Early Stem Cell Engraftment Predicts Late Cardiac Functional Recovery
Preclinical Insights From Molecular Imaging

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**Background**—Human cardiac progenitor cells have demonstrated great potential for myocardial repair in small and large animals, but robust methods for longitudinal assessment of their engraftment in humans is not yet readily available. In this study, we sought to optimize and evaluate the use of positron emission tomography (PET) reporter gene imaging for monitoring human cardiac progenitor cell (hCPC) transplantation in a mouse model of myocardial infarction.

**Methods and Results**—hCPCs were isolated and expanded from human myocardial samples and stably transduced with herpes simplex virus thymidine kinase (TK) PET reporter gene. Thymidine kinase-expressing hCPCs were characterized in vitro and transplanted into murine myocardial infarction models (n=57). Cardiac echocardiographic, magnetic resonance imaging and pressure-volume loop analyses revealed improvement in left ventricular contractile function 2 weeks after transplant (hCPC versus phosphate-buffered saline, P<0.03). Noninvasive PET imaging was used to track hCPC fate over a 4-week time period, demonstrating a substantial decline in surviving cells. Importantly, early cell engraftment as assessed by PET was found to predict subsequent functional improvement, implying a “dose–effect” relationship. We isolated the transplanted cells from recipient myocardium by laser capture microdissection for in vivo transcriptome analysis. Our results provide direct evidence that hCPCs augment cardiac function after their transplantation into ischemic myocardium through paracrine secretion of growth factors.

**Conclusions**—PET reporter gene imaging can provide important diagnostic and prognostic information regarding the ultimate success of hCPC treatment for myocardial infarction. *(Circ Cardiovasc Imaging. 2012;5:481-490.)*

**Key Words:** cardiac progenitor cells ■ cell therapy ■ positron emission tomography ■ stem cells

After myocardial infarction, the limited proliferative ability of the surviving cardiac cells renders the damaged heart susceptible to morbidity sequelae such as unfavorable remodeling and heart failure. Although therapeutic options have advanced in the last several decades, the morbidity and mortality associated with ischemic cardiomyopathy remains unacceptably high. In recent years, stem and progenitor cells have emerged as exciting new tools to alleviate left ventricular dysfunction alongside existing pharmacological and device treatment options. In particular, human cardiac progenitor cells (hCPCs) demonstrate enormous potential for future clinical translation of cardiac cell therapy. hCPCs are a clonogenic, self-renewing population that are preprogrammed for differentiation into cells of all 3 cardiac lineages in vitro: myocytes, smooth muscle cells, and endothelial cells. In addition, hCPCs can be derived autologously from a small sample of myocardial tissue, thereby avoiding concerns of immunologic rejection. Numerous small and large animal studies have concluded that transplantation of CPCs into ischemic myocardium yields significant improvement in cardiac function after myocardial infarction.
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Results from early clinical trials of bone marrow-derived cell therapy for cardiovascular disease have yielded promising but variable results, whereas results of early clinical trials of hCPC therapy are still forthcoming. Many questions remain regarding the optimal cell type, dose, and timing of any such cell therapy. Robust evaluation of these parameters has been greatly aided by the development of noninvasive imaging methods for longitudinal assessment of stem cell engraftment and proliferation. Direct labeling of cells using radiotracers or magnetic particles allows short-term visualization of transplant location, but death of the cells may lead to erroneous signal from neighboring phagocytes or interstitium. The temporal limitations of these physical methods for labeling cells have been overcome by techniques for stable labeling of cells with various reporter genes, thereby enabling quantitative assessment of cell survival and proliferation. In this regard, bioluminescence imaging (BLI) in conjunction with luciferase reporter genes has been used previously to monitor the survival of stem cell transplants. However, BLI will not be applicable in humans because its low energy photons (2–3 eV) are easily attenuated within the deeper tissues of larger animals.

Positron emission tomography (PET), on the other hand, is a clinically applicable noninvasive imaging modality that readily allows for the monitoring of cell homing in humans. Various generations of PET thymidine kinase (TK) reporter genes have been developed in recent years to optimize our collective ability to monitor various cellular processes, including transcriptional regulation, protein–protein interactions, and cell trafficking. Here we sought to compare the various generations of PET TK reporter genes for their potential use in monitoring clinical hCPC therapy. Furthermore, we sought to determine whether early PET imaging would provide prognostic information about the ultimate success of cell therapy trials. Our results indicate that an imaging component of future clinical cell therapy trials will yield significant insight into the determinants of successful treatment.

Methods

Isolation, Culture, and Characterization of hCPCs

The study was conducted with institutional approval from the Stanford Investigational Research Board. The hCPCs were isolated based on a previously described protocol using magnetic separation in conjunction with a Sca-1 antibody. In brief, human fetal hearts were collected after elective abortion by a commercial vendor (StemExpress, Placerville, CA). Fetal hearts underwent Langendorff perfusion with Tyrode solution containing fetal calf serum (Gibco), 10 ng/mL basic fibroblast growth factor, 5 ng/mL epithelial growth factor, 5 ng/mL insulin-like growth factor, and 5 ng/mL hepatocyte growth factor.

Lentiviral Vector Construction and Transduction of hCPCs With Firefly Luciferase and Green Fluorescent Protein Double Fusion Reporter Genes

See the online-only Data Supplement Methods for details.

Effect of TK Variants on hCPC Viability, Proliferation, and Differentiation Potential

See the online-only Data Supplement Methods for details.

Microarray Hybridization and Data Analysis of Various TK-hCPC Lines

See the online-only Data Supplement Methods for details.

In Vitro [18F]-FHBG Accumulation Assay

See the online-only Data Supplement Methods for details.

