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Safe Genetic Modification of Cardiac Stem Cells Using a Site-Specific Integration Technique

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Abstract

Background—Human cardiac progenitor cells (hCPCs) are a promising cell source for regenerative repair after myocardial infarction. Exploitation of their full therapeutic potential may require stable genetic modification of the cells *ex vivo*. Safe genetic engineering of stem cells, using facile methods for site-specific integration of transgenes into known genomic contexts, would significantly enhance the overall safety and efficacy of cellular therapy in a variety of clinical contexts.

Methods and Results—We employed the phiC31 site-specific recombinase to achieve targeted integration of a triple fusion reporter gene into a known chromosomal context in hCPCs and human endothelial cells (hECs). Stable expression of the reporter gene from its unique chromosomal integration site resulted in no discernible genomic instability or adverse changes in cell phenotype. Namely, phiC31-modified hCPCs were unchanged in their differentiation propensity, cellular proliferative rate, and global gene expression profile when compared to unaltered control hCPCs. Expression of the triple fusion reporter gene enabled multimodal assessment of cell fate *in vitro* and *in vivo* using fluorescence microscopy, bioluminescence imaging (BLI), and positron emission tomography (PET). Intramyocardial transplantation of genetically modified hCPCs resulted in significant improvement in myocardial function two weeks after cell delivery, as assessed by echocardiography (P = 0.002) and magnetic resonance imaging (P = 0.001). We also demonstrated the feasibility and therapeutic efficacy of genetically

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modifying differentiated hECs, which enhanced hindlimb perfusion (P<0.05 at day 7 and 14 after transplantation) on laser Doppler imaging.

Conclusions—The phiC31 integrase genomic modification system is a safe, efficient tool to enable site-specific integration of reporter transgenes in progenitor and differentiated cell types.

Keywords

cell therapy; stem cells; imaging; cardiovascular disease

INTRODUCTION

Cardiovascular disease remains one of the leading causes of morbidity and mortality in the United States and globally. After a myocardial infarction, the limited proliferative ability of the surviving myocardial cells renders the heart susceptible to morbid sequelae such as unfavorable remodeling and ischemic cardiomyopathy. Recently, stem cell therapy has emerged as a promising adjunct to existing pharmacologic and device treatment options [1, 2]. In particular, human cardiac progenitor cells (hCPCs) are attractive candidates for clinical translation of cell therapy [3]. hCPCs can be autologously derived, obviating the need for immune suppression after transplantation, and are pre-programmed for differentiation into the three principal cardiovascular lineages: cardiomyocytes, vascular endothelial cells, and smooth muscle cells. Pre-clinical studies in small and large animals have thus far affirmed the beneficial effect of CPC transplantation on recipient myocardial function [4–7]. More recently, phase I clinical trials demonstrated that infusion of hCPCs resulted in a mean improvement of ~8% in left ventricular ejection fraction (LVEF) in treated patients compared to controls after 4 months [8].

Despite these promising initial results, little is known regarding important questions such as the optimal cell dose or delivery method, the rate of *in vivo* cellular survival, proliferation, engraftment and differentiation, or the mechanism(s) of potential therapeutic benefit in clinical hCPC trials. Translation of stem cell therapy has also been limited by poor engraftment and survival [9, 10] as well as unpredictable differentiation *in vivo* [11, 12]. In recent years, stem cell imaging has emerged as a valuable tool to quantitatively address some of these variables, and its implementation in clinical trials could add novel insight into the determinants of successful stem cell therapy [13]. Genetic engineering of stem cells is currently being explored as a means to image transplanted cells as well as overcome the aforementioned challenges. Stable *ex vivo* gene transfer into stem cells prior to their transplantation can be used to enhance stem cell homing [14], survival [15], imaging [16], and targeted ablation [16, 17]. However, genetic engineering is limited by many of the challenges facing the gene therapy field at large. In particular, several current gene therapy approaches rely on retrovirus, lentivirus, or transposon elements, which catalyze random transgene integration into many potential sites. Randomly integrating vectors are not safe for clinical use because insertional mutagenesis can cause oncogenic activation and malignant transformation of cells [18]. Safe techniques for site-specific integration of transgenes into the human genome are therefore needed to overcome these shortcomings.

The phiC31 integrase is a site-specific recombinase that can be used to integrate transgenes into specific loci of the human genome. Specifically, the phiC31 integrase catalyzes recombination between *attB* and *attP* attachment sites, which are DNA recognition sequences of substantial size (~17bp). This enzyme can therefore be used to integrate a transgene-containing plasmid carrying an *attB* site into pseudo-*attP* sites in the human genome. These specific loci of the human genome are known to produce robust, long-term transgene expression without risking proto-oncogene activation [19]. Here we employed this site-specific integration system to safely derive hCPC clones capable of long-term *in vivo*

monitoring by using positron emission tomography (PET) or bioluminescence imaging (BLI). The resulting hCPCs are safe for use in humans because of their precisely known genetic makeup. Importantly, in addition to reporter gene imaging, this technique can be used to introduce a variety of genetic constructs safely and efficiently for enhanced cardiac stem cell therapy.

