Long-Term Outcome of Administration of c-kit\textsuperscript{POS} Cardiac Progenitor Cells After Acute Myocardial Infarction

Transplanted Cells Do not Become Cardiomyocytes, but Structural and Functional Improvement and Proliferation of Endogenous Cells Persist for at Least One Year

Xian-Liang Tang,* Qianhong Li,* Gregg Rokosh, Santosh K. Sanganalmath, Ning Chen, Qinghui Ou, Heather Stowers, Greg Hunt, Roberto Bolli

Rationale: Cardiac progenitor cells (CPCs) improve left ventricular remodeling and function after acute or chronic myocardial infarction. However, the long-term (>5 weeks) effects, potential tumorigenicity, and fate of transplanted CPCs are unknown.

Objective: To assess the outcome of CPC therapy at 1 year.

Methods and Results: Female rats underwent a 90-minute coronary occlusion; 4 hours after reperfusion, they received intracoronarily vehicle or 1 million male, syngeneic CPCs. One year later, CPC-treated rats exhibited smaller scars and more viable myocardium in the risk region, along with improved left ventricular remodeling and regional and global left ventricular function. No tumors were observed. Some transplanted (Y-chromosome\textsuperscript{POS}) CPCs (or their progeny) persisted and continued to proliferate, but they failed to acquire a mature cardiomyocyte phenotype and were too few (4–8% of nuclei) to account for the benefits of CPC therapy. Surprisingly, CPC transplantation triggered a prolonged proliferative response of endogenous cells, resulting in increased formation of endothelial cells and Y-chromosome\textsuperscript{NEG} CPCs for 12 months and increased formation, for at least 7 months, of small cells that expressed cardiomyocytic proteins (α-sarcomeric actin) but did not have a mature cardiomyocyte phenotype.

Conclusions: The beneficial effects of CPCs on left ventricular remodeling and dysfunction are sustained for at least 1 year and thus are likely to be permanent. Because transplanted CPCs do not differentiate into mature cardiomyocytes, their major mechanism of action must involve paracrine actions. These paracrine mechanisms could be very prolonged because some CPCs engraft, proliferate, and persist at 1 year. This is the first report that transplantation of any cell type in the heart induces a proliferative response that lasts at least 1 year. The results strongly support the safety and clinical utility of CPC therapy. (Circ Res. 2016;118:1091-1105. DOI: 10.1161/CIRCRESAHA.115.307647.)

Key Words: myocardial infarction ■ stem cells ■ ventricular function ■ ventricular remodeling

The adult heart contains a pool of c-kit\textsuperscript{POS}/CD45\textsuperscript{NEG} cells that have been suggested to possess the properties of cardiac progenitor cells (CPCs).\textsuperscript{1} Recent evidence indicates that the salubrious effects of transplanted (exogenous) CPCs are not mediated by differentiation into cardiomyocytes but, rather, by paracrine actions.\textsuperscript{2,3} Regardless of the mechanism of action, a plethora of experimental studies from multiple laboratories have consistently shown that administration of CPCs promotes cardiac repair, attenuates left ventricular (LV) dysfunction, and improves LV remodeling in various animal models of both acute and chronic postmyocardial infarction (post-MI) heart failure.\textsuperscript{2,4-8} In our laboratory, we have found that transplantation of CPCs exerts a panoply of salutary effects on LV structure and function in rat\textsuperscript{9,10} and pig model of chronic ischemic cardiomyopathy and in murine models of acute MI.\textsuperscript{12-14}

In This Issue, see p 1041
Editorial, see p 1045
A major limitation of all of the aforementioned studies, however, is that the follow-up after cell therapy was relatively short (4–6 weeks); consequently, the long-term (>1 year) effects of CPCs on LV function and structure in experimental models of ischemic cardiomyopathy remain unknown. Similarly, the safety of CPC therapy remains uncertain because potential adverse effects, such as tumor formation, would not be expected to be evident within the 4- to 6-week follow-up of the studies performed to date; a longer time frame is needed to assess this possibility. For the same reason, the long-term fate of transplanted CPCs beyond the first 4 to 6 weeks is essentially unknown.