Surgical Model of Myocardial Infarction and hCPC Delivery

In adult female SCID Beige mice (Charles River Laboratories, Wilmington, MA), myocardial infarction was induced by ligation of the left coronary artery under 1.5% to 2% inhaled isoflurane anesthesia and confirmed by myocardial Blanching and electrocardiographic changes. Animals were initially randomized into 5 experimental groups receiving hCPCs (n=60) or 1 control group receiving phosphate-buffered saline (n=15). Four of the 5 experimental groups received hCPCs that stably expressed the different variants of the PET TK reporter gene shown in Figure 1A. The PET TK reporter gene variants were the original wild-type herpes simplex virus-1 (HSV) thymidine kinase (wt-tk), semimarrow variant 39 HSV-thymidine kinase (sr39-tk), A168H mutant HSV-tk (A168H), and truncated mitochondrial thymidine kinase type 2 (Δmtk2; A168H and Δmtk2 were courtesy of Dr Juri Gelovani at M.D. Anderson). Animals were randomized to the groups as follows: wt-tk-hCPCs (n=5), sr39-tk-hCPCs (n=17), A168H-hCPCs (n=20), and Δmtk2-hCPCs (n=5). One experimental group received untransduced hCPCs (n=10). All 5 experimental groups were injected with 1×10^6 cells using a 31-gauge Hamilton syringe immediately after myocardial infarction. In all groups, the volume of injection was 20 μL at 3 sites near the peripheral border zone. All surgical procedures and injections were performed by a single experienced and blinded investigator (Y.G.).

[18F]-FHBG PET Imaging

Small animal microPET imaging (Vista system; GE Healthcare, Chalfont St Giles, UK) was performed in a subset of the animals (n=40 hCPCs and n=10 phosphate-buffered saline) on Days 1, 7, 14, 21, and 28 postoperatively. Mice were fasted for 3 hours before radioisotope injection. Animals were then injected with approximately 7400 kBq (200 mCi) of [18F]-FHBG radiotracer through the tail vein. At 60 minutes after injection, animals were anesthetized with inhaled 2% isoflurane. Images were acquired, reconstructed by filtered back projection, and analyzed using image software AMIDE (SourceForge, Inc, Mountain View, CA) by a blinded investigator (M.H.). Three-dimensional regions of interest were drawn encompassing the heart. For each region of interest, counts/mL/min were then converted to counts/g/min and divided by the injected dose to obtain the image region of interest derived [18F]-FHBG percentage injected dose per gram of heart (%ID/g).

BLI of hCPC Engraftment for Confirmation of PET Data

See the online-only Data Supplement Methods for details.
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Analysis of Left Ventricular Function With Echocardiogram and MRI
See the online-only Data Supplement Methods for details.

Analysis of Left Ventricular Function With Pressure-Volume Loops
See the online-only Data Supplement Methods for details.

Analysis of In Vivo Gene Expression Using Laser Capture Microdissection
Mouse hearts were removed after perfusion with 20 mL phosphate-buffered saline, embedded in optimal cutting temperature, and immediately frozen in liquid nitrogen. For laser capture microdissection, 7 tissue sections of the left ventricle were prepared on polyethylene naphthalate membrane-coated slides (MicroDissect GmbH, Leica, Germany). The slides were then thawed briefly and air-dried for 5 minutes before dissection. Green fluorescence observed under laser microscopy was used as a landmark for microdissection. Green fluorescent protein (+) (GFP+) cells, indicating transplanted hCPCs, were dissected using a Leica AS LMD6000 system (Leica, Germany). GFP− recipient myocardium was also isolated for normalization. The dissected tissues were placed into the caps of microcentrifuge tubes with 5 μL of lysis enhanced buffer and collected by centrifugation at 8000 g for 5 minutes. Total RNA extraction and reverse transcription of these samples were performed using a commercial One Step reverse transcription–polymerase chain reaction kit (Invitrogen, Carlsbad, CA).

Histological Examination
See the online-only Data Supplement Methods for details.

Statistical Analysis
Statistics were calculated using SPSS 12.0 (SPSS Inc, Chicago, IL), Stata Release 9.2 (StataCorp LP, College Station, TX), and R 2.14.2 (R Foundation for Statistical Computing, Vienna, Austria). Unless specified, descriptive statistics included mean and SD. One-way analysis of variance with post hoc testing was used as indicated in figure legends. Differences between the high and low cell engraftment groups in percent fractional shortening as evaluated by echocardiography, ejection fraction as evaluated by cardiac MRI, and cell engraftment as evaluated using PET were tested with a mixed effects regression on number of days posttransplantation, cellular engraftment (high versus low or hCPC versus phosphate-buffered saline), and their interaction with mouse as a random effect. An autoregressive model of order 1 correlation structure was used to model the dependence of within-mouse measurements. Significance of time and treatment effect was assessed through a likelihood ratio test, comparing with the null model with no time or treatment effect. Correlations among PET signal, ejection fraction, and percent fractional shortening were assessed by Spearman rank correlation. Differences were considered significant at probability values of <0.05 unless they were subject to a Bonferroni-adjusted critical probability value as indicated in the figure legends.
Results

Isolation, Expansion, and Features of hCPCs Isolated From Human Myocardial Biopsy Specimens

We first isolated Sca-1+ hCPCs from human fetal hearts and clonally expanded them using a previously described protocol (see the online-only Data Supplement Methods). The identity of the isolated cells was validated by cell surface marker (online-only Data Supplement Figure I) and gene expression profiling (online-only Data Supplement Figure II) before stable transduction with a double-fusion firefly luciferase (Fluc) and GFP reporter gene cassette. GFP+ cells were fluorescence-activated cell sorter isolated and expanded to generate stable Fluc+/GFP+hCPC lines that can be quantitatively tracked in vivo using BLI (online-only Data Supplemental Figure III).