METHODS

Isolation, culture, and characterization of human cardiac progenitor cells

Human cardiac progenitor cells (hCPCs) were isolated based on a previously described protocol using magnetic separation in conjunction with a Sca-1 antibody [20–22]. The study protocol was approved by the Stanford Institutional Review Board (IRB). In brief, human fetal hearts (StemExpress, Diamond Springs, CA) were perfused using a Langendorff apparatus using Tyrode's solution containing collagenase and protease. hCPCs were isolated by magnetic cell sorting (MACS, Miltenyl Biotec, 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). For isolation of human endothelial cells (hECs), please see Supplemental Methods for details. All hCPCs (unmodified and genetically-modified) used for *in vitro* experiments and intramyocardial injection were at passage 10–16.

Flow cytometry analysis

Please see Supplemental Methods for details.

Plasmid constructs and generation of site-specific integrated cell lines

The donor construct pDB-FRT was generated by replacing the cassette coding GFP in a donor vector pDB2 [23] containing an *attB* site with a triple fusion gene [24]. The plasmid pCSI expressing phiC31 integrase has been described [25]. To construct the donor plasmid pDB-FRT, a triple fusion gene coding firefly luciferase (Fluc), monomeric red fluorescent protein (mRfp), and herpes simplex virus truncated thymidine kinase (HSVttk) was cloned downstream of the CMV promoter. The hCPCs and hECs were grown and transfected with Fugene6 according to the manufacturer's instructions using 3 μl of reagent per 1 μg of DNA. Transfections yielding integrase-derived clones used 10 ng of pDB-FRT and 5 μg of pCSI. After 24 h, cells were split 1:30 to three 10 cm plates. At 48 h after transfection, cells were selected in medium containing 350 μg/ml of G418 (Invitrogen). Selection was continued for 10–14 days, at which time colonies became visible. Individual colonies were picked with a pipet tip and transferred to individual wells of a 24-well plate. Surviving colonies were expanded for PCR analysis. To screen for site-specific integrations, a nest PCR method using primers targeting 11 most frequent integration sites was used as described previously [26]. Briefly, a set of 11 primers were used for the first round PCR to amplify the integration genes from the genomic DNA extracted from individual clones. The clones with site-specific integration demonstrated the expected size second round PCR product and were selected for further characterization and functional studies.

Effect of reporter gene expression on hCPC viability, proliferation, and differentiation

Please see Supplemental Methods for details.

In vitro [^{18}F]-FHBG accumulation assay

Please see Supplemental Methods for details.

Microarray hybridization and data analysis

Please see Supplemental Methods for details.

Surgical model of mice myocardial infarction and hCPC delivery

In adult female SCID Beige mice (Charles River Laboratories, Wilmington, MA), myocardial infarction (MI) was induced by ligation of the left coronary artery under 1.5–2% inhaled isoflurane anesthesia, and confirmed by myocardial blanching and EKG changes. Animals were randomized into experimental groups receiving phiC31-hCPCs (n=30) or unmodified hCPCs (n=15) and a control group receiving PBS (n=15). All experimental animals were injected with 1×10^6 cells using a 31-gauge Hamilton syringe immediately after MI. In all groups, the volume of injection was 20 μl at 3 sites near the peri-infarct border zone. All surgical procedures and injections were performed by a blinded experienced microsurgeon (Y.G.).

In vivo optical bioluminescence imaging (BLI) of cell survival in transplanted animals

Please see Supplemental Methods for details.

[^{18}F]-FHBG positron emission tomographic (PET) imaging of cell survival in transplanted animals

Please see Supplemental Methods for details.

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 (P.K.N.) blinded to the group designation. Analysis of M-mode images was performed using Siemens built-in software. Left ventricular volume at end of diastolic diameter (EDD) and end-systolic diameter (ESD), and volume blood at end of diastolic (BED) were measured and used to calculate left ventricular ejection fraction 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 [27].

Delivery of human endothelial cells (hECs) in a hindlimb ischemia model

Please see Supplemental Methods for details.

Laser Doppler perfusion imaging

Blood flow to the ischemic or normal (nonischemic) hindlimb was assessed using a PeriScan PIM3 laser Doppler system (Perimed AB, Sweden) as described previously [28]. Each animal was prewarmed to a core temperature of 37°C, and hindlimb blood flow was measured pre- and post-operatively on day 0 and again on days 3, 7, and 14. Regions of interest of the ischemic or nonischemic hindlimb were drawn in a standard fashion. The level of perfusion in the ischemic and normal hindlimbs was quantified using the mean pixel value within the

region of interest, and the relative changes in hindlimb blood flow were expressed as the ratio of the left (ischemic) over right (normal) mean pixel value.

Histological examination

Please see Supplemental Methods for details.

