Rejuvenation of Human Cardiac Progenitor Cells With Pim-1 Kinase

Sadia Mohsin, Mohsin Khan, Jonathan Nguyen, Monique Alkatib, Sailay Siddiqi, Nirmala Hariharan, Kathleen Wallach, Megan Monsanto, Natalie Gude, Walter Dembitsky, Mark A. Sussman

Rationale: Myocardial function is enhanced by adoptive transfer of human cardiac progenitor cells (hCPCs) into a pathologically challenged heart. However, advanced age, comorbidities, and myocardial injury in patients with heart failure constrain the proliferation, survival, and regenerative capacity of hCPCs. Rejuvenation of senescent hCPCs will improve the outcome of regenerative therapy for a substantial patient population possessing functionally impaired stem cells.

Objective: Reverse phenotypic and functional senescence of hCPCs by ex vivo modification with Pim-1.

Methods and Results: C-kit–positive hCPCs were isolated from heart biopsy samples of patients undergoing left ventricular assist device implantation. Growth kinetics, telomere lengths, and expression of cell cycle regulators showed significant variation between hCPC isolated from multiple patients. Telomere length was significantly decreased in hCPC with slow-growth kinetics concomitant with decreased proliferation and upregulation of senescent markers compared with hCPC with fast-growth kinetics. Desirable youthful characteristics were conferred on hCPCs by genetic modification using Pim-1 kinase, including increases in proliferation, telomere length, survival, and decreased expression of senescence markers.

Conclusions: Senescence characteristics of hCPCs are ameliorated by Pim-1 kinase resulting in rejuvenation of phenotypic and functional properties. hCPCs show improved cellular properties resulting from Pim-1 modification, but benefits were more pronounced in hCPC with slow-growth kinetics relative to hCPC with fast-growth kinetics. With the majority of patients with heart failure presenting advanced age, infirmity, and impaired regenerative capacity, the use of Pim-1 modification should be incorporated into cell-based therapeutic approaches to broaden inclusion criteria and address limitations associated with the senescent phenotype of aged hCPC. (Circ Res. 2013;113:1169-1179.)

Key Words: aging ■ cell cycle proteins ■ heart failure ■ telomere lengthening

The long-held belief of the heart as post mitotic organ has been toppled by demonstration of myocyte turnover throughout life and the existence of adult cardiac progenitor cells. Human cardiac progenitor cells (hCPCs) are multipotent and capable of forming the 3 requisite cardiogenic lineages needed to form myocardium. hCPCs isolated from failing human hearts augment cardiac function and myocardial regeneration in the clinical setting after adoptive transfer into pathologically injured myocardial tissue as demonstrated in the Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO) trial.

The SCIPIO phase I trial was designed primarily to address feasibility and safety end points rather than therapeutic benefit, making the observation of functional and structural improvement in patients even more remarkable. However, a significant unresolved issue in SCIPIO, as well as other early clinical trials for autologous cellular treatment of heart failure, is the relatively narrow inclusion/exclusion criteria of patient eligibility to participate. Concurrent presentation of heart failure with comorbidities of chronic disease (eg, diabetes mellitus, obesity, hypertension,), habitual stressors (eg, smoking, poor diet, alcoholism), or genetic predisposition contributes to impaired reparative capacity, leaving patients with such conditions with little hope to benefit from current gains in cellular regenerative therapy. The ability of hCPC to maintain cardiac repair is significantly compromised by several factors, including genetics, progression and disease pathogenesis, heart load, medication, and aging.

Advanced age is a primary risk factor...
Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>hCPCs</td>
<td>human cardiac progenitor cells</td>
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<tr>
<td>hCPCs-eGFP</td>
<td>human cardiac progenitor cells expressing enhanced green fluorescent protein</td>
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<tr>
<td>hCPCs-F</td>
<td>fast-growing human cardiac progenitor cells</td>
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<tr>
<td>hCPCs-Pim</td>
<td>Pim-1–modified human cardiac progenitor cells</td>
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<tr>
<td>hCPCs-S</td>
<td>slow-growing human cardiac progenitor cells</td>
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<td>LVAD</td>
<td>left ventricular assist device</td>
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for developing heart failure, and hCPCs in aged patients with heart failure are characteristically senescent with limited replicative capacity blunting cellular replacement and accelerate the aging process caused by the accumulation of large and poorly contracting myocytes.11

Reparative ability of CPCs can be significantly enhanced by molecular interventional approaches to increase survival, proliferation, engraftment, commitment, and persistence.13,14 Superior regeneration and functional improvement mediated by human CPCs modified with Pim-1 kinase relative to identically expanded but nonmodified CPCs were demonstrated using cells isolated from an aged patient with heart failure at the time of left ventricular assist device (LVAD) implantation in an murine myocardial infarction model.14 Pim-1 kinase enhances proliferation,15 metabolic activity,14 differentiation15 and involves in neovascularogenesis16 and neomyogenesis that forms an integral part of cardiac repair and regeneration response. Pim-1 also serves as a prosurvival role by preserving mitochondrial integrity17,18 and antagonizing intrinsic apoptotic cascades.19 Moreover, Pim-1 preserves telomere length and telomerase activity consistent with a youthful cellular phenotype.14 Although ex vivo genetic modification of hCPC to augment regenerative potential is a viable option, reproducibility of Pim-1–mediated cellular enhancement in the context of a heterogeneous patient population with heart failure remains to be demonstrated.