Expression of TK PET Reporter Genes Does Not Affect the Phenotype of hCPCs

Fluc+/GFP+hCPCs were stably transduced with 4 variants of the PET TK reporter gene that had been chosen for further evaluation (Figure 1A). They were the original wild-type HSV thymidine kinase (wt-tk), semirandom variant 39 HSV-thymidine kinase (sr39-tk), A168H mutant HSV-tk (A168H), and truncated mitochondrial thymidine kinase type 2 (Δhtk2).17–20 sr39-tk was developed in 2000 by random sequence mutagenesis of the nucleoside binding region of wt-tk and selected for an enhanced ability to convert the prodrug acyclovir and/or ganciclovir into cytotoxic agents.24 A168H was later developed in 2006 by engineering of a single conserved residue of the wt-tk gene based on its crystal structure.25 Δhtk2 was derived from human mitochondrial thymidine kinase type 2 by truncation of the N-terminal nuclear localization signal in 2007.20 Although all variants of TK have been developed with an aim of enhanced sensitivity and specificity, there have not yet been direct comparisons of their use as reporter genes for cardiac stem cell therapy. To address this question, we stably transduced Fluc+/GFP+hCPCs with the various TK reporter genes and observed no discernible adverse effects on cell proliferation (Figure 1B), cell viability (online-only Data Supplement Figure IV), gene expression profile (online-only Data Supplement Figure V), or karyotype (online-only Data Supplement Figure VI). Most importantly, stably transduced hCPCs maintained their ability to differentiate into all 3 cardiac lineages, as evidenced by expression of α-actinin, CD31, or α-smooth muscle actin (Figure 1C).

Comparison of [18F]-FHBG Radiotracer Uptake Rates by Different TK Variants

We wished to assess the use of PET reporter gene imaging in the setting of clinical stem cell transplantation trials and thus opted to use the [18F]-FHBG radiotracer in conjunction with TK reporter gene variants. [18F]-FHBG is a positron-emitting nucleoside analog used in conjunction with TK for imaging of gene expression. The safety, pharmacokinetics, and dosimetry of [18F]-FHBG have been studied in rats, rabbits, and...
human volunteers, and the US Food and Drug Administration has approved its use as an investigational new drug (Investigational New Drug #61880) for clinical trials.²⁶ Radiotracer uptake into hCPCs was quantified in vitro over a 120-minute time course using a gamma counter (Figure 2A), and confirmed qualitatively using microPET imaging acquisition (Figure 2B). Robust uptake was highest in the hCPCs stably expressing the A168H and sr39-tk variants (cells to media uptake ratio of 7.80±0.20 and 6.40±0.19, respectively). Next, we confirmed the ability of various TK variants to uptake radiotracer in an in vivo setting by transplanting TK-expressing hCPCs intramyocardially into SCID beige mice (Figure 2C–D). PET signal intensities were compared after normalization for any experimental variation in cell number by concurrently measuring BLI signals (because the parent hCPC line also stably expressed the Fluc-GFP double fusion reporter gene). hCPCs expressing A168H or sr39-tk displayed the highest PET signal intensities, confirming our in vitro observations (1.34±0.06 and 1.16±0.08% ID/g, respectively). hCPC lines stably expressing the sr39-tk and A168H TK variant were then expanded for all subsequent experiments.

### Transplantation of hCPC Improves Left Ventricular Contractility

Adult SCID beige mice were subjected to left anterior descending artery ligation followed by injection with either 1×10⁶ hCPCs (n=37 from sr39-tk and A168H cohorts) or phosphate-buffered saline (n=10). To determine the effect of hCPC transplant on myocardial left ventricular (LV) function, we carried out echocardiographic (Figure 3A) and MRI (Figure 3C) assessments over the 4-week study period. Compared with control animals injected with saline, hCPC-treated animals demonstrated a statistically significant improvement in fractional shortening (Figure 3B) as well as ejection fraction (Figure 3D) at Day 14 posttransplant, as assessed by echocardiography and MRI, respectively. A statistically significant difference in ejection fraction was also detected between the hCPC-treated and untreated phosphate-buffered saline controls at Week 4 posttransplant. However, no statistically significant differences in fractional shortening were observed between the hCPC-treated and untreated phosphate-buffered saline controls at Week 4 posttransplant.
Temporal Kinetics of hCPC Survival in Ischemic Myocardium

We next sought to quantitatively assess the dynamics of cellular engraftment using PET imaging. We carried out serial PET imaging of the hCPC-treated animals and observed loss of PET signal intensity over time, reflecting death of the transplanted cells over the 28-day study period (Figure 4A–B). These results were confirmed independently using BLI of Fluc expression within the same animals (online-only Data Supplement Figure VII).