Statistical analysis

Statistics were calculated using SPSS 12.0 (SPSS Inc, Chicago, Ill). Descriptive statistics included mean and SD. One-way analysis of variance and 2-way repeated-measures analysis of variance with post hoc testing were used. Differences were considered significant at *P*-values of <0.05.

RESULTS

phiC31 integrase mediates site-specific integration of the triple fusion reporter gene

A donor vector containing the triple fusion reporter gene was designed as shown in Figure 1A. The triple fusion reporter gene encodes firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and herpes simplex virus type truncated thymidine kinase (HSVttk) to enable multimodal imaging using bioluminescence, fluorescence, and positron emission tomography (PET), respectively. This vector was co-transfected with the integration vector containing the phiC31 integrase into 293 cells to validate the site-specific integration system. The transfected cells were cultured in neomycin for three weeks to select stably-integrated cell clones. Genomic DNA was isolated from these clones, and primers for three typical integration sites were used to screen for site-specific integration of the triple fusion reporter transgene (Supplemental Figure 1). As expected, only the cells transfected with phiC31 integrase showed an appropriately-sized PCR product, indicating unique transgene insertion at the intended genomic site.

Isolation and derivation of genetically modified human cardiac progenitor cells

We first isolated Sca-1^+ hCPCs from human fetal hearts and clonally expanded them using a previously described protocol [21]. The purity of the isolated progenitor cells was confirmed by cell surface marker expression (Supplemental Figure 2). Then, one 60-mm cell culture dish containing approximately 1×10^6 hCPCs was co-transfected with the donor and integration plasmids as shown in Figure 1A. After 3 weeks of growth in selection media containing neomycin, an average of 57 colonies was obtained (See Supplemental Methods). Control transfections of the donor plasmid only (without the integration plasmid) yielded only 1–2 neomycin-resistant colonies, suggesting that transient expression of the phiC31 integrase substantially improved the rate of site-specific recombination. We then screened the genomic DNA of hCPCs for integration using a set of 44 pairs of primers [19]. The genomic DNA of pooled neomycin-resistant hCPCs reliably demonstrated integration in 3 well-characterized integration sites on chromosomes 19, 21, and X (19q13, 21q21 and Xq22; Figure 1B; See Supplemental Methods). We screened 20 arbitrarily selected clones with 8p22, 19q13, 21q21, and Xq22 primers. From these 20 clones, we obtained 2 clones with a single integration: clone 16 contained a single integration at the 19q13 site and clone 7 contained a single integration at the 21q21 site (Figure 1C). Of note, only single integration sites were found in the clones obtained using phiC31 integration, whereas clones derived in parallel using lentiviral vectors displayed *multiple* integration sites, as evaluated by Southern blotting (Supplemental Figure 3). We then chose the hCPC clone with known integration at the 19q13 site (Figure 1D) for subsequent functional experiments (designated phiC31-hCPCs).

Site-specific integration of the triple fusion reporter gene does not alter hCPC phenotype and enables robust transgene expression

Expression of the reporter gene from the "safe harbor" locus on chromosome 19 did not affect the cellular phenotype of hCPCs. Namely, integration of the reporter gene using phiC31 caused no discernible adverse effects on cellular proliferation (Supplemental Figure 4), karyotype (Supplemental Figure 5), or gene expression profile (Supplemental Figure 6). Of note, hCPC clones derived using lentivirus did display a relative deficit in cellular proliferation capacity at 72 and 96 hours compared to untransduced hCPCs (Supplemental Figure 4). Most importantly, derivation of hCPC clones using phiC31 did not appreciably affect the differentiation propensity of hCPCs. phiC31-hCPCs retained expression of the Mef2C and Nkx2.5 cardiac transcription factors, as well as their ability to differentiate into cardiomyocyte, smooth muscle, and endothelial cell lineages, as evidenced by expression of the α-actinin, smooth muscle actin (SMA), or CD31 markers, respectively (Figure 2A, Supplemental Figure 7). The gene expression profile of phiC31-hCPCs, as assessed by transcriptome microarray, was no different than that of untransduced hCPCs (Figure 2B). Finally, expression of the triple fusion reporter gene (Fluc-mRFP-HSVttk) from the 19q13 site was stable and robust. Fluc activity correlated linearly with cell number ($R^2 = 0.99$) as shown in Figure 2C and Supplemental Figure 8. Bioluminescent signal intensity did not decrease appreciably over multiple passages (Supplemental Figure 9), indicating that "gene silencing" played a negligible role in interpretation of the results. Uptake of 9-[4-[^{18}F]fluoro-3-(hydroxymethyl)butyl]guanine ([^{18}F]-FHBG) radiotracer increased linearly over time in both phiC31-modified hCPCs and lentivirally-modified hCPCs, signifying robust HSVttk activity (Figure 2D, Supplemental Figure 8). As expected, HSVttk activity was diminished in phiC31-modified hCPCs containing a single integration copy of the reporter gene, compared to lentivirally-modified hCPCs that contained multiple randomly integrated copies of the reporter gene (Supplemental Figure 3).