Another fundamental issue that remains to be clarified is the mechanism(s) that underlies the beneficial effects of CPCs. Differentiation of transplanted CPCs into myocytes and vascular cells has been thought to play an important role but, as alluded to above, in our previous studies in models of ischemic cardiomyopathy, we did not observe formation of adult myocytes from transplanted cells; however, because in all of these studies animals were followed-up for only 4 to 5 weeks after cell therapy, the possibility that differentiation of transplanted CPCs into mature myocytes may occur at a later time cannot be ruled out. Because we did not observe any evidence of differentiation of exogenous CPCs, we have proposed that these cells produce their salubrious effects, at least in part, by activating the pool of endogenous CPCs; however, this possibility has not been substantiated.

Thus, several fundamental issues remain to be addressed regarding CPC therapy. From a translational standpoint, it is important to assess the long-term safety of CPC therapy and establish, in a rigorous and well-controlled experimental model, whether the cardiac reparative benefits of CPC administration are transient or permanent. From a conceptual standpoint, understanding the mechanism of action of exogenous CPCs requires an effort to illuminate the long-term fate of the transplanted cells and to establish whether or not their salubrious effects are mediated by differentiation of the transplanted cells into mature cardiac myocytes.

This study was undertaken to elucidate these issues. Specifically, using a well-characterized rat model and a rigorously blinded study design, we sought to determine i) whether the effects of CPCs on LV function and remodeling after MI persist for 1 year, a period that is significantly longer than the follow-up periods previously examined; ii) if so, whether the salutary actions of CPCs are mediated by differentiation of the cells into adult myocytes; iii) whether transplantation of exogenous CPCs elicits proliferation of endogenous CPCs and, if so, how long this response lasts; and iv) whether administration of CPCs is associated with tumor formation over a 1-year follow-up. To directly compare the results of the present investigation with those of our previous studies in rats, we used a similar rat model and the same dose of CPCs as in that study. To elucidate whether transplantation of CPCs promotes cell proliferation and to delineate its time course, 5-bromo-2′-deoxyuridine (BrdC) was administered to different subsets of animals during the 3rd, 7th, or 12th month after MI.

Methods

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth Edition, Revised 2010) and with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine (Louisville, KY).

Isolation and Expansion of c-kitPOS CPCs

c-kitPOS CPCs were prepared using a modification of the method described by Beltrami et al. Briefly, adult male Fischer 344 rats (4–6 months of age) were anesthetized with sodium pentobarbital and heparinized. The heart was harvested, rinsed with ice-cold Krebs–Henseleit perfusion buffer, perfused in a retrograde fashion with Krebs–Henseleit perfusion buffer at 37°C, 5 mL/min, for 6 to 8 minutes to wash out the blood, and then perfused with digestion buffer (1 mg/mL of collagenase II in Krebs–Henseleit perfusion buffer) for 10 minutes. The digested heart was cut into ≈1 mm³ cubes, which were then triturated until cells were completely dissociated. The nonmyocyte population was separated from myocytes by gravity sedimentation, counted (number of cells determined), and centrifuged at 600g for 10 minutes at room temperature. The cell pellet was resuspended, and the cells were seeded onto two 150-mm dishes in Ham’s F-12K (Kaighn’s) medium with 5% fetal bovine serum (HyClone), 10 ng/mL basic fibroblast growth factor (Peprotech), and 10 ng/mL leukemia inhibitory factor (Chemicon; rat CPC medium). After 6 days of expansion, c-kitPOS cells were sorted with magnetic beads using a rabbit anti-kit antibody (H-300, Santa Cruz). The recovery of c-kitPOS CPCs was determined by flow cytometric analysis (BD LSRII, Becton–Dickinson) with an R-phyceroerythrin–conjugated monoclonal anti-kit antibody (Pharmingen). In the sorted CPCs, expression of c-kit was repeatedly determined by flow cytometric analysis in cell passages 1 through 6. C-kitPOS CPCs from passages 4 to 6 were used in all in vivo studies of post-MI CPC transplantation.