Early PET Imaging Predicts Subsequent Myocardial Functional Improvement

In light of the variable results of cell-based therapy in recent clinical trials, we decided to evaluate whether a high degree of early cellular engraftment was predictive of late therapeutic benefit. Using PET imaging data obtained on Day 1 after cell transplant as a surrogate measure of cell engraftment, we stratified the hCPC transplant recipients in our cohort into high and low engraftment groups (Figure 5A). A basic finite mixture model of Gaussian distributions inferred 2 equal-sized groups (P<0.001) with a cutoff at 0.22% ID/g (mean±SD of the 2 groups: 0.18±0.02% ID/g versus 0.27±0.02% ID/g). Substantial variation in PET signal intensity was observed at Day 1 posttransplant with no correlation to initial LV function measured on Day 1. Variable engraftment is likely attributed to imperfections in the intramyocardial delivery of hCPCs and corresponds to the degree of variation expected in a clinical setting, where initial retention may range from 1% to 19% of delivered cells depending on the mode of delivery.11 Initial PET signal intensity at Day 1, as a surrogate measure of engraftment success, correlated well with LV function as assessed by echocardiography at Week 1 (P=0.64) and correlated best with LV function at Week 2 (P=0.76; Figure 5B). Cardiac MRI assessment of myocardial function at week 2 also correlated well to initial PET signal intensity at Day 1 (P=0.80; P<0.0001; n=34). Over the course of the 4-week study period, the low cell engraftment group did not demonstrate any statistically significant improvements in LV function when compared with untreated animals (P=0.68). However, there was a significant difference in the trend over time between the high and low cell engraftment groups (P<0.001; Figure 5C). The results of the stratification into high and low engraftment groups were confirmed independently by cardiac MRI assessment of LV ejection fraction (online-only Data Supplement Figure VIII) and pressure-volume loop analysis of end-systolic volume and end-systolic pressure (online-only Data Supplement Figure IX). We then independently confirmed a positive correlation between cellular engraftment and ventricular wall thickness using gross histological and immunohistochemical staining (Figure 6). Therefore, the number of surviving hCPCs at early time points directly correlates with the degree of functional improvement at later time points in a “dose–effect” relationship. These results underscore the use of robust methods for quantitative monitoring of cellular engraftment to better understand the complex determinants of successful cell therapy.27

hCPC Expression of Paracrine Growth Factors In Vitro and In Vivo

Although some studies have demonstrated a therapeutic benefit of stem cell therapy on myocardial contractility in large animals6,5 and humans,8,8 the mechanism(s) by which stem cells might exert their beneficial effects has remained somewhat controversial. Hypotheses include paracrine stimulation of recipient myocardium, direct cardiomyogenesis, and mechanical mitigation of wall strain. In support of the role that paracrine secretion may play in augmenting cardiac function, we found that hCPCs grown in hypoxic culture conditions upregulated expression of multiple antiapoptotic growth factors, including vascular endothelial growth factor A, fibroblast growth factor 2, and epithelial growth factor (online-only Data Supplement Figure X). However, we also sought to address this question by examining the effect of transplantation on CPC phenotype using immunohistochemistry and laser capture microdissection. Laser capture microdissection allows isolation of hCPCs after their transplantation into recipient myocardium, thereby allowing evaluation of the in vivo transcriptional program of hCPCs (Figure 7A).
We found that hCPCs isolated from recipient myocardium by laser capture microdissection had significantly upregulated their expression of growth factor transcripts in vivo, particularly vascular endothelial growth factor A, vascular endothelial growth factor B, fibroblast growth factor 2, and connective tissue growth factor (Figure 7B). In contrast, immunohistochemical staining of transplanted GFP + CPCs was negative for markers of cardiac or endothelial differentiation such as α-actinin and CD31 (online-only Data Supplement Figure XI). We examined the cardiac differentiation potential of CPCs more thoroughly by assessing transplanted CPCs over time using laser capture microdissection and an array of cardiac markers. Transcript markers of early cardiac differentiation such as GATA4 and TBX5 as well as definitive cardiomyocyte markers such as MYL2, TNNT2, MYH6, and ACTN2 were all negative in >95% of the transplanted GFP + CPCs (online-only Data Supplement Figure XII). Therefore, our findings support the hypothesis that hCPC transplantation improves cardiac functional recovery due to paracrine secretion of growth factors rather than direct cardiomyogenesis. Although studies thus far have inferred the behavior of stem/progenitor cells based on their in vitro response to hypoxia, we have demonstrated here that hCPCs upregulate transcription of antiapoptotic growth factors in vivo after their transplantation into ischemic myocardium. Our results are in agreement with those of other groups suggesting that paracrine secretion plays the dominant role in ameliorating left ventricular dysfunction after transplantation of cardiac stem cells.28,29

Discussion

Several parameters for hCPC identification are currently found in the literature, including isolation of c-kit+ cells,30 cardiospheres-derived cells,31 Sca-1+ cells,22 and Isl1+ cells.32 These various hCPC formulations have all demonstrated a degree of efficacy in animal studies, although evaluation of the degree of overlap between these various populations and their comparative efficacy is needed in the future. We tested 4 different variations of the TK PET reporter gene in conjunction with the [18F]-FHBG PET reporter probe to monitor Sca-1+ hCPC therapy. The transcriptome and differentiation capacity of hCPCs was not affected by stable expression of the TK reporter gene. hCPC survival in murine ischemic myocardium was subsequently monitored using BLI and PET and was noted to decline substantially over the 4-week study period. Nonetheless, hCPC therapy transiently ameliorated the negative consequences of myocardial infarction, as demonstrated by echocardiographic, cardiac MRI, and pressure-volume loop evaluation of LV function. Importantly, early PET imaging assessment of the success of hCPC engraftment at Day 1 was found to correlate positively with subsequent LV functional improvement at Day 14. The mechanism by which hCPCs exert their functional benefits include upregulation of paracrine growth factor expression in treated hearts.
Clinical trials of stem cell therapies for myocardial repair have become a reality in recent years. Preliminary results from the SCIPIO trial have demonstrated that autologous c-kit\(^+\) Lin\(^-\) cardiac stem cells can enhance myocardial function in patients who have had myocardial function up to 1 year after treatment.\(^{10}\) A second Phase I study has also reported encouraging preliminary data (CADUCEUS; CArdiocyte and DErived AuToologous stem CElls to Reverse ventricular dySfunction).\(^{33}\) Two recent meta-analyses of studies evaluating bone marrow-derived cell therapy for myocardial repair have also reported a modest yet significant 3% to 4% augmentation in LV ejection fraction.\(^{8,9}\) However, the mechanism(s) by which stem cells exert their beneficial effects has remained controversial. In agreement with others,\(^{28}\) the mechanism of functional benefit in our study appears to involve upregulated expression and secretion of various growth factors.