Intramyocardial transplantation of human cardiac progenitor cells improves left ventricular contractility

We next tested the efficacy of hCPC transplantation in ameliorating the effects of myocardial infarction in mice. Adult NOD SCID mice were subjected to LAD artery ligation followed by injection with 1×10^6 phiC31-hCPCs (n=30), 1×10^6 unmodified-hCPCs (n=15), or PBS control (n=15). Myocardial left ventricular (LV) function after hCPC transplantation was assessed by echocardiography (Figure 3A) and cardiac MRI (Supplemental Figure 10A) over the 8-week study period. At week 2 post-transplant, phiC31-hCPC-treated hearts demonstrated a statistically significant improvement in fractional shortening (24.9±1.34% vs. 19.8±0.99%; P=0.0017; Figure 3B) and ejection fraction (54.3±1.82% vs. 45.6±1.51%; P=0.0008; Supplemental Figure 10B) compared to PBS controls. However, no statistically significant differences in contractility were observed between the hCPC-treated and untreated PBS controls at week 8 post-transplant. As expected, the phiC31-hCPC treated group did not display statistically significant differences from the unmodified hCPC-treated group at any of the tested time points.

Molecular imaging of human cardiac progenitor cell survival in ischemic myocardium

We then evaluated the temporal kinetics of hCPC engraftment using BLI and PET. We carried out serial acquisitions concurrently over the 8-week study period, and observed loss of both BLI and PET signal intensities over time, reflecting death of the transplanted cells (Figure 4A). When normalized to BLI signal intensity at day 1, the percentage of cells that survived was 22.7 ±11.5% at day 7, 15.7 ± 7.7% at day 14, 10.6 ± 5.06% at day 28, and 0.8 ± 0.4% at day 56 (Figure 4B). These results were confirmed using PET imaging (Figure 4C and 4D), and are in agreement with the results of other groups showing death of the majority of transplanted stem cells within 4–6 weeks after intramyocardial delivery [9, 29–31]. To

confirm the results of our PET and BLI data, we also measured cell engraftment using real-time quantitative polymerase chain reaction (RT-qPCR). To this end, we utilized human-specific primers against Alu sequences to quantify the number of human genomes present within murine hearts at 14 and 28 days after transplantation. Our results using RT-qPCR were concordant with data obtained using PET and BLI. Namely, at days 14 and 28 after transplantation, 12.12 ± 3.33% and 6.36 ± 2.35% of unmodified hCPCs had successfully engrafted into murine myocardium. Similarly, at days 14 and 28 after transplantation, 11.37 ± 3.55% and 6.65 ± 2.46% of phiC31-hCPCs had successfully engrafted (Supplemental Figure 11).

Isolation and site-specific genetic modification of differentiated human endothelial cells

hCPCs represent a multipotent cell type with significant proliferative potential, which may account for the relative ease with which site-specific hCPC integrants can be obtained. We therefore wished to demonstrate site-specific integration in another cell type; in particular, a more differentiated cell source with therapeutic potential such as human endothelial cells (hECs). We generated human ECs containing site-specific integration of the triple-fusion reporter gene by co-transfection of donor and integration vectors (Figure 1A). The transfected cells were clonally expanded and screened for integration at known pseudo-*attP* sites [19]. One out of 20 clones was shown to have a single integration at the 8p22 site and was expanded for further characterization and functional experiments phiC31-hECs were identical to untransduced hECs in their phenotype, as assessed by their expression of the endothelial cell marker CD31 (>90%), LDL uptake, and ability to form tubules *in vitro* (Supplemental Figure 12).

Transplantation of human endothelial cells improves ischemic limb perfusion

To assess cellular engraftment over time, we used BLI to evaluate the temporal kinetics of hEC survival (Figure 5A). Normalized to day 1, the BLI signals were 37.6±5.5% at day 4, 17.7±3.5% at day 7, and 11.3±2.1% at day 14 (Figure 5B). These results suggest acute death of hECs during the first week, but relatively stable engraftment thereafter. We subsequently carried out laser Doppler blood perfusion (LDBP) monitoring to examine whether hECs delivery improved ischemic hindlimb perfusion (Figure 5C). The hindlimb perfusion ratio (ischemic/control hindlimb) was significantly improved in the hindlimbs transplanted with hECs compared to PBS at day 7 (0.41±0.051 vs. 0.32±0.035, $P<0.005$) and day 14 (0.54±0.071 vs. 0.41±0.036; $P<0.005$) after transplantation (Figure 5D). The hindlimb perfusion ratios of phiC31-hECs and the unmodified hECs were similar. These data show that phiC31-hECs, despite their genetic modification, can be safely transplanted *in vivo* and retain their therapeutic capability to restore perfusion.

