight at 4°C in TNB. The following day the slides were washed three times in 1 x TN and secondary antibodies were applied for 2 hours at room temperature in TNB. Several signals required detection using a Tyramide Signal Amplification (TSA) kit (Perkin Elmer) following manufacturers recommendations. Samples requiring multiple Tyramide amplifications on one slide were quenched in 3% H2O2 for 20 minutes at room temperature between detection of peroxidase, washed then incubated with Streptavidin horseradish peroxidase (HRP) 1:100 in TNB for 1 hour, washed and developed with tyramide substrate 1:50 for 10 minutes. Once slides were developed in tyramide substrate they were washed and coverslipped using VectaShield mounting media. In order to visualize nuclei, slides were treated with To-pro3 Iodide (Topro), Molecular Probes, at 1:10,000 for 20 minutes in 1X TN, washed and cover slipped. Micrographs were acquired using a Leica TCS-SP2 confocal laser scanning microscope.

c-kit, Ki-67, Pim-1, and GFP required an additional Tyramide amplification. Kits were obtained from Perkin-Elmer (Sat701001) and were performed according to the manufacturer’s protocol. All stainings were accompanied by a section not given primary antibody as a negative control, when inspected these slides had no staining indicating specific staining for that primary antibody and not non-specific signal from the secondary antibody or subsequent Tyramide amplification. Quantitation was performed on the left ventricles of hearts in paraffin wax. Three sections per heart were counted with each section being at least 15µm away from the last to ensure that the same cell was not counted twice. For the Numb and Adaptin counts over five hundred c-kit+/phistone+ cells were counted per mouse line over 3 hearts each.

Western Blot Analysis
Whole cell lysates isolated from cardiac progenitor cells were prepared in 1X SDS sample buffer. Lysates were sonicated briefly then boiled for 5 minutes and loaded or stored at -80°C.
The amount of protein was estimated using Bradford protein quantitation assay and equal amount of protein were loaded into an Invitrogen 4-12% Tris-Glycine mini-gel and run at 200V for 1 hour on an invitrogen electrophoresis apparatus. Separated proteins were transferred to a PVDF membrane pre-incubated in methanol then blocked for 1 hour with 5% dry milk in TBS-T (50 mmol/liter Tris-HCl (pH 7.6)/150 mM NaCl/ 0.1% Tween 20). After transfer the membrane was probed with primary antibodies overnight at 4°C with gentle agitation in blocking buffer. Primary antibodies consisted of GAPDH (Invitrogen), GFP (Invitrogen), Pim-1 1:500 (Cell Signaling Technology), c-Myc (Sigma), Cyclin E (Abcam) and pT145-p21 (Santa Cruz). The next day blots were washed with TBS-T three times, probed with fluorescent, HRP, or alkaline phosphatase-conjugated secondary antibodies 1:2000 in blocking solution (Jackson Labs) for 2 hours at room temperature. Following three washes with TBS-T blots were scanned using a Typhoon 9410 (GE Healthcare) and signal quantitated using ImageQuant software.

Adult cardiac progenitor cell isolation and MTT Assay
CPCs are isolated using two mice per preparation they are anesthetized using ketamine-xylazine solution then the heart was cannulated through the aortic arch and perfused at 37°C in oxygenated basic buffer (J-MEM, 0.7 g/L hepes, 1.25g/L taurine, 20units/L insulin, Pen/Strep/Glutamine, Amphotericin, Gentamicin, pH 7.3) on a Radnotti apparatus. The heart was then digested for 12 minutes at 37°C in 320units/mL of collagenase II in oxygenated basic buffer. Afterwards, the heart was then minced in basic buffer +0.5% BSA and the cardiomyocytes pelleted for 1 min at 100g and discarded. Remaining cells in the supernatant were passed through a 25mM filter and pelleted. The cell pellet was resuspended and incubated with anti-c-kit (CD117) Miltenyi beads in PBS + 0.5% BSA; washed and then isolated on a magnetic column to extract c-kit+ CSCs according to manufacturer’s instructions. CSCs are cultured according to standard tissue culture protocols in CSC media (DMEM/F12, 10% Embryonic Stem Cell-Grade FBS, PSG, Insulin-Transferrin-Selenium, 1000u/mL LIF, 40ng/mL EGF, 20ng/mL bFGF). On average less than one hundred cells are originally plated, a small percentage of these survive and are expanded by serial passaging. Cells were passaged at least ten passages before experiments were performed. MTT reagent was obtained from Fluka (catalog #88417) cells were plated at 5000 cells per well in 96-well flat bottom plates. Cells were incubated with 10µl of a 5mg/ml solution of MTT reagent for 4-5 hours, then incubated with 100µl of stopping reagent (.1N HCl + 10% SDS) overnight at 37°C. Plates were read on a spectrophotometer at 570nm to measure signal.

Quercetagetin administration: 5000 CPCs were treated with 10µM of inhibitors of Pim-1 kinase activity (R&D Chemicals cat. #1030) at three days after plating, cycling was assessed by MTT and Cyquant assay.