Surgical Preparation and Experimental Protocol

The protocol and dose of CPCs were similar to those used in our previous studies. Female Fischer 344 rats (age, 3 months; weight, 175±20 g) were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg) and ventilated with a rodent respirator (Harvard Apparatus). Anesthesia was maintained with isoflurane inhalation, and body temperature was kept at 37°C with a heating pad. After administration of antibiotics, the chest was opened and the heart exposed. All animals underwent a 90-minute occlusion of the left anterior descending coronary artery followed by reperfusion, after which the chest was closed. Four hours after reperfusion, rats were reanesthetized, the chest reopened, and a thin catheter (Intracath, 22G, Becton Dickinson) was advanced into the aortic root via the LV apex. The aorta and the pulmonary artery were occluded briefly with a snare for two 20-s intervals, 10 minutes apart, and vehicle or CPCs were injected into the aortic root during the occlusion. CPCs were suspended in sterile Plasma-Lyte A solution (1×10⁶ CPCs diluted in 1 mL). All rats were euthanized 1 year later (Figure 1). In phase A, rats were used to assess LV function and structure and for histological studies (Figure 1A). To identify newly formed cells, in phase B, BrdC (MP Biomedicals, LLC), a more water-soluble precursor of 5-bromo-2′-deoxyuridine (BrdU), was infused subcutaneously with Alzet mini-osmotic pumps (Durect Corp., CA) during the 3rd, 7th, or 12th month after reperfusion (33 mg/kg/d for 1 month; Figure 1B).
Experimental Protocol

**Phase A: Analysis of LV function and structure**

<table>
<thead>
<tr>
<th>50-min Occlusion</th>
<th>0</th>
<th>4 h</th>
<th>48 h</th>
<th>3 months</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echo (BSL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echo (48 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echo (3 mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echo (6 mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemodynamic study (Millar catheter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthanasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Phase B: Analysis of BrdU incorporation**

<table>
<thead>
<tr>
<th>50-min Occlusion</th>
<th>0</th>
<th>4 h</th>
<th>2 months</th>
<th>3 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracoronary administration (vehicle or CPCs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BrdC infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthanasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BrdU analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>50-min Occlusion</th>
<th>0</th>
<th>4 h</th>
<th>6 months</th>
<th>7 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracoronary administration (vehicle or CPCs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BrdC infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthanasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BrdU analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Experimental protocol. Rats were subjected to a 90-minute coronary occlusion followed by reperfusion; 4 hours after reperfusion, they received intracoronary infusion of either vehicle or CPCs. Rats were followed-up for 12 months.

A. Analysis of LV function and structure. Echocardiographic studies were performed at baseline, 48 hours, and 3, 6, and 12 months after MI. Hemodynamic studies were performed immediately before euthanasia at the 12-month follow-up. Hearts were perfused and fixed with formalin for histology and Y-chromosome analysis.

B. Analysis of BrdU incorporation. Rats received a 1-month BrdC infusion via an osmotic pump starting at 2, 6, or 11 months after intracoronary CPC administration; animals were euthanized for assay of BrdU incorporation at 12 months after CPC administration. BrdC indicates 5-bromo-2′-deoxyuridine; CPC, cardiac progenitor cell; LV, left ventricular; and MI, myocardial infarction.