DISCUSSION

In recent years, stem cell therapy has emerged as a promising approach to promote tissue repair after myocardial infarction. Despite encouraging initial results, clinical translation of cellular therapy has been impeded by several challenges, including poor engraftment [32], malignant transformation [33], or acute death of the transplanted cells [34]. Innovative approaches to overcome these challenges have relied on genetic modification of the delivered stem cells to enable expression of transgenes. Stable expression of such transgenes can enable a variety of valuable enhancements to cellular therapy, including enhanced vasculogenesis [35], improved cell survival [15, 36], cellular tracking using clinical imaging modalities [37], and the availability of a “safety switch” to induce apoptosis in the event of cellular misbehavior [17]. However, commonly employed gene therapy vectors such as lentivirus and retrovirus can produce random integrations that are subject to the

unpredictable effects of insertional mutagenesis. Hence site-specific integration techniques are needed to safely translate gene therapy into the clinical setting.

In recent years, stem and progenitor cells have emerged as excited new tools for ameliorating left ventricular dysfunction following myocardial infarction. hCPCs hold considerable potential in this regard, as they are a clonogenic, self-renewing population that is pre-programmed for differentiation into cells of all three cardiac lineages *in vitro*: myocytes, smooth muscle cells, and endothelial cells. Several parameters for hCPC identification are currently found in the literature, including isolation of c-kit$^+$ cells [3], cardiosphere-derived cells [38], Sca-1$^+$ cells [39], and Isl1$^+$ cells [40]. 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 still needed. We employed Sca-1$^+$ hCPCs in this study, as we and others have found that hCPCs isolated using the Sca-1 antibody consistently provide high yields of homogeneous cells with identical morphology, differentiation, and surface marker expression [39, 41, 42]. However, the human equivalent for murine Sca-1 has not yet been identified. Presumably, the Sca-1 antibody cross-reacts with a human protein to yield a homogeneous population, but further work is needed to elucidate the identity of this epitope.

Here, we employed the phiC31 integrase to achieve site-specific integration of a triple fusion reporter gene into a known chromosomal context in hCPCs and hECs. This proof-of-principle study demonstrates the utility of the phiC31 integration technique in producing both progenitor cells (hCPCs) and differentiated cells (hECs) that have been genetically modified to stably express a transgene of interest. Moreover, the techniques described herein are easily applied in any context with just basic molecular biology expertise and plasmid manipulation tools [43]. Genomic modification of hCPCs did not appreciably alter the progenitor cell phenotype, as assessed by global transcriptome microarray analysis and immunohistochemical analysis of differentiating progenitor cells. Indeed, phiC31-modified hCPCs retained their ability to improve myocardial contractility by a statistically significant margin compared to controls when assessed two weeks after intramyocardial injection. Likewise, phiC31-modified hECs maintained their ability to improve ischemic hindlimb perfusion after genomic modification.

We have the found the phiC31 integrase technique a facile and efficient approach for generating site-specific integrants in progenitor and differentiated cells. Alternate site-specific integration techniques are significantly less efficient than phiC31-integrase mediated recombination because they often rely on successful activation of mammalian cells' homologous recombination machinery. One such technique involves the introduction of a double-strand break at the desired genomic location by a tandem pair of zinc-finger nucleases (ZFNs) [44]. Although ZFNs allow targeting of a wider variety of genomic loci, their design and validation require extensive time and expertise. In addition to verifying proper targeting, cells' exposure to ZFNs must be minimized in order to prevent any off-target cleavage events. The phiC31 integrase system is easily implemented using common plasmid manipulation and molecular biology techniques.