Irrespective of the relative contribution of the multiple complementary mechanisms of stem cells’ effects, the ultimate success of any given cell therapy will likely depend on the successful delivery and engraftment of a sufficient number of transplanted cells. Cell-based myocardial repair strategies have thus far remained hindered by poor engraftment (<10%) of all cell types, including mesenchymal stem cells,\(^{34}\) bone marrow mononuclear cells,\(^{35}\) human embryonic stem cell-derived cardiomyocytes,\(^{36}\) and cardiosphere-derived cells.\(^{5}\) Robust methods for longitudinal monitoring of cell engraftment will therefore play a necessary and vital role

Figure 6. Ex vivo histological confirmation of in vivo PET imaging data. A, hCPCs engrafted within the recipient myocardium at Day 14 after injection. Higher levels of GFP\(^+\) hCPCs are identified within the hearts with higher Day 1 PET signal. B, Myocardial wall thickness in representative thin sections of gross hearts at Day 14 after injection. C, Significant enhancement of myocardial wall thickness in the hearts engrafting high levels of hCPCs (one-way ANOVA with Bonferroni correction; \(P<0.0001; n=12/\text{group}\)). PET indicates positron emission tomography; hCPCs, human cardiac progenitor cell; GFP, green fluorescent protein; ANOVA, analysis of variance.

Figure 7. In vivo growth factor gene expression response of hCPCs to myocardial ischemia. A, Laser capture microdissection (LCM) was used to identify and isolate GFP\(^+\) hCPCs at Day 14 after injection for RNA extraction. B, qPCR of transplanted hCPCs isolated using LCM reveals upregulation of VEGF-A, VEGF-B, and CTGF growth factor genes in vivo as compared with control samples isolated from a remote zone of the same heart (n=5/group). hCPCs indicates human cardiac progenitor cells; GFP, green fluorescent protein; qPCR, quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; CTGF, connective tissue growth factor.
in better understanding the complex determinants of successful cell therapy. In the present study, we optimized PET reporter gene imaging as a surrogate marker of the success of hCPC engraftment and demonstrated the role of PET imaging in providing important prognostic information regarding which recipients were likely to functionally benefit from hCPC transplant in subsequent follow-up. Due to the considerable biological complexity of any potential therapeutic cell product and its interaction with the host as well as the relatively simple current techniques for delivery, clinical application of cell therapy will likely demonstrate wide variability in the engraftment of administered cells. Early assessment of engraftment success using the clinically applicable imaging modalities demonstrated here could significantly and positively impact the course of care, potentially identifying which patients are candidates for additional rounds of cell therapy administration due to undertreatment. The positive correlation of Day 1 PET signal with Week 2 left ventricular contractility as described here also implies a theoretical “dose–response” relationship for hCPC therapy and bodes well for its future success as a treatment option alongside pharmacological therapies. Even more favorable results may be achieved in the future using strategies aimed at prolonging cell survival such as prosurvival cocktails or genetic modification, whereby the incremental benefits can also be monitored by imaging of cell fate. As the field of cardiac cell therapy continues to mature, an improved understanding of the determinants of success has become crucial, and noninvasive imaging approaches as outlined here are likely to play a significant role in this effort.

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Disclosures
None.

References
Human cardiac progenitor cell transplantation is an exciting new therapy to ameliorate cardiac dysfunction after myocardial infarction. However, much remains to be understood regarding the parameters for successful cell therapy, including the optimal cell dose, engraftment rate, long-term survival, and delivery route. Molecular imaging techniques can be used to monitor the retention, engraftment, and delivery of stem cells after their delivery in vivo. In this study, we stably modified human cardiac stem cells expressing Pim-1 kinase. This enabled us to label the cells and track their survival and take-up in the myocardium using positron emission tomography (PET).

CLINICAL PERSPECTIVE

Human cardiac progenitor cell transplantation is an exciting new therapy to ameliorate cardiac dysfunction after myocardial infarction. However, much remains to be understood regarding the parameters for successful cell therapy, including the optimal cell dose, engraftment rate, long-term survival, and delivery route. Molecular imaging techniques can be used to monitor the retention, engraftment, and delivery of stem cells after their delivery in vivo. In this study, we stably modified human cardiac progenitor cells to express variants of the thymidine kinase reporter gene, thereby enabling use of positron emission tomography to monitor cellular engraftment and survival after intramyocardial delivery. We found that human cardiac progenitor cell transplantation resulted in a modest improvement in myocardial contractility and that this improvement in contractility correlated with the number of engrafted cells at early time points. Importantly, we showed that cellular engraftment at early time points can provide valuable prognostic information regarding the ultimate success of human cardiac progenitor cell transplantation at later time points. In light of the variable response to cell transplantation seen in recent clinical cell therapy trials, molecular imaging techniques may prove pivotal to ensuring adequate cellular delivery and optimizing patient outcomes.
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SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

Isolation and culture of cardiac progenitor cells from human hearts. Human fetal tissue and atrial biopsies were obtained with approval by the Stanford IRB committee. The human cardiac progenitor cells (hCPCs) were isolated based on a previously described protocol using magnetic separation in conjunction with a Sca-1 antibody.$^{1-3}$ Human fetal hearts were subjected to Langendorff perfusion with Tyrode’s solution containing collagenase and protease. Atrial biopsies were minced into small pieces followed by collagenase treatment. After cardiomyocyte depletion of the cell suspension, hCPCs were isolated by magnetic cell sorting (MACS, MiltenylBiotec, Sunnyvale, CA) using Sca-1-coupled magnetic beads, according to the manufacturer’s protocol. Sca-1$^+$ cells were eluted from the column by washing with PBS supplemented with 2% FBS and cultured on 0.1% gelatin-coated dishes in M199 (Gibco)/EGM-2 (3:1) supplemented with 10% FCS (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml epithelial growth factor (EGF), 5 ng/ml insulin-like growth factor (IGF-1), and 5 ng/ml hepatocyte growth factor (HGF).