There is more to aging than the chronologic measure of time passage. Cellular aging is reflected by a constellation of molecular and phenotypic changes consistent with acquisition of senescence. Biological age of a cell is most commonly assessed by telomere length, which is affected by multiple factors, including mitotic activity, environmental stress, and genetic damage. Clear correlations between cardiovascular disease risk and telomere length highlight the importance of studying characteristics of biological aging on a molecular level to comprehend critical relationships between cellular phenotype, functional behavior, and preservation/restoration of myocardial health.20,21 Shortening of telomeres is associated with cellular arrest, acquisition of a senescent phenotype, and even cell death22 that all contribute to the loss of regenerative potential with age. Indeed, aging itself can be considered, in part, a stem cell–mediated disease.23 The loss of regeneration and reparative capacity inevitably leads to inability to cope with chronic stress or acute pathological injury. Reduction in hCPC ability to repair myocardium attributable to patient age, and pathology represents a serious problem and needs to be addressed before extending the beneficial effect of hCPC-based cell therapy for the treatment of heart failure. Patients with heart failure requiring LVAD implantation represent the target population most likely to experience impaired adult hCPC functional capacity; therefore, this study focuses on characterization of cells isolated from these patients. Results shown here demonstrate that hCPC isolated from multiple patients with varying age and disease pathogeneses possess variable growth kinetics, telomere lengths, and concomitant differences in expression of senescence markers. hCPC carrying the cumulative effects of age and disease can be rejuvenated by modification with Pim-1 kinase and can be promoted to drive a youthful phenotype to antagonize characteristics of cellular senescence.

Methods

Human Cardiac Stem Cell Isolation

hCPCs were isolated from heart tissue obtained from patients undergoing surgery for LVAD implantation as described previously.14 Briefly, tissue was minced in small pieces and digested in collagenase 150 U mg/mL (Worthington Bio Corp, Lakewood, NJ) for 2 hours at 37°C. Cells were incubated with c-kit–labeled beads (Miltenyi Biotec, St Auburn, CA) and sorted according to the manufacturer’s protocol. The pellet was suspended in hCPC media: Hams F12 (Fisher Scientific), 10% PBS, 1% PSG, 5 mU/mL human erythropoietin (Sigma Aldrich), 10 ng/mL basic FGF (Peprotech), and incubated at 37°C in CO₂ incubator. Adherent cells were seeded and expanded clonally. C-kit expression in hCPC is 87% to 89% measured by fluorescence activated cell sorting analysis (Online Figure IA). hCPC from multiple patients showed differences in growth kinetics. Two hCPC lines with distinct proliferation potentials were chosen for further experiments. H10-004 exhibited the slowest growth rate and is referred to as hCPC-S, whereas H10-014 exhibited the fastest growth rate referred to as hCPC-F. Fetal CPCs were isolated from fetal heart samples (second trimester) obtained from Novogenix laboratories.

Generation of Lentiviral Vectors and Transduction of hCPC

hCPCs expressing enhanced green fluorescent protein (hCPC-eGFP) and hCPC-Pim were transduced with lentivirus for eGFP and Pim-1 and eGFP as previously described.14 hCPC-S and hCPC-F were modified with lentiviral vectors Lv-eGFP and Lv-eGFP+Pim1 (Online Figure IB), and efficiency of modification after lentiviral transduction was 77.1% and 79.4% with Lv-eGFP and Lv-eGFP+Pim, respectively, as measured by flow cytometric analyses for eGFP (Online Figure IC). Pim-1 overexpression in hCPC was confirmed by immunoblot analysis (Online Figure ID).

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from hCPC and treatment using Quick-RNA MiniPrep (Zymo Research) according to manufacturer’s protocol. cDNA was prepared using iScript cDNA Synthesis Kit (Bio Rad). Real-time polymerase chain reaction (PCR) was performed on samples in triplicate using iQ SYBER Green (Bio Rad). Primer sequences are listed in Online Table I.