In summary, our report establishes for the first time the feasibility of site-specific genetic integration in human cardiac progenitor cells and human endothelial cells using non-viral vectors. This type of genetic engineering of human stem cells enables a multitude of improvements to overcome many of cellular therapy's current limitations. The ease, efficiency, and safety of the phiC31 integrase system make it an appealing approach to such attempts at stable genetic modification of therapeutic cell populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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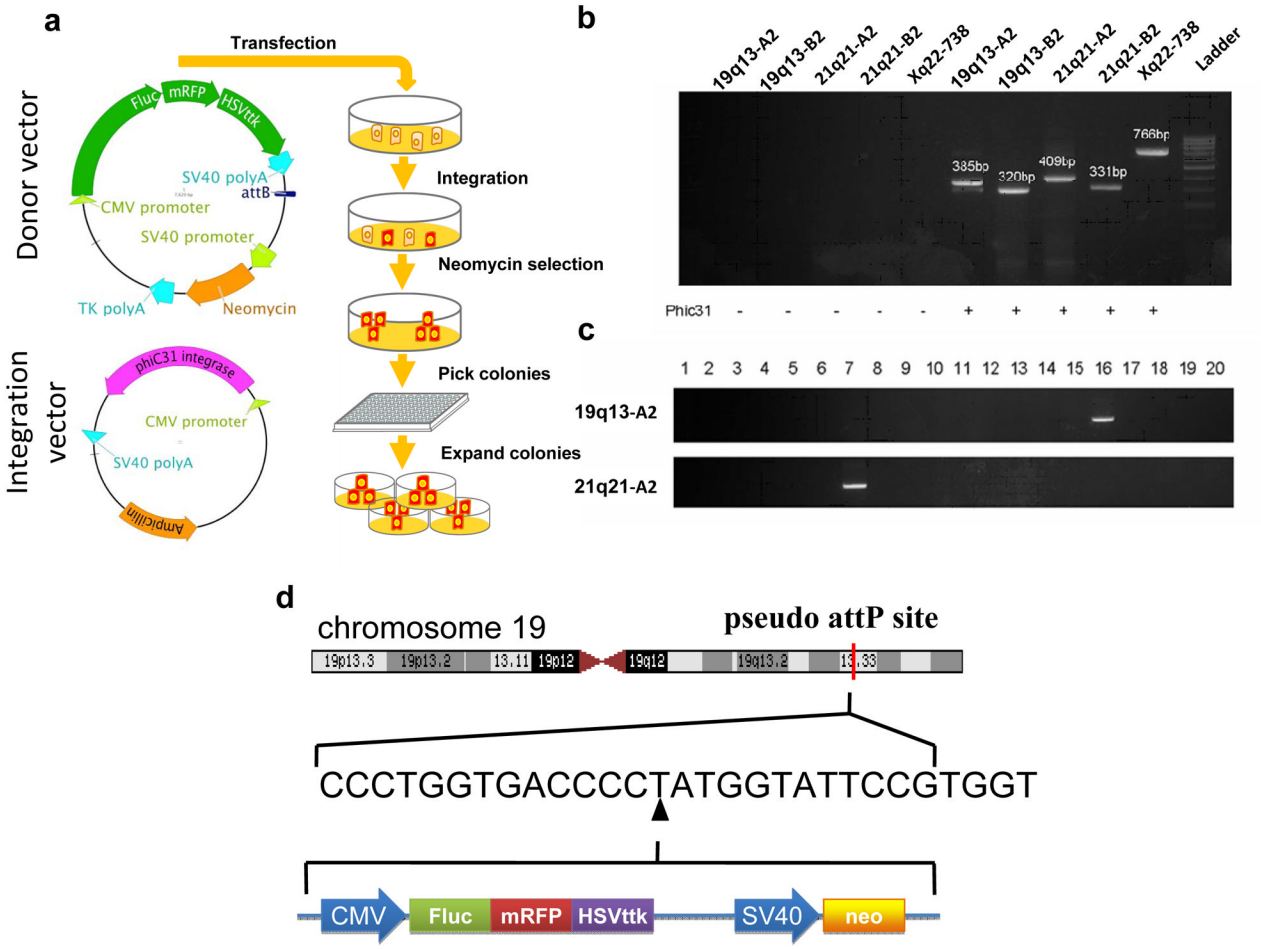

Figure 1. Site-specific integration of triple fusion reporter gene into human cardiac progenitor cells

(A) An integration vector plasmid encoding the phiC31 integrase (purple) and a donor vector plasmid encoding the triple fusion (TF) reporter gene (green) were initially co-transfected into hCPCs. Next, positive integrants were identified using neomycin selection. Individual colonies grown from single cells were then picked and expanded for further characterization. **(B)** Genomic DNA of the neomycin-resistant hCPC population was isolated and subjected to PCR analysis with a known set of primers to identify integration at specific loci located on chromosomes 19, 21, and X. **(C)** Twenty individually expanded hCPC colonies grown from single cells were screened for integration at loci on chromosomes 19 and 21. Clone #7 revealed a single integration of the triple fusion reporter gene at a site on chromosome 21, while Clone #16 revealed a single integration at a site on chromosome 19. **(D)** Site-specific integration of the triple fusion reporter gene occurs uniquely at a “safe harbor” site located on chromosome 19.

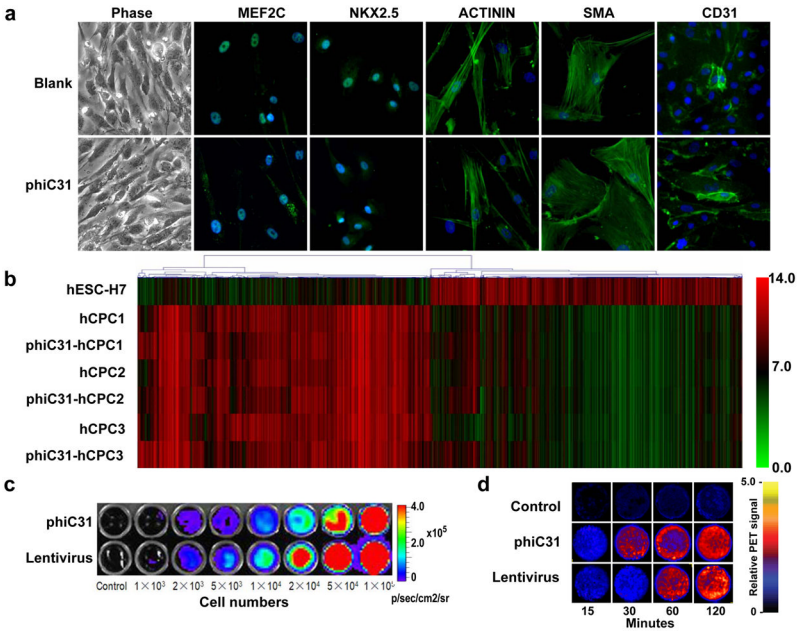

Figure 2. ***In vitro*** **comparison of triple fusion reporter gene-expressing hCPCs derived using site-specific phiC31 integrase recombination versus random lentiviral infection**