Flow cytometry analysis. Cultured hCPCs were trypsinized and 300,000 cells were used for fluorescence activated cell sorting (FACS) analysis. The cells were washed twice in wash buffer (1% FBS/PBS/0.05M azide) and resuspended in 100 µl wash buffer containing antibody. The cells were incubated on ice in the dark for 30 minutes, washed four times with cold wash buffer, resuspended in 250 µl wash buffer, and analyzed using a Beckman Coulter Cytomics FC500 FACS. Antibodies used were fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated against CD14, CD34, CD45, CD133, CD105 (endoglin), Sca-1, and isotype control IgGs, all from Pharmingen BD.
**Lentiviral vector construction.** Schematic of lentiviral constructs used in this study are presented in Figure 1A. Lentiviral vectors were cloned using the Enzyme Free Lentivector Kit (System Biosciences, Menlo Park, CA). Four primers were designed for each TK reporter gene to be cloned. Primer 1 (forward) and 2 (reverse) were for amplification. Primers 3 and 4 contained the adaptor sequences GAGGCAGCAGAGACCG and CGAACAGAGAGACCG plus the sequence of primers 1 and 2, respectively. The wt-tk and sr39-tk cDNA sequences were kindly provided by Dr. Sanjiv Gambhir (Stanford University School of Medicine) \(^4\). ΔhTK2 and A168H cDNA sequences were kindly provided by Dr. Juri Gelovani (University of Texas, MD Anderson Cancer Center) \(^5,6\). The lentivirus was produced by transfecting 293T packaging cells with Clone-it™ Enzyme free Lentivectors using Lipofectamine 2000 (Invitrogen) in 100-mm dishes. Two 3-dimensional supernatants were collected. The collected supernatant was filtered using Amicon Ultra-15 centrifugal filter devices (Millipore, County Cork, Ireland), and stored at -80°C.

**Quantitative polymerase chain reaction (q-PCR) assay.** The transduced hCPCs were analyzed by Quantitative PCR using an ABI StepOnePlus® instrument (Applied Biosystems). The primer pairs which were used to detect puromycin and endogenous standard glyceraldehydes-3-phosphate dehydrogenase (GAPDH) are as followed: 5’-ACCGAGTACAAGCCACACG-3’ (PURO-F), 5’-TTGCGATGTCAGCCCG-3’ (PURO-R), 5’CCATGGAGAAGGCTGGGG-3’ (GAPDH-F), and 5’CAAAGTTTCTATGGATGACC-3’ (GAPDH-R). Expression of puromycin was normalized to GAPDH, and results were expressed as change in C\(_t\) (\(\Delta C_t\)) values. PCR reactions were performed on the ABI 7900HT system.
Effect of reporter gene expression on hCPC viability, proliferation, and differentiation potential. The effect of introducing the various TK reporter genes on hCPC viability and proliferation was tested using the Trypan blue exclusion assay and the DHL cell proliferation assay kit (Anaspec), respectively. To test differentiation potential, hCPCs were cultured in the presence of cell culture media supplemented with specific differentiation factors. To induce differentiation into cardiomyocytes, cells were treated with 5 μM 5-azacytidine (Sigma) for 72 hours in differentiation medium (Iscove’s Modified Dulbecco’s Medium/Ham’s F12 (1:1) (Gibco)) supplemented with L-glutamine (Gibco), 2% horse serum, nonessential amino acids, insulin-transferrin-selenium supplement, and 10-4 M ascorbic acid (Sigma). After induction, the medium was changed every three days. For smooth muscle differentiation, CPCs were cultured in poly-D-lysine coated plates in differentiation medium containing 35% Iscove’s modified Dulbecco’s medium with 10% FBS/65% Dulbecco’s modified Eagle medium-Ham F-12 mix containing 2% B27, 0.1 mmol/l 2-mercaptoethanol, 10 ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN), 20 ng/ml basic fibroblast growth factor, 40 nmol/l cardiotrophin-1, 40 nmol/l thrombin (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin G, 100 g/ml streptomycin, and 2 mmol/l glutamine. For endothelial differentiation, hCPCs were cultured on fibronectin-coated plates with EGM-2 medium (Lonza, Switzerland) with an extra 20 ng/ml of VEGF (R&D Systems). Then the cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as previously described.