and at 48 hours and 3, 6, and 12 months after treatment. The anterior chest was shaved, and rats were placed in the left lateral decubitus position. Body temperature was maintained between 36.9°C and 37.3°C. Echocardiographic images were obtained using an HP SONOS 7500 ultrasound system equipped with a L12-5 linear broadband and a S12 phased array transducers fitted with a 0.3-cm standoff. The heart was imaged in the parasternal short-axis view at the level of the papillary muscles to obtain LV wall thickness and ejection fraction, and in the parasternal long-axis view to measure LV end-systolic and end-diastolic volumes. All measurements were averaged in 3 consecutive cardiac cycles and analyzed off-line by a single blinded observer using the COMPACS image analysis software. All calculations were derived using standard formulas. LV end-systolic and end-diastolic diameters were measured from M-mode tracings obtained at the mid-papillary level and analyzed according to modified American Society for Echocardiography standards (posterior wall leading-edge to leading-edge and anterior wall trailing-edge to trailing-edge).18

**Hemodynamics**

Hemodynamic studies were performed at 1 year after MI, just before euthanasia.9,10 Rats were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated. Anesthesia was maintained with 1% isoflurane, and the core temperature was kept at 37°C with a heating pad throughout the study. A 2F microtip pressure–volume catheter (SPR-869, Millar Instruments) was inserted into the right carotid artery and advanced into the LV cavity. The right jugular vein was cannulated for fluid administration. After 20 minutes of stabilization, the pressure–volume signals were recorded continuously with an ARIA pressure–volume conductance system (Millar Instruments) coupled with a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a personal computer. Pressure–volume relations were assessed by transiently compressing the inferior vena cava with a cotton swab. Parallel conductance from surrounding structures was calculated by injecting a small bolus of 15% NaCl through the jugular vein. LV end-diastolic pressure, dP/dt max, end-systolic elastance, and preload recruitable stroke work were calculated using the PVAN software program (Millar).9,10

**Morphometry and Histology**

After the hemodynamic measurements, a polyethylene catheter filled with phosphate buffer (0.2 mol/L; pH, 7.4) and heparin (100 IU/mL) was advanced to the ascending aorta via the right carotid artery. In rapid succession, the heart was arrested in diastole by injecting 1.0 mL of a mixture of cadmium chloride (100 mmol/L)/potassium chloride (3 mol/L) through the aortic catheter. The heart was then excised and perfused retrogradely with phosphate buffer for ≈3 minutes to flush out residual blood in the coronary circulation, followed by perfusion with 10% neutral buffered formalin solution for 15 minutes. Perfusion pressure was maintained between 60 and 80 mmHg, whereas end-diastolic pressure was kept at 8 mmHg. After perfusion–fixation, the atria and right ventricle were dissected from the left ventricle. The LV weight was measured. The heart was cut into 4 transverse slices (≈2-mm thick), which were processed; embedded in paraffin; sectioned at 4-µm intervals; and stained with Masson’s trichrome, picrosirius red, or antibodies against specific cell markers. Images were acquired digitally and analyzed using NIH ImageJ (1.46r). From the Masson’s trichrome-stained images, morphometric parameters including risk area, scar area, and LV wall thickness in the risk were measured in each section.9,10 In accordance with our previous work,9 the risk region was defined as the sum of the LV segments containing the infarct scar and the 2 border zones (defined as the regions that straddle 0.5 mm on either side of the lateral borders of the scar). To quantify both the degree of LV dilation and the degree of infarct wall thinning, the LV expansion index was calculated.9 Myocardial collagen content was quantitated on the picrosirius red-stained sections by determining collagen density (as percentage of area) under polarized light.9

**Immunohistochemistry**

Immunohistochemistry was performed in formalin-fixed, paraffin-embedded, 4-µm-thick heart sections. CPC proliferation was assessed by immunofluorescent staining of nuclei for BrdU. Myocytes were stained with α-sarcomeric actin (α-SA) antibodies, and myocyte membranes were stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (Sigma) to facilitate the identification of individual myocytes for analysis of myocyte density.
To determine vessel density, heart sections were stained with biotinylated Isolecitin B4 (IB4; Sigma). Newly formed CPCs were evaluated by immunofluorescent staining of cell membranes for c-kit and CD45 (Santa Cruz) and staining of nuclei for BrdU on the same heart sections.