Protein Isolation and Immunoblot Analysis

hCPCs were plated in 6-well dishes (50000 cells/well). Samples were harvested in 50 μL of sample buffer, boiled, and sonicated. Protein lysates were run on 4% to 12% NuPage Novex Bis Tris gel (Invitrogen), transferred on to a polyvinylidene fluoride (PVDF) membrane, blocked in 8% skim milk in Tris-buffered saline Tween-20 solution for 1 hour at room temperature, and then incubated with primary antibodies overnight at 4°C. Membranes were incubated with secondary antibodies for 1 hour at room temperature after several washes with Tris-buffered saline Tween-20 solution. Fluorescence signal was detected and quantified using Typhoon fluorescent scanner together
with Image Quant 5.0 software (Amersham Biosciences). Antibodies are listed in Online Table II.

CyQuant, Viability, and MTT Assays
CyQuant assay involves plating cells in quadruplicate (2000 cells/well) in a 96-well plate and incubation with MTT reagent (Sigma Aldrich) or CyQuant reagent (Life Technologies) as previously described. Viability assay was measured by trypan blue staining. Population doubling times were calculated using reading from CyQuant and viability assays and a population doubling time online calculator (http://www.doubling-time.com/compute.php).

Cell Death Assay
hCPC were plated in a 6-well dish (30,000 cells/well) and incubated in hCPC medium with 2.5% serum overnight and then treated with 30 μmol/L H2O2 for 3 hours the following day. Cell death was confirmed by visualizing the cells under a light microscope before collection. Cells were harvested and stained with Annexin-V (Life Technologies) according to manufacturer’s protocol. Data were acquired with the BD fluorescence activated cell sorting Canto and analyzed by FlowJo or fluorescence activated cell sorting Diva software (BD Biosciences).

Telomere Length Measurement (Quantitative Fluorescence In Situ Hybridization)
Telomere length was analyzed by quantitative fluorescent in situ hybridization (quantitative fluorescence in situ hybridization) and confocal microscopy. PNA probe (Dako) was used to label telomeres according to manufacturer’s protocol. Telomere staining was scanned using Leica LCS confocal and relative fluorescent unit was measured by Leica LCS software. To control for experimental variation, all experimental slide sets were scanned using identical settings on the confocal microscope. A minimum of 200 hCPC were analyzed per line.

Telomere Repeat Amplification Protocol Assay
Telomerase activity was assessed by telomere repeat amplification protocol assay using quantitative PCR according to the manufacturer’s protocol (TRAPEze RT; Chemicon S7710) and as described in the Materials in the Online Data Supplement.

Telomere Length Measurements (RT-PCR)
Telomere length was measured by real-time PCR detection system by modified monochrome multiplex quantitative PCR method as described previously. In a duplex PCR detection method, albumin was simultaneously amplified with telomere template to normalize for different amounts of DNA per well and sample. The detailed protocol and thermal cycling profile are described in the Materials in the Online Data Supplement.

Senescence-Associated β-Galactosidase Staining
hCPC were cultured in 2-chamber permanox slides, and senescent cells were detected using a senescence-associated β-galactosidase assay (Abcam #ab65351) according the manufacturer’s protocol. Images were obtained using Olympus IX70 microscope.

Figure 1. Characterization of human cardiac progenitor cell (hCPC) isolated from multiple patients. A, hCPCs show variation in population doubling times as measured by CyQuant and viability assay cell counts from multiple patients (n=3). B, Differences in proliferation rates are observed in multiple hCPC lines (n=3). C, Telomere lengths in multiple hCPC lines show variability as measured by real-time polymerase chain reaction (n=6). D, Percentage of dead cells measured by Annexin-V staining showed variability in multiple hCPC lines when exposed to 30 μmol/L of H2O2 challenge (n=3). Black bar represents hCPC with slow-growth kinetics (hCPC-S), maroon bars represents hCPC with medium growth kinetics, green bars represents hCPC with fast-growth kinetics (hCPC-F), purple bar represent hCPC isolated from fetal heart samples (Fetal CPC). *P<0.05, **P<0.01. Significance values are calculated for hCPC-S versus hCPC-F groups.
Measurement of Cell Morphology
hCPC were plated in a chamber slides, and images were obtained using Olympus IX70 microscope. Cell morphology was measured by tracing the outline of the cells using ImageJ software. Twenty different fields of view were used to quantify cell morphology per cell line.

Statistical Analysis
All data were expressed as a mean±SEM. Comparison between 2 groups was performed using Student t test or multiple groups by 1- or 2-way ANOVA. P value <0.05 was considered as statistically significant. Statistical analysis was performed using GraphPad prism version 5.0 software.