(A) The phiC31-hCPCs show unaltered Mef2C and Nkx2-5 cardiac progenitor gene expression and can differentiate into cardiomyocytes, smooth muscle and endothelial cells similar to unmodified control hCPCs. **(B)** The phiC31-hCPCs and the unmodified control hCPCs have similar global gene expression patterns as assessed by transcriptome microarrays and are different from the human embryonic stem cell line (hES-H7). **(C)** Representative bioluminescence imaging (BLI) of known numbers of hCPCs derived using site-specific phiC31 recombination or random lentiviral integration. **(D)** Representative microPET images of known numbers of hCPCs during the [^{18}F]-FHBG accumulation assay.

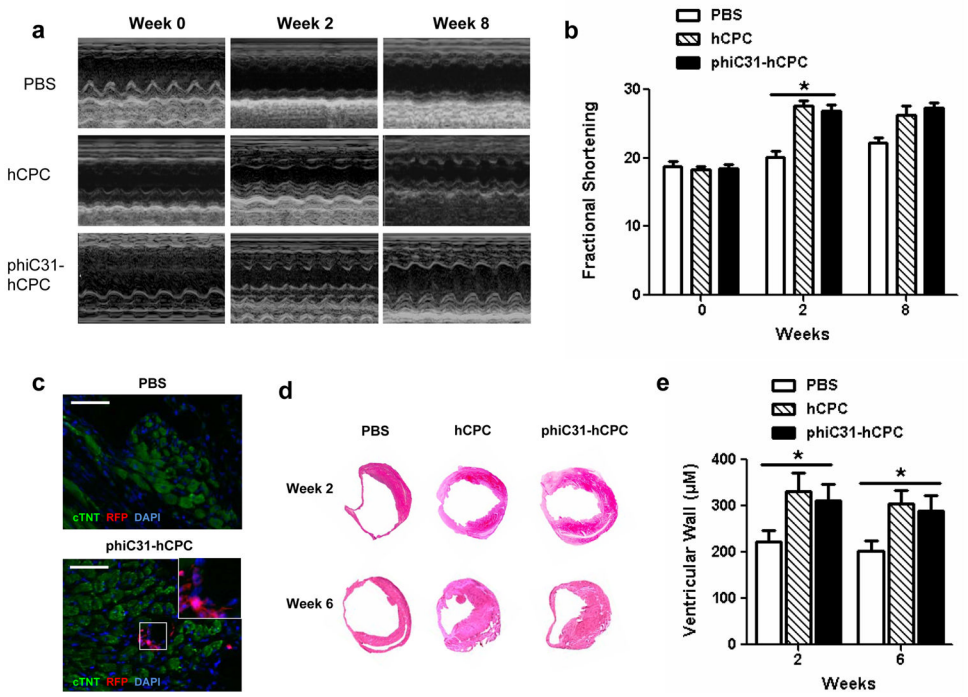

Figure 3. Improvement in myocardial contractility after hCPC transplant as assessed by echocardiography and MRI

(A) Representative M-mode echocardiogram demonstrates functional improvement at week 2 in the hCPC group. **(B)** The fractional shortening of hCPC groups shows significant improvement compared with PBS control group at week 2 ($P < 0.001$). There was no significant difference between the phiC31-hCPC and unmodified hCPC groups. **(C)** Representative sections of the peri-infarct zone in the area of intramyocardial hCPC or PBS injection demonstrates the presence of phiC31-hCPCs. phiC31-hCPCs stained positive for the RFP antibody in the recipient myocardium at day 14 after injection. **(D)** Representative HE staining of the heart sections show myocardial wall thickness of gross hearts at week 2 and week 6 after injection. **(E)** Myocardial wall thickness of the hCPC groups are significantly increased compared to PBS control group at week 2 after transplantation. There was no significant difference between the phiC31-hCPC and unmodified hCPC groups. All hCPCs (unmodified and phiC31-modified) were transplanted intramyocardially at passage 10–16.