Fluorescence In Situ Hybridization Analysis
To check the fate of the transplanted male CPCs, single-labeled Y chromosome was detected by fluorescence in situ hybridization according to the modified manufacturer’s protocol (ID Laboratories, London, ON). Serial tissue sections from paraffin-embedded heart blocks were cut to 4-μm thickness. Heart tissue sections on coated slides were deparaffinized in xylene and rehydrated gradually, followed by an antigen retrieval procedure. After treatment with pepsin and air-drying, a denatured rat Y chromosome probe was added to each section and covered with coverslip. After overnight hybridization at room temperature, the coverslip was removed, and serial washings were performed. Male and female rat heart tissue sections were used as positive and negative controls, respectively, in the fluorescence in situ hybridization staining procedure. The fate of transplanted CPCs in the heart was assessed by double staining with a green fluorochrome-labeled Y-chromosome probe and a specific monoclonal antibody against the cardiac-specific marker α-SA (Sigma). Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). To minimize autofluorescence, slides were incubated with 0.1% Sudan Black B (Sigma), rinsed in phosphate-buffered saline, and then mounted with ProLong Gold antifade reagent (Invitrogen). Immunohistochemical images were imaged by confocal microscopy and quantitatively analyzed by Image J (1.46, NIH). In each heart, Y-chromosomePOS nuclei, Y-chromosomePOS nuclei/total nuclei and total nuclei were counted in 25 confocal images (6 from the scar region, 14 from 2 border zones, and 5 from the noninfarcted region), and the results were corrected for the Y-chromosome density detected in normal male myocardium. For Y-chromosomePOS nuclear analysis, an average of 1235 nuclei was counted in a 0.352 mm² area per rat heart, and the results were normalized to male heart Y-chromosome density.

Statistical Analysis
All data are expressed as means±SEM. Intragroup and intergroup comparisons of the echocardiographic and BrdU data were performed using a two-way ANOVA with repeated measures followed by the Student’s t test with Bonferroni correction. Intergroup comparisons of morphometric, histological, immunohistochemical, and hemodynamic data were performed by one-way ANOVA followed by the Student’s t test with the Bonferroni correction. All analyses were conducted with SigmaStat 3.5. Values of P<0.05 were considered significant.

Results
Characterization of Rat c-kitPOS CPCs
C-kitPOS CPCs to be used for transplantation were isolated by magnetic-activated cell sorting from a nonmyocyte cell population obtained from syngeneic rat hearts. The recovery of c-kitPOS cells ranged from 0.2 to 3.0% of the total cell population, averaging 0.71±0.17% (n=18). After expansion and sorting, c-kitPOS CPCs accounted for 80% of cells (Figure 2A and 2B) and the percentage of c-kitPOS cells remained stable through 4 passages in culture (Figure 2C).

Exclusions
A total of 142 rats were enrolled in this study. Four normal (noninfarcted) rats were used as a reference for comparison; these animals did not undergo MI, received BrdC infusion for 1 month, and had age equivalent to the 12th month subgroups in the cohorts subjected to MI (Figure 1). The remaining 138 rats were subjected to MI and studied in 2 consecutive phases (A and B [Figure 1]). In phase A, 90 rats (40 in the vehicle-treated and 50 in the CPC-treated group) were used to assess the long-term effects of CPCs, both with respect to LV function and structure and with respect to tumor formation (Figure 1). Of these 90 rats, 22 (10 in the vehicle group and 12 in the CPC-treated group) died at various times during the 1 year follow-up, whereas 68 (30 in the vehicle-treated and 38 in the CPC-treated group) survived till the end of the 1-year follow-up. The 1-year survival rate was similar between the 2 groups (Online Figure I, Online Data Supplement). Postmortem examination of the 50 CPC-treated rats (12 of which died during follow-up, whereas 38 survived till the end of the 1-year follow-up) did not yield any evidence of tumor formation. Among the 68 rats that survived for 1 year, a subset of 26 (12 in the vehicle-treated and 14 in the CPC-treated group) were used for serial echocardiographic analyses and for pathological/histological studies.