Results
Characterization of hCPC Isolated From Multiple Patients
hCPC were isolated from multiple patients undergoing LVAD implantation. Population doubling times ranging from 28.1 to 21.5 hours were observed in the hCPC-S versus hCPC-F lines, respectively, as calculated by population doubling time. Growth kinetics are 30% faster in hCPC-F as compared with hCPC-S measured by population doubling time (Figure 1A; P<0.05). Growth rate of the hCPC-F is similar to the 21.2-hour doubling time for fetal CPC used as a standard control of healthy stem cells (Figure 1A). Similarly, increased proliferation rates were also observed using a CyQuant DNA labeling assay, with hCPC-F exhibiting 60% and 90% greater labeling than hCPC-S, respectively (Figure 1B; P<0.05). Similarly, 55.2% increase in telomere length was observed in hCPC-F as compared with hCPC-S (Figure 1C; P<0.01). Telomere length measurements in hCPC showed variation from 2.1 kbp observed in hCPC-S to 3.8 kbp measured in hCPC-F. Telomere length in fetal CPC is substantially longer than adult CPC lines at 8.3 kbp (Figure 1A). Similarly, increased prolife-ration rates were also observed using a CyQuant DNA labeling assay, with hCPC-F exhibiting 60% and 90% greater labeling than hCPC-S, respectively (Figure 1B; P<0.05). Similarly, 55.2% increase in telomere length was observed in hCPC-F as compared with hCPC-S (Figure 1C; P<0.01). Telomere length measurements in hCPC showed variation from 2.1 kbp observed in hCPC-S to 3.8 kbp measured in hCPC-F. Telomere length in fetal CPC is substantially longer than adult CPC lines at 8.3 kbp (Figure 1C). Telomere lengths were measured at passage 6 in hCPC lines and fetal CPC. Increased cell death was observed in hCPC-S (26.6%) compared with hCPC-F (21%) after apoptotic stimulation (Figure 1D; P<0.05). Fetal CPC exhibited 19.5% susceptibility to apoptotic challenge (Figure 1D). Collectively, these results indicate that concomitant changes in telomere length, population doubling time, and proliferation rates in hCPC can be used as readout for biological age of hCPC.

Cell Cycle Regulator Profile in Human CPC
Profiling of cell cycle inhibitors, such as p16 and p53, was assessed by quantitative PCR analyzed in multiple hCPC lines. Transcript level increases of p53 (P<0.01; Figure 2A) and p16 (P<0.05; Figure 2C) were elevated in hCPC-S relative to hCPC-F as evidenced by lower ΔCT values (ΔCT values are inversely proportional to mRNA levels). Also, diminished p53 expression and the absence of detectable p16 signal was observed in fetal hCPC (Figure 2A and 2C). mRNA level differences coincide with changes in protein expression, with significant increases of both p53 (3.2-fold; P<0.001) and p16 (2.1-fold; P<0.05) in hCPC-S relative to hCPC-F, whereas lowest expression was consistently observed in fetal CPC as confirmed by immunoblot analysis (Figure 2B–2D). Propropyliferative markers were higher in hCPC-F versus hCPC-S as evidenced by increases in mRNA for Cyclin D1 (P<0.01; Figure 2E) and Pim-1 (P<0.001; Figure 2G) when comparing hCPC-F with hCPC-S, respectively. Increased mRNA of Cyclin D1 and Pim-1 is evidenced by lower ΔCT values in hCPC-F as compared with hCPC-S. Concomitantly, increased protein expression of Cyclin D1 is observed in hCPC-F compared with hCPC-S (2.1-fold; P<0.001; Figure 2F).

Table. Clinical Profile of Patients Used for hCPC Cell Isolation

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<th>Sex</th>
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<td>√</td>
<td>1 Pk/d for 30 y</td>
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EF indicates ejection fraction; hCPC, human cardiac progenitor cells; hCPC-F, hCPC with fast-growth kinetics; hCPC-S, hCPC with slow-growth kinetics; and NYHA, New York Heart Association.
Increased Pim-1 expression is observed in hCPC-F compared with hCPC-S as evidenced by immunoblot analysis (2.4-fold; P<0.05; Figure 2H). Fetal CPC showed highest expression for both Cyclin D1 and Pim-1 (Figure 2F and 2H). Collectively, these results demonstrate the inherent variability between multiple hCPC lines with respect to expression of phenotypic markers indicative of senescence and proliferative status, both of which correlate with the biological properties of the cells in vitro (Figure 1).

**Replicative Senescence in Human CPC**

Replicative senescence indicative of lowered proliferative potential was evident in hCPC-S compared with hCPC-F, with increased senescence-associated β-galactosidase–positive cells after serial passaging (Figure 3A). hCPC-S acquired β-gal+ expression at a higher rate than hCPC-F by passage 7 (63.3% versus 24.3%, respectively), whereas control fetal hCPCs were only 5.6% β-galactosidase positive at the same passage number (P<0.001; Figure 3B). Subsequent passage-induced mitotic arrest was reached at passage 9 for hCPC-S with 61.9% more β-gal+ cells compared with hCPC-F (at passage 9), as hCPC-F continued to proliferate until passage 15 (Figure 3C). Acquisition of β-gal+ expression indicative of senescence coincides with morphometric changes in hCPC. Specifically, hCPC-S become flat and lose normal morphology before mitotic arrest quantitated as decrease...
in length:width ratio ($P < 0.001$; Figure 3D) and increase in cell roundness ($P < 0.01$; Figure 3E) at passage 9 compared with hCPC-F. Taken together, these results are consistent with hCPC-S predisposition toward replicative senescence indicative of mitotic exhaustion caused by biological aging (as shown in Figures 1 and 2).