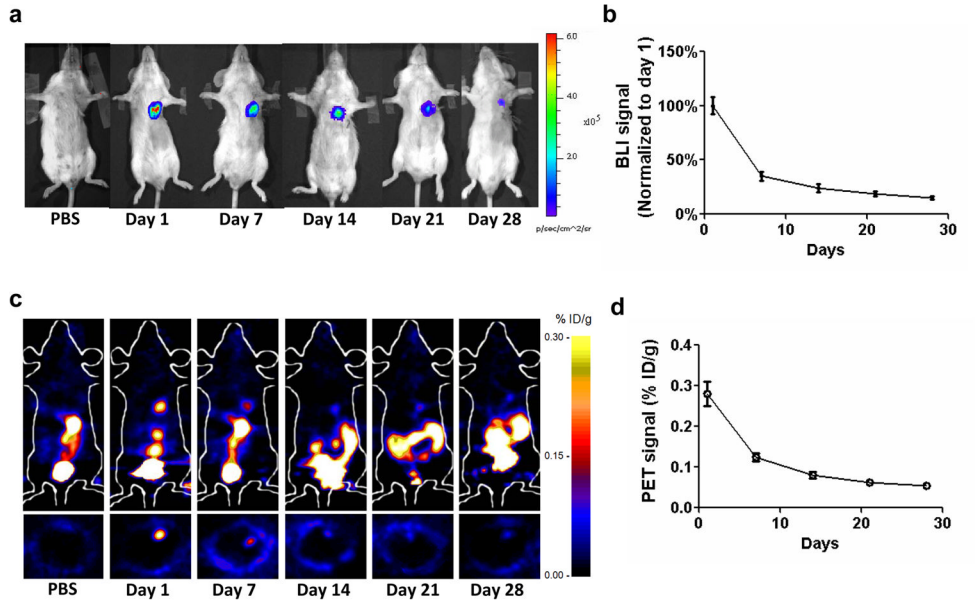

Figure 4. Temporal kinetics of hCPC survival in infarcted hearts

(A, C) Longitudinal BLI and PET imaging of a representative adult female SCID mouse injected with 1×10^6 hCPCs stably expressing the triple fusion reporter gene. **(B, D)** Progressive decrease in PET and BLI signal intensity over the 28 day time course. **(B)** Normalized to day 1, BLI signals were 22.7 ± 11.5% on day 7, 15.7 ± 7.7% on day 14, and 10.6 ± 5.06% on day 28. **(D)** Normalized to day 1, PET signals were 44.7 ± 3.2% on day 7, 22.4 ± 1.6% on day 14, 15.3 ± 1.1% on day 21, and 13.2 ± 0.6% on day 28.

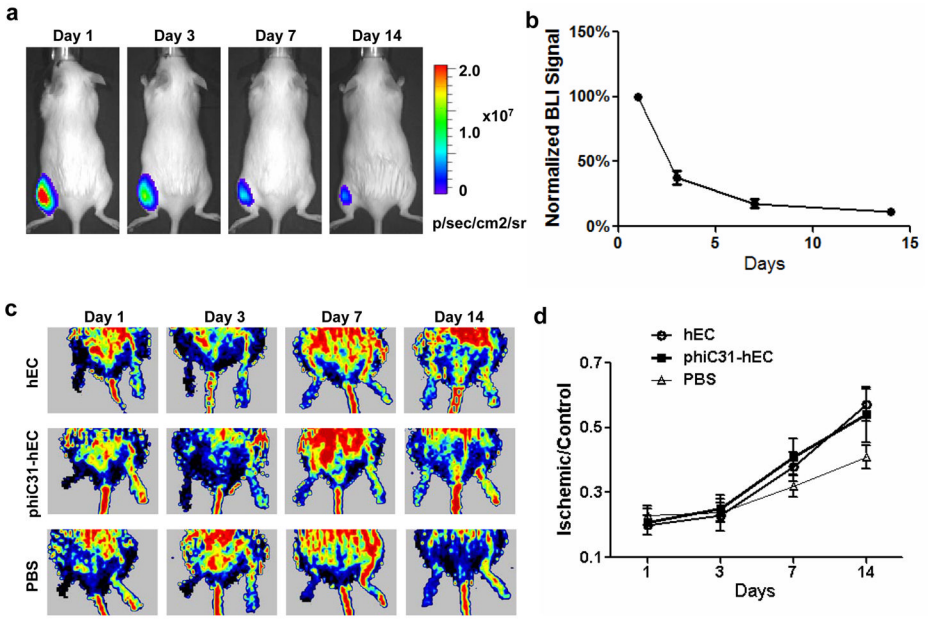

Figure 5. Improvement in hindlimb perfusion after transplantation of human endothelial cells

(A) Localization and survival of phiC31-hECs in the ischemic hindlimb after intramuscular delivery as assessed by BLI. **(B)** Quantification of BLI signal in the hindlimb after cell delivery. **(C)** Representative laser Doppler perfusion images of an adult female SCID beige mouse injected with 1×10^6 unmodified hECs or 1×10^6 phiC31-hECs at 1, 3, 7, and 14 days after cell delivery showing improved blood flow for the treatment group relative to PBS controls. **(D)** The mean hindlimb perfusion ratio (ischemic/control hindlimb) was significantly improved in hEC-treated mice compared to the PBS-treated mice (**P*<0.05, transplant groups vs. control groups at day 14 post-transplant). There was no significant difference between the phiC31-hEC and unmodified hEC groups (n=5 per group).