In phase B, 48 rats (24 in the vehicle-treated and 24 in the CPC-treated group) were used for studies of BrdU incorporation (Figure 1). Twelve rats died during the period of BrdC infusion (6 in the vehicle-treated and 6 in the CPC-treated group); therefore, 6 rats completed the protocol in each of the 3 subgroups (BrdC infusion during the 3rd, 7th, and 12th month after MI) in both the control and treated groups.

Body Weight, LV Size, and Gross Pathology
Although body weight was similar between the 2 groups at baseline, the gain in body weight was significantly greater in the CPC group at 3, 6, and 12 months after MI (Table 1). Postmortem measurements of LV size showed that the length and weight of the left ventricle were significantly increased in both groups compared with normal controls of similar age (Table 1); however, LV length and weight were significantly less in the CPC-treated versus the vehicle-treated group,
concomitant with a reduced LV/body weight ratio, indicating less LV hypertrophy (Table 1). Gross pathological examination did not reveal any tumors.

Morphometric Analysis
Despite similar risk regions (35.7±1.8% of LV weight in the vehicle-treated group versus 35.3±2.6% in the CPC-treated group; Table 2 and Figure 3B), in CPC-treated rats, scar size was reduced (11.6±1.2% of LV weight versus 16.6±1.1% in the vehicle group, P<0.01; Figure 3A and 3B), whereas the amount of viable tissue within the risk region was increased (65.8±3.0% of risk region weight versus 53.6±2.4% in the vehicle group, P<0.01; Table 2 and Figure 3A and 3B), suggesting that the infusion of CPCs resulted in formation of new myocardium in the infarcted region. The total amount of viable tissue in the left ventricle, expressed in absolute units, was similar in CPC- and vehicle-treated hearts (914.3±29.8 and 995.8±30.3 mg, respectively); however, because total LV weight tended to be higher in vehicle-treated hearts, the percentage of LV weight that was accounted for by viable tissue was slightly, but significantly, greater in CPC-treated hearts (88.4±1.2% versus 83.4±1.1% in the vehicle group; P<0.01; Table 2 and Figure 3B). In addition, CPC-treated rats exhibited an increase in the thickness of the infarcted LV wall and a decrease in the LV expansion index (P<0.05 for both; Figure 3A and 3B).

Because myocardial fibrosis plays a key role in the pathology of LV remodeling after MI,22,23 we examined whether CPCs attenuated myocardial collagen deposition. Collagen content in the risk region was significantly less in CPC-treated rats compared with vehicle-treated rats (P<0.05) although there was no significant difference between the 2 groups in the posterior wall (noninfarcted region; Online Figure II, Online Data Supplement). This reduced collagen deposition in the myocardium may have contributed, at least in part, to the functional benefits of CPC therapy.

Myocyte and Vessel Density
Myocyte density and myocyte nuclear density were assessed by staining myocytes with α-SA antibodies and myocyte membranes with FITC-conjugated wheat germ agglutinin to facilitate counting individual myocytes (Online Figure IV, Online Data Supplement). Of these 2 parameters, myocyte density may be a more reliable indicator of the number of myocytes per area unit because it is independent of the number of nuclei per cell. We deliberately excluded from the counts small cells that lacked the morphology of mature cardiomyocytes. In the risk region, both myocyte density and myocyte nuclear density were significantly higher in CPC-treated rats compared with vehicle-treated rats. Similar trends were observed in the noninfarcted region, but the differences were not statistically significant (Online Figure IV, Online Data Supplement). Although other explanations are possible, increased myocyte density in CPC-treated hearts is consistent with formation of new myocytes.