### Increased Telomere Length and Tert Activity in Pim-1–Modified hCPC

Variation in hCPCs as evidenced by proliferative capacity, replicative senescence, and telomere length (Figures 1–3) prompted further studies to assess reversibility of biological age and to drive youthful phenotypic characteristics. Previous studies by our group have demonstrated the ability of Pim-1 kinase to enhance myocardial regeneration\(^\text{13,14}\) and to increase transiently telomere length.\(^\text{25}\) In addition, Pim-1 kinase expression is higher in fetal hCPCs and hCPC-F compared with hCPC-S (Figure 2), establishing a correlation between Pim-1 expression and youthful phenotypic characteristics of hCPCs. On the basis of these results, we performed the genetic modification of hCPC-S and hCPC-F to overexpress Pim-1 kinase with subsequent analysis of telomere length and Tert activity.

Pronounced white foci indicative of extended telomere length were observed in Pim-1–modified hCPC by quantitative fluorescence in situ hybridization analysis (Figure 4A and 4E) and are represented as relative fluorescent unit. Telomere length was significantly increased by 70% ($P < 0.001$; Figure 4B) in Pim-1–modified hCPC-S compared with hCPC-F (30%; $P < 0.01$; Figure 4F) when both cell lines were compared with respective control eGFP-modified hCPC. Quantitative fluorescence in situ hybridization was corroborated by PCR-based assessment of telomere length, showing a 2.2-fold increase in telomere length with Pim-1–modified hCPC-S ($P < 0.001$; Figure 4C) and 1.3-fold telomere length increase in Pim-1–modified hCPC-F ($P < 0.01$; Figure 4G) relative to respective eGFP controls. Similarly, a nonsignificant increase in telomere repeat amplification protocol activity is observed after Pim-1–modified hCPC-S (Figure 4D) and hCPC-F (Figure 4H) relative to respective eGFP controls. Collectively, these data indicate that Pim-1 modification elongates telomere length and promotes a youthful phenotype in hCPC, with greater enhancement of hCPCs possessing advanced biological age characteristics.

### Increased Proliferation, Metabolic Activity, and Survival After Pim-1 Modification

Genetic modification with Pim-1 significantly decreased population doubling time by 1.3-fold in hCPC-S ($P < 0.001$) compared with 1.1-fold decrease in hCPC-F ($P < 0.05$; Figure 5A). Concurrently, proliferation rate increased by 1.4-fold in hCPC-S ($P < 0.001$) and 1.2-fold ($P < 0.05$) in hCPC-F after Pim-1 modification with respect to eGFP-modified hCPC control as determined by CyQuant assay. Increased metabolic activity was exhibited by Pim-1–modified hCPC-S (30.7% higher)
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Pim-1 Antagonizes Human CPC Senescence

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and hCPC-F (13.9% higher) compared with eGFP controls at day 3 as measured by MTT assay (Figure 5C). Similarly, enhanced survival was demonstrated in Pim-1-modified hCPC-S and hCPC-F in response to apoptotic challenge as evidenced by respective decreases of 1.4-fold ($P<0.01$) and 1.6-fold ($P<0.001$) in Annexin-V+ cells (Figure 5D).

**Reversal of Senescent Phenotype After Ex Vivo Gene Manipulation With Pim-1 in hCPC**

Senescence-associated β-galactosidase–positive staining was decreased after Pim-1-modification in hCPC-S (32.9%; $P<0.001$) and hCPC-F (23.5%; $P<0.01$; Figure 6A). Decreases in both cell length/width ratio and roundness indicative of cell flattening and a postmitotic phenotype were most prominently displayed in hCPC-S by passage 9 (Figure 6B and 6C) at which point the cells were no longer capable of further passages because of loss of mitotic activity. In comparison, hCPCs-F maintain mitotic phenotypic properties at passage 9 and are capable of expansion until passage 14, indicating delayed senescence relative to hCPC-S. Restoration of growth-associated–rounded morphology to hCPC-S was conferred by Pim-1 modification that extended hCPC-S growth from passage numbers 9 to 15 before mitotic arrest (Figure 6D).