Progenitor Cell Counts
The numbers of c-kit+ cells were counted in hearts at time points described in the text. Three left ventricular sections at least 15 µm apart were counted in at least three hearts and the average number of c-kit+ cells was then determined per heart. The number of c-kit+ cells was standardized to the total area of the heart using Leica LCS Lite (Leica AG) software.

Cycling Progenitor Cell Counts
The number of cycling progenitor cells was measured by counting the number of cells positive for Ki-67, PCNA, and c-kit in the left ventricle. The percentage of Ki-67+ and PCNA+ cells co-localizing with c-kit+ cells was determined as described above.

Myocardial Infarction and Bromodeoxyuridine (BrdU) Injections
Myocardial infarction was induced in NTG and Pim-wt male mice between 8 and 10 weeks of age. Mice were anesthetized with isoflurane (Abbot, Chicago, IL) prior to permanent occlusion of the left anterior descending coronary artery. Cyanosis and akinesia of the affected tissue confirmed infarction. 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. Lack of anterior wall motion in conjunction with at least 40% decrease in EF and FS were required for study inclusion. Forty-eight hours after MI mice were given intra-peritoneal injections of BrdU (Sigma), at 50-mg/kg-body weight for 5 days twice a day. On the seventh day after MI mice were sacrificed as described in the immunohistochemistry section. The
percentage of BrdU+ cells co-localizing with c-kit were analyzed as described above in these hearts 7 days post MI.

**TUNEL Labeling**
Slides were deparaffinized then labeled for TUNEL using the in situ Cell Death Detection Kit, TMR red (Roche Applied Science), manufacturer's instructions were followed except tdt enzyme was diluted ½ with TUNEL dilution buffer (Roche Applied Science).

**Telomere Length Measurements**
Telomere length was analyzed by in situ hybridization (Q-FISH) and confocal microscopy. PNA probe was purchased from DAKO (K5325) and manufacturers protocol was followed. Telomere signal was acquired in each nucleus and divided by Topro 3 signal to account for differences in nuclear size.
Supplement Figure I. Pim-wt CPCs possess enhanced markers of proliferation. pThr145 p21 and cyclin E levels in CPCs isolated from NTG and Pim-wt hearts. *p<0.05 by Student’s t-test.

Supplement Figure II. Pim-1 overexpression does not alter mitotic spindle fiber assembly. Immunocytochemistry of NTG and Pim-wt CPCs fixed during metaphase and anaphase. CPCs are stained with Pim-1 (green), Numa (red), Tubulin (purple), and DNA (blue, Sytox,Molecular Probes).
Supplemental Figure III. Cardiac cell cycling during postnatal development. Quantitation of PCNA+/GATA4+ cells in the left ventricles of developing myocardium. Four views of at least 100 cells per view were analyzed in 3 hearts per group. Over 1000 cells were analyzed per sample group, *p<0.05.
Supplement Figure IV. Pim-1 overexpression is cardioprotective after myocardial infarction. A-C. Quantitation of surviving myocardium in the left ventricular free wall (LVFW) assessed by immunohistochemistry staining for desmin. Pim-wt hearts have significantly smaller infarct sizes at all time points *p<0.05. D-F. Quantitation of Tunel+ CPCs 7, 10, and 21 days after infarction. G. Example of sham operated echo and echo of mouse included in the study three days after infarction.
Supplement Figure V. Cycling CPCs post infarction.
A. Representative confocal images of CPCs from sections of infarct zones within a NTG or Pim-wt heart 7 days post infarction or B. 10 days post infarction. Samples were immunolabeled with c-kit (red), Ki-67 (green), Tropomyosin (blue) and Topro (white). Coincidence of Ki-67 and c-kit is shown for each image (inset in yellow box). Yellow arrows indicate co-localization of Ki-67 and c-kit; yellow arrowheads indicate co-localization of Ki-67 and Tropomyosin.
Supplement Figure VI. Pim-1 stimulates cardiomyocyte cycling. A. Representative confocal images of cardiomyocytes (tropomyosin+) from sections of infarct zones within a NTG or Pim-wt heart 3 weeks post infarction stained for Ki-67 (green), Tropomyosin (red), and Topro (blue). B. Quantitation of PCNA+ myocytes per mm² at 7 days, 10 days C, and 21 days D after infarction. E. Quantitation of Ki-67+ myocytes per mm² 3 weeks after infarction *p<0.05. F. Telomere length measurements 3 weeks after infarction, all myocyte sizes are in µm². * p<0.05 versus NTG of equivalent size, #p<0.01 versus Pim-wt 401-600µm². Significance between two groups was determined by Student’s t-test.
Supplement Figure VII. Pim-1 promotes BrdU incorporation. A. Confocal micrographs of sections stained for BrdU (green), Tropomyosin (red), and Topro (blue) at low magnification and B at high magnification. Coincidence of BrdU and Tropomyosin c-kit is shown (inset in yellow box). Yellow arrows represent BrdU+/Tropomyosin+ cells. C. and D. Quantitation of the number of BrdU+ nuclei (C) and BrdU+ /Tropomyosin+ myocytes (D) in NTG and Pim-wt 7-week-old hearts. *p<0.05 and ** p<0.01 vs. NTG by students t-test.

Table I. Numbers of Mice used in experiments:

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<th>Procedure/Experiment</th>
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