Vascular density was assessed by staining tissue with FITC-conjugated isolectin B4 in rats that received BrdU infusion during the 12th month after MI (Online Figure III, Online Data Supplement). Presumably as a result of scarring, vascular density was significantly less in the risk region than in the noninfarcted region of both vehicle- and CPC-treated hearts (Online Figure III, Online Data Supplement). In the noninfarcted region, vascular density tended to be greater in CPC-treated than in vehicle-treated rats, but the difference was not statistically significant (Online Figure III, Online Data Supplement). In the risk region, total vascular density was similar; however, further analysis revealed that the density of BrdU<sup>PSS</sup> vessels and BrdU<sup>PSS</sup> endothelial cells was significantly higher in CPC-treated rats than in vehicle-treated rats (Online Figure IIID to IIIF, Online Data Supplement). No such differences were observed in the noninfarcted region. These data suggest that treatment with CPCs increased the turnover of endothelial cells in the risk region.

Interestingly, the distribution of vessel diameter was significantly different between the vehicle-treated and the CPC-treated groups: the former exhibited a greater number of vessels between 10 and 50 μm in diameter, both in the risk region and in the noninfarcted region (Online Figure IIIG to IIIK, Online Data Supplement).

Echocardiographic and Hemodynamic Analysis
The echocardiographic data are summarized in Figure 4A and 4B. At 48 hour after MI, all parameters of LV function and

### Table 1. Body Weight and Postmortem Measurements of LV Weight and LV Length

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age-Matched Normal Controls (n=4)</th>
<th>Vehicle-Treated (n=12)</th>
<th>CPC-Treated (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g) at baseline</td>
<td>171.5±3.8</td>
<td>196.3±4.1†</td>
<td>188.2±3.4‡</td>
</tr>
<tr>
<td>% gain from baseline at 3-month follow-up</td>
<td>...</td>
<td>7.9±2.3</td>
<td>18.7±1.5†</td>
</tr>
<tr>
<td>% gain from baseline at 6-month follow-up</td>
<td>...</td>
<td>20.0±3.2</td>
<td>27.1±1.7*</td>
</tr>
<tr>
<td>% gain from baseline at 12-month follow-up</td>
<td>...</td>
<td>37.7±3.1</td>
<td>47.6±2.8*</td>
</tr>
<tr>
<td>LV weight (g) at the end of the 12-month follow-up</td>
<td>0.72±0.02</td>
<td>1.00±0.03‡</td>
<td>0.91±0.03*‡</td>
</tr>
<tr>
<td>LV length (mm) at the end of the 12-month follow-up</td>
<td>10.5±0.6</td>
<td>15.1±0.4‡</td>
<td>13.0±0.5‡</td>
</tr>
<tr>
<td>LV weight/body weight ratio</td>
<td>0.27±0.01</td>
<td>0.37±0.01‡</td>
<td>0.33±0.01*‡</td>
</tr>
</tbody>
</table>

Data are means±SEM. CPC indicates cardiac progenitor cell; and LV, left ventricular.
*P<0.05.
†P<0.01 vs vehicle-treated.
‡P<0.05 vs age-matched normal controls.
dimensions, measured by echocardiography, were similar in the control and treated groups (Figure 4B), indicating that the extent of injury was comparable.

As expected, vehicle-treated rats exhibited a progressive increase in LV end-systolic and end-diastolic volumes (Figure 4B), indicative of post-MI LV remodeling. Remarkably, both of these changes were essentially abrogated in CPC-treated rats, in which LV end-systolic and end-diastolic volumes did not increase appreciably from 48 hours to 12 months post MI; as a result, LV volumes were significantly less in treated than controls rats at 3, 6, and 12 months post MI. At 12 months, both groups exhibited greater end-systolic and end-diastolic volumes than normal (noninfarcted), age-matched rats, but the increase was less in CPC-treated rats (Figure 4B). In the CPC-treated group, the thickness of the infarcted (anterior) LV wall in diastole increased after administration of CPCs, so that at 3, 6, and 12 months after MI, it was significantly greater than in vehicle-treated rats although at 12 months it was still less than in normal (noninfarcted) rats. End-diastolic thickness of the posterior (noninfarcted) wall did not differ between the 2 groups.