**Discussion**

Stem cell therapy for cardiac repair holds great promise, but the ability of cardiac stem cells to repair damaged myocardium declines with age. Ex vivo expansion of hCPCs from pathologically challenged hearts is required in autologous therapy to expand the stem cell pool and select for proliferative cells before reinfusion, but in vitro demands on cell proliferation may exhaust the mitotic potential of isolated cells and may render them predisposed to undergo replicative senescence and compromised functional impact. Shortening of telomere length has been linked to both senescence and cell death, further highlighting concerns related to expansion of hCPCs with telomeres compromised by biological aging. Heterogeneous origin of
disease pathologies along with differences in individual genetic and environmental makeup can adversely affect cardiac stem cell efficiency to repair damaged myocardium, limiting the beneficial effect of stem cell therapy. hCPCs derived from patients with advanced biological age and severe concurrent clinical features will require rejuvenation to reverse the deleterious effects of aging and disease. Findings in this study demonstrate that hCPCs isolated from multiple patients with heart failure exhibit clear differences in growth rates, telomere lengths, and expression of senescent markers, representing biological age rather than chronologic age as a key determinant of cellular phenotype. Genetic modification with Pim-1 kinase exhibits remarkable capacity to rejuvenate hCPCs with advanced biological age with enhanced proliferation, increased telomere lengths, and decreased susceptibility to replicative senescence. Telomere lengths of the 2 hCPC lines modified with Pim-1 were significantly longer, supporting the functional capacity of Pim-1 to reverse the phenotype of biological age in hCPCs. Pim-1 modification is a novel and effective way to augment hCPC function, extending the benefits of autologous stem cell therapy for the repair of damaged hearts to a large segment of the population with dissimilar age and disease pathogeneses.

Myocardial repair processes in the heart are supported by CPCs located within cardiac niches. Many intrinsic and extrinsic factors regulate CPC turnover and replenishment within the niche, thus affecting CPC reparability in response to myocardial injury. Accumulation of age-related changes, such as DNA damage, telomere attrition, epigenetic deregulation, and environmental stress, impair CPC function in the heart. Heart-related pathologies primarily occur in the aged population and compromise repair capability of endogenous CPC pool. Senescent CPCs are limited in their capacity to expand and to generate de novo cardiomyocytes, resulting in diminished cell turnover and acceleration of myocardial aging. Indeed, CPCs isolated from multiple patients with varying age and disease pathologies have varying growth kinetics, telomere length (Figure 1), and expression of cell cycle regulators (Figure 2). However, telomere lengths from fetal CPC are the longest, and there is a clear distinction between the telomere length of samples derived from patients with LV AD and those derived from fetal samples. In addition to the chronological age, patients with heart failure display distinct disease pathogeneses and a combination of genetic and environmental factors that can impact CPC function. Analysis of patient characteristics and their respective

Figure 5. Enhanced proliferation, metabolic activity, and survival after Pim-1 modification of human cardiac progenitor cells (hCPCs) with fast- and slow-growth kinetics (hCPC-F and hCPC-S, respectively). A, Increased population doubling time calculated by CyQuant and viability assay cell counts after Pim-1 modification (n=3). B and C, Increased proliferation rate and metabolic activity in genetically modified hCPC-S and hCPC-F with Pim-1 (n=3). D, Percentage of Annexin-V+ cells are measured by fluorescence activated cell sorting analysis after apoptotic stimuli of 30 μmol/L H2O2 shows increased cell survival after Pim-1 modification (n=3). *P<0.05, **P<0.01, ***P<0.001. Significance values are calculated for enhanced green fluorescent protein (eGFP) vs Pim in both hCPC groups.
CPCs revealed fast-, medium-, and slow-growing CPCs with growth rates inversely related to expression of senescent markers (Figure 2). hCPCs examined from patients with heart failure represent the target population that would benefit most from regenerative cell therapy, yet all of the samples analyzed fell short of exhibiting phenotypic characteristics comparable with fetal hCPCs used as the gold standard of a cardiogenic cell. The choice of fetal hCPCs for comparison was based on the rationale that the rapidly developing and highly mitotic environment of the fetal heart represents the characteristic regenerative potential desired with an adoptively transferred cell population intended to form myocardium. Ideally, the goal would be to introduce cells with maximal regenerative potential similar to a fetal cell with minimal risk for oncogenic transformation as is inherent with iPS or embryonic stem cell approaches. The fetal CPCs exhibit replicative senescence in culture at 19 passages, which was markedly prolonged relative to any of the hCPC patient-isolated lines without genetic modification to express Pim-1 kinase.