In vitro $[^{18}F]$-FHBG accumulation assay. TK-expressing hCPCs were seeded in 150-mm tissue culture dishes (Falcon) at a concentration of $1 \times 10^6$ cells per dish in triplicate and grown until 50%-60% confluent. Then the medium was replaced with 14 mL of medium containing F-18-9-
(4-fluoro-3-hydroxymethylbutyl)guanine ([\(^{18}\)F]-FHBG) radiotracer (92.5 kBq/mL at time 0). The cells were subsequently incubated for 15, 30, 60, and 120 min before the monolayers were scraped, transferred into 15-mL tubes, and centrifuged at 1,000 \(\times\) g for 2 min. A 100 µl aliquot of supernatant was transferred to a pre-weighted scintillation tube, and the rest was removed by aspiration before the cell pellet was snap-frozen on dry ice. The frozen pellets were transferred to pre-weighted scintillation vials, weighed, and thoroughly resuspended in 0.5 mL of Soluene-350 (Perkin Elmer). Radioactive \(\gamma\)-emissions of the medium and the cell pellets were measured on a \(\gamma\)-counter (Cobra Quantum; Packard) to quantify [\(^{18}\)F]-FHBG uptake. Activity ratios of the cell pellet to medium ([dpm/g cells]/[dpm/g medium]) were calculated and plotted against time.

**Microarray hybridization and data analysis.** Total RNA samples were hybridized to Affymetrix GeneChip Human Gene 1.0 ST Arrays, and then normalized and annotated by the Affymetrix® Expression Console™ software. The Pearson Correlation Coefficient was calculated for each pair of samples using the expression level of transcripts which showed a standard deviation of greater than 0.2 among all samples. For hierarchical clustering, we used Pearson correlation for average linkage clustering.

**In vivo optical bioluminescence imaging (BLI).** Bioluminescence was measured at day 1, day 7, day 14, day 21 and day 28 using an In Vivo Imaging System 50 (IVISTM; Caliper, Hopkinton, MA) at 37°C. Mice were injected (150 mg/kg body weight, i.p.) with D-luciferin (Caliper) immediately prior to anesthesia. Mice were anesthetized with 2% isoflurane and imaged at 5-min exposure times. Bioluminescent signals were analyzed using Living Image® 2.50.2 software (Caliper) to identify regions of interest, quantitate light emission, and subtract
background luminescence.

**Analysis of left ventricular function with echocardiogram and magnetic resonance imaging (MRI).** Echocardiography was performed before (day -7) and after (day 2, week 1, week 2, week 3 and week 4) the LAD ligation. The Siemens-Acuson Sequoia C512 system equipped with a multi-frequency (8-14 MHZ) 15L8 transducer was used by an experienced operator (D.N.) blinded to the group designation. Analysis of M-mode images was performed using Siemens built-in software. Left ventricular contractility was assessed by measuring left ventricular end-diastolic diameter (EDD), end-systolic diameter (ESD), and volume blood at end of diastole (BED), and calculating left ventricular fractional shortening by the formula: LVFS = (EDD-ESD)/BED. The MRI imaging was performed on a Sigma 3.0T Excite HD scanner (GE Healthcare Systems, Milwaukee, Wisconsin) with a Mayo Clinic T/R MRI coil (Mayo Clinic Medical Devices, Rochester, Minnesota) in a subset of animals (n=5/group). Mice were anesthetized with 2% isoflurane with oxygen (1 L/min) and placed in the prone position for imaging. A small animal electrocardiogram and respiratory gating system (Small Animal Instruments, Stony Brook, New York) was used to acquire images as previously described 8.

**Analysis of left ventricular function with pressure-volume (PV) loops.** At the end of the study (week 8), invasive hemodynamic measurements were performed in a subset of the animals (n = 10/group). Briefly, after midline neck incision, a 1.4 conductance catheter (Millar Instruments, Houston, Texas) was introduced into the left ventricle through the right carotid artery. After stabilization, the signals were continuously recorded at a sampling rate of 1,000/s using PV conductance system coupled to a PowerLab/4SP analog to digital converter (AD
Instruments, Colorado Springs, Colorado). Data were analyzed by using a cardiac PV analysis program (PVAN 3.4, Millar Instruments) and Chart/Scope Software (AD Instruments).

In vitro expression of cytokine proteins by hCPCs after exposure to hypoxia. hCPCs grown in culture were subjected to a 48 hr hypoxia treatment (5% O₂), after which the supernatant was collected and exposed to the angiogenesis cytokine array (Panomics) according to the manufacturer’s instructions. In three separate experiments (each containing duplicate spots), the arrays were hybridized and imaged together. Expression intensities were calculated by adding the total pixel intensity for each spot. Normalization within each array was achieved using positive control spots (8 per array). Protein expression levels were compared between hCPCs grown under normoxic and hypoxic conditions.

Histological examination. Mice were sacrificed and left ventricular (LV) tissue was obtained at 4 weeks after MI. Tissue samples were embedded into OCT compound (Miles Scientific, Elkhart, IN). Frozen sections (5 μm thick) were processed for immunostaining. Anti-cardiac Troponin T antibody (Thermo Scientific) and anti-GFP antibody (Thermo Scientific) were used. To quantify the left ventricle (LV) infarct size, H&E staining was performed in each group (n=5/group). For each heart, eight to ten sections from apex to base (1.2 mm apart) were analyzed. Images were taken for each section to calculate the fibrotic and non-fibrotic areas as well as ventricular and septal wall thickness. The NIH Image J software was used to quantify the infarct zones.
Supplemental Figure 1
Supplemental Figure 2

relative gene expression
Supplemental Figure 3

A

Control 1×10^3 2×10^3 4×10^3

6×10^3 8×10^3 1×10^4 2×10^4

B

R^2=0.98

Photons/sec/cm^2/sr

0 5.0×10^3 1.0×10^4 1.5×10^4 2.0×10^4 2.5×10^4

Cell numbers
Supplemental Figure 4
**Supplemental Figure 5**

A

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B

**A168H**

![Graph showing data distribution for A168H](image)

**sr39-tk**

![Graph showing data distribution for sr39-tk](image)

**Δhtk2**

![Graph showing data distribution for Δhtk2](image)

**wt-tk**

![Graph showing data distribution for wt-tk](image)
Supplemental Figure 6
Supplemental Figure 7
Supplemental Figure 9

A

![Graphs showing pressure-volume relationships for control, low cell engraftment, and high cell engraftment conditions.](image)

B

![Bar charts comparing ESV, EDV, ESP, and EDP across control, low cell engraftment, and high cell engraftment conditions.](image)

Supplemental Figure 9
Supplemental Figure 11
Supplemental Figure 12
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Flow cytometry analysis of cell surface marker expression of cultured hCPCs. Histogram plots are shown with the isotype control in black and the specified marker in gray. hCPCs demonstrate the expected Sca-1^+, CD105^+, CD45^−, CD133^−, CD14^−, and CD34^− profile.