hCPCs for autologous therapy will depend on expansion of patient-derived cells in vitro, which expose hCPCs to the detrimental effects of high-pressure mitotic growth and concomitant replicative senescence and senescent characteristics (Figure 3). The capacity of Pim-1 kinase to enhance myocardial regeneration together with improved CPC survival, proliferation, and commitment to cardiac lineages has been demonstrated. Furthermore, Pim-1 modification augmented telomere length and cell doubling time transiently in aged murine CPCs. However, in our initial study, we selected a hCPC line for adoptive transfer based on maximal expression of Pim-1 but had not addressed the potential for Pim-1 modification to result in varying outcomes based on the biological age of patient hCPCs. Although encouraged by our initial study with Pim-1–engineered hCPCs, the question of how broadly applicable this modification strategy remained unanswered and was highlighted in an editorial accompanying the article. This subsequent report advances initial findings by showing beneficial effects of Pim-1 modification evident in divergent hCPC populations with varying phenotypes isolated from multiple patients. The present study performed an unbiased sampling of tissue samples from LVAD recipients, isolated their hCPCs, and then chose the cells with the hCPC-F and hCPC-S growth kinetics to represent the widest possible variation in phenotype as a platform for Pim-1 modification for rejuvenation of cells. Salutary effects of Pim-1 modification on proliferation (Figure 5) and telomere lengths (Figure 4) were consistent with suppression of p53 and p16 and blunting of the senescent phenotype (Figure 6). Importantly, Pim-1 modification exhibited beneficial effects in fast- and slow-growing hCPCs, the maximum increase in proliferation and telomere lengths was observed in slow-growing hCPCs, supporting the postulate that Pim-1 modification enhances phenotypic youthfulness in hCPCs regardless of initial differences in age or comorbidities. Indeed, the greatest benefit was observed in hCPCs isolated from patients that displayed advanced aging and the worse clinical features. Last, but not least, Pim-1 modification evoked a response consistent with restoring the hCPCs to a young phenotype but is capable of undergoing

Figure 6. Pim-1 ameliorates human cardiac progenitor cell (hCPC) senescence. A, Percentage of senescence-associated β-galactosidase–positive cells in hCPC modified with Pim-1 at passage 9. B and C, Length:width ratio and roundness of hCPC show well-maintained normal morphology after Pim-1 modification measured by ImageJ software. D, Graphical representation of passage numbers indicating growth arrest in hCPC with slow-growth kinetics (hCPC-S; Pim-1), and hCPC with fast-growth kinetics (hCPC-F; Pim-1) after replicative senescence. E and F, Decrease in expression of senescence-associated markers measured by p16 and p53 gene expression after Pim-1 modification (n=3) ***P<0.001. Significance values are calculated for GFP expressing vs Pim in both hCPC groups.
replicative senescence in a time frame comparable with the fetal hCPCs rather and did not exhibit a transformed behavior that would be unacceptable for clinical therapeutic use.

Clinical trials for heart repair have recently shown that hCPCs from patients are capable of mediating increases in ejection fraction and reducing scar size.\textsuperscript{6,7} However, narrow inclusion criteria in these trials leave this question for debate whether initial beneficial results with hCPC transplantation are only applicable to a limited patient population with heart failure. Patients with heart failure show remarkable diversity in the pathogenesis of the disease. Various factors ranging from diabetes mellitus,\textsuperscript{8} hypertension, smoking, stress, and life choices compound disease pathology. Importantly, incidence of heart failure coincides with increased age that further contributes to development of heart failure. Prerequisite for a successful, broadly applicable cell-based therapy for the repair of damaged myocardium demands a wide spectrum strategy to reduce the deleterious effects of aging, disease pathologies, and environmental factors. Pim-1 modification of age-exhausted or senescent hCPCs rejuvenates the phenotypic characteristics of the cells and accounts for our previous findings demonstrating the superior regenerative potential of Pim-1–engineered cells.\textsuperscript{9,10} Modification of hCPCs by Pim-1 kinase is a viable strategy to rejuvenate hCPCs isolated from a diverse population with heart failure, opening up the possibility of extending hCPC-based cell therapy for the treatment of heart failure in a large segment of patients from various forms of cardiovascular disease.

Acknowledgments

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Disclosures

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References

depletion of the cardiac stem cell pool and is rescued by restoration of progenitor cell function. Circulation. 2010;121:276–292.


What Is Known?

- Aging and disease compromise cardiac progenitor cell (CPC) function.
- Pim-1 modification of CPCs enhances survival, proliferation, and lineage commitment capacity of adoptively transferred CPCs, improving myocardial regeneration after infarction.

What New Information Does This Article Contribute?

- CPCs isolated from multiple patients exhibit differences in growth kinetics and telomere length consistent with biological aging.
- Pim-1 modification rejuvenates aged and diseased CPC pool by increasing telomere length and decreasing cell cycle inhibitors.
- Beneficial effects of Pim-1 modification are more pronounced in human CPCs with slow-growth kinetics.