Supplemental Figure 2. Gene expression profiling of hCPC lines transduced with various thymidine kinase reporter genes in comparison to untransduced control hCPCs as well as human embryonic stem cells (hESC). Upregulation of cardiac-specific genes is observed in relation to hESC (H7 line), with no significant differences in gene expression noted between the various hCPC lines transduced with different TK reporter genes.

Supplemental Figure 3. Fluc+/GFP^+ hCPCs demonstrate a linear correlation between bioluminescence imaging (BLI) signal and number of viable cells, allowing for quantitative longitudinal BLI of hCPC transplant in living animals.

Supplemental Figure 4. Viability of hCPCs as assessed by Trypan blue exclusion assay was not affected by stable transduction of thymidine kinase variants. Cell viability values were normalized to those obtained on day 0 of cell culture.

Supplemental Figure 5. Microarray analysis of hCPC transcriptome before and after stable expression of thymidine kinase variants. Stable transduction with thymidine kinase reporter genes does not affect the gene expression profile of hCPCs. (A) Cross-correlation coefficients of paired microarray comparisons are >0.97 for all tested hCPC lines. A negative control human
embryonic stem cell line (hES) was included for comparison. (B) Scatter plots depicting gene expression fold-changes between paired cell lines. No significant variations in gene expression are noted among the various stably transduced hCPC lines.

**Supplemental Figure 6.** Normal karyotypes of the hCPC lines used in this study. Normal 46,XY karyotype of parent Fluc+/GFP+hCPC line (left). Normal 46,XY karyotypes of hCPC lines stably expressing the A168H, sr39-tk, Δhtk2, and wt-tk variants (right). Stable expression of thymidine kinase reporter gene does not result in any detectable genomic instability.

**Supplemental Figure 7.** Temporal kinetics of hCPC engraftment monitored using bioluminescence imaging. Representative images of adult SCID beige female mice injected with 1x10^6 hCPCs stably expressing the sr39-tk, Δhtk2, or wt-tk PET reporter gene variants are shown over a period of four weeks. A substantial decline in BLI signal intensity was observed over the study period, as shown in Figure 4, confirming the results of our PET imaging data acquisition and analysis.

**Supplemental Figure 8.** Echocardiographic and MRI assessment of cardiac contractility segregated into high vs. low engraftment groups. (A) Representative M-mode echocardiogram of hearts in the high vs. low cell engraftment categories. (B) Representative cardiac MR images of hearts in the high vs. low engraftment categories. (C) Significant improvement in left ventricular ejection fraction (vertical axis) in the high cell engraftment group compared to PBS controls at week 2 (Linear mixed model with high vs low engraftment, time, and group X time interaction as fixed effects, mouse as random effect. P<0.001; n=37 total, n=19 in the high cell engraftment
Supplemental Figure 9. Invasive hemodynamic monitoring confirms results of echocardiographic and cardiac MR analyses. (A) Pressure-volume (PV) loop recordings of mice in the high and low cell engraftment groups along with PBS controls at week 8 after injection. (B) Significant improvement in end-systolic volume (ESV), end-diastolic volume (EDV), and end-systolic pressure (ESP) is seen in the high cell engraftment group, when compared with PBS controls. One-way ANOVA; $P = 0.004$, $P = 0.009$, and $P = 0.002$, respectively. (n=10/group). Dunnett’s Multiple Comparison Test was also performed ($P<0.05$ for high cell engraftment vs PBS).

Supplemental Figure 10. Growth factor expression of CPCs increases dramatically in response to hypoxia. (A) Raw image of the growth factor antibody array. The media of CPCs grown in normoxic or hypoxic (5% $O_2$) culture conditions for 48 hours was exposed to the antibody array. A key to the antibody array is displayed to the right. (B) Quantification of three antibody array experiments reveals significant increases in the secretion of growth factors such as VEGF-A, FGF2, and EGF in CPCs grown under hypoxic conditions.

Supplemental Figure 11. Immunohistochemical analysis of transplanted GFP$^+$ hCPCs reveals no evidence of significant differentiation of transplanted cells into cardiomyocyte or endothelial cells lineages. Co-localization is not observed between GFP and $\alpha$-actinin, a cardiomyocyte marker (top right panel) or between GFP and CD31, and endothelial cell marker (bottom right panel).
Supplemental Figure 12. Cardiac lineage transcript expression over time in transplanted cardiac progenitor cells shows no evidence for significant direct cardiac differentiation. (A) Transcriptional markers of early cardiac commitment, such as GATA4 and TBX5, were only marginally positive in isolated GFP+ CPCs. (B) Transcriptional markers of committed cardiomyocytes, such as TNNT2, MYH6, and Actinin2 were positive in <5% of isolated cells. (C) Endothelial cell transcripts such as VCAM1, ICAM1, and vWF were also only marginally positive in isolated GFP+ cells. (D) Transplanted cells were also largely negative for smooth muscle markers such as SMA.
REFERENCES