Myocardial regeneration and repair mediated by human CPCs is compromised by biological aging. Impairment of CPCs function is further accelerated by chronic stress or disease conditions. Here, we report that human adult CPCs isolated from multiple patients with heart failure undergoing left ventricular assist device implantation vary with respect to growth kinetics, all of which were inferior when compared with human fetal CPCs. Fetal CPCs were used as a gold standard in the study intended to represent a highly cardiogenic cell that would be ideal to promote cardiomyogenesis and myocardial repair. Pim-1 modification of human adult CPCs reversed their senescent phenotype as evidenced by lengthening of the telomeres, improved growth kinetics, and delayed replicative senescence. Genetic engineering with Pim-1 modification was beneficial for all CPCs tested although CPCs with slow-growth kinetics benefited the most by restoration of youthful phenotypic characteristics. Therefore, the efficacy of CPC transfer therapy is expected to be improved by Pim-1 modification that rejuvenates CPCs impaired by age and chronic disease. The ability of Pim-1 modification to rejuvenate compromised CPCs may enable stem cell treatment availability to patients currently considered as poor candidates for therapeutic intervention because of advanced age or chronic comorbidities presenting together with heart failure.
Rejuvenation of Human Cardiac Progenitor Cells With Pim-1 Kinase
Sadia Mohsin, Mohsin Khan, Jonathan Nguyen, Monique Alkatib, Sailay Siddiqi, Nirmala Hariharan, Kathleen Wallach, Megan Monsanto, Natalie Gude, Walter Dembitsky and Mark A. Sussman

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Supplemental Material:

Modification of hCPCs:

For lentiviral infection, hCPC were plated in 6-well dish (50,000 cells/ well), serially transduced with lentivirus (multiplicity of infection (MOI) of 20 followed by MOI of 10 two days after first infection) to obtain hCPC expressing enhanced green fluorescent protein (eGFP) together with Pim-1 or eGFP alone. Engineered hCPCs were expanded and analyzed using flow cytometry to validate high percentage eGFP expression.

Telomere Length Measurements (Q-FISH)

Telomere length was analyzed by quantitative in situ hybridization (Q-FISH) and confocal microscopy. PNA probe was purchased from DAKO (K5325) and measurements were done according to manufacturer’s protocol. Briefly, slides were deparaffinized and rehydrated, followed by antigen retrieval using 10mM Citrate buffer pH 6.0. Slides were subsequently washed with PBS and treated for 9 minutes with Proteinase K (Dako, S3020). Following another PBS wash slides were dehydrated with a series of cold ethanol then allowed to dry. PNA probe was placed on the section and denatured at 80°C for 10 minutes and hybridized at 37°C overnight. Slides were washed two times in Wash Buffer at 65°C and counterstained with sytox blue. Telomere signal was acquired in each nucleus using Leica software and divided by sytox blue signal to account for differences in nuclear size.

Telomere length (Rt-PCR)

Telomere lengths measured by modified monochrome multiplex Quantitative PCR method on a Real time PCR machine. In this PCR method Albumin is amplified with the telomere template at the same time as described in Weischer M et al. Briefly, 20ng of DNA, 1X syber green with albumin and telomere primer were combined in a reaction volume of 10ul and the samples were atleast run in a set of 6 replicates. The thermal cycle used Stage 1:15 min at 95°C, Stage 2: 2 cycles of 15 s at 94°C, 15s at 49°C and stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15s at 73°C with signal acquisition, 10 s at 84°C, 15 s at 87°C with signal acquisition, stage 4: 1 cycle of 0.05 s at 65°C with signal acquisition.

Telomerase Activity

Telomerase activity was assessed by quantitative PCR according to the manufacturer’s protocol (TRAPeze RT, Chemicon S7710). Briefly, hCPCs were harvested in CHAPS buffer and centrifuged at 4°C. 1 μg of lysate was obtained then incubated in a solution containing reverse transcriptase reaction mix and Taq polymerase at 30°C for 30 minutes. Control cells provided by the manufacturer were used as positive control and serial dilutions of control template TSR8 was employed for quantification. CHAPS buffer in the absence of protein lysates and heat-inactivated lysates were used as negative control. PCR cycling conditions and data analysis were performed according to the manufacturer’s protocol.
Online Figure I:

**Human CPCs over expressing Pim-1** A) c-kit expression in hCPC-S and hCPC-F by FACS analysis. B) Lentiviral vectors for eGFP and eGFP Pim-1. C) Percentage GFP in hCPC (eGFP) and hCPC (Pim) D) Immunoblot analysis of hCPC for over expression of Pim-1 after lentiviral transduction in hCPC-S and hCPC-F.
**Online Table I:**

**List of primers**

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## Online Table II:

### List of antibodies

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Online Figure I:  
**Human CPCs over expressing Pim-1**  
A) c-kit expression in hCPC-S and hCPC-F by FACS analysis  
B) lentiviral vectors for eGFP and eGFP Pim-1.  
C) Percentage GFP in hCPC-S and hCPC-F after modification with (eGFP) and (Pim-1)  
D) Immunoblot analysis of hCPC for over expression of Pim-1 after lentiviral transduction in hCPC-S and hCPC-F.