At 48 hours after MI, the degree of regional and global LV systolic dysfunction, assessed by systolic thickening fraction, fractional shortening, and LV ejection fraction, was similar in the 2 groups (Figure 4B), indicating that the extent of injury sustained during coronary occlusion/reperfusion was comparable. In control rats, these parameters of regional and global LV systolic function exhibited a progressive deterioration throughout the 12-month follow-up, consistent with the development of post-MI cardiomyopathy (Figure 4B). In rats treated with CPCs, however, the deterioration in both regional and global LV function was attenuated (Figure 4B). For example, thickening fraction in the anterior (infarcted) LV wall was significantly greater in treated than vehicle-treated rats at 6 months (78.6±3.0% versus 66.7±4.0%, respectively; \( P<0.05 \)) and 12 months of follow-up (71.1±3.3% versus 57.6±3.7%, \( P<0.05 \); Figure 4B). The preservation of thickening fraction in the anterior LV wall of CPC-treated rats was such that at 12 months this parameter did not differ significantly from normal (noninfarcted) rats (Figure 4B). Similarly, LV ejection fraction was significantly greater in treated than control rats at 3 months (76.0±1.5% versus 66.0±2.9%, respectively; \( P<0.01 \)), 6 months (73.2±2.6% versus 65.4±2.2%, \( P<0.01 \)), and 12 months (68.4±2.1% versus 60.6±2.8%, \( P<0.01 \)).

### Table 2. Morphometric Analysis

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Treated (n=12)</th>
<th>CPC-Treated (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV weight at postmortem measurement (mg)</td>
<td>995.8±30.3</td>
<td>914.3±29.8</td>
</tr>
<tr>
<td>Area measured by trichrome image analysis (mm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV area</td>
<td>48.2±3.1</td>
<td>47.6±2.4</td>
</tr>
<tr>
<td>Risk area</td>
<td>17.3±1.6</td>
<td>17.0±1.7</td>
</tr>
<tr>
<td>Scar area</td>
<td>8.0±0.8</td>
<td>5.4±0.6*</td>
</tr>
<tr>
<td>Nonrisk area</td>
<td>30.8±1.9</td>
<td>30.6±1.7</td>
</tr>
<tr>
<td>Weight calculated from area measured by trichrome analysis (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk region weight</td>
<td>352.8±16.1</td>
<td>322.4±27.0</td>
</tr>
<tr>
<td>Scar tissue weight</td>
<td>162.5±9.3</td>
<td>103.3±9.4†</td>
</tr>
<tr>
<td>Nonrisk region weight</td>
<td>643.0±31.8</td>
<td>591.9±31.6</td>
</tr>
<tr>
<td>Viable tissue weight within risk region</td>
<td>190.3±14.3</td>
<td>219.1±26.5</td>
</tr>
<tr>
<td>Total viable tissue in LV</td>
<td>833.3±33.2</td>
<td>811.0±33.6</td>
</tr>
<tr>
<td>Percentages calculated from weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk region weight (% of LV weight)</td>
<td>35.7±1.8</td>
<td>35.3±2.6</td>
</tr>
<tr>
<td>Scar tissue weight (% risk region weight)</td>
<td>46.4±2.4</td>
<td>34.2±3.0†</td>
</tr>
<tr>
<td>Scar tissue weight (% of LV weight)</td>
<td>16.6±1.1</td>
<td>11.6±1.2†</td>
</tr>
<tr>
<td>Viable tissue weight (% risk region weight)</td>
<td>53.6±2.4</td>
<td>65.8±3.0†</td>
</tr>
<tr>
<td>Viable tissue weight (% of LV weight)</td>
<td>83.4±1.1</td>
<td>88.4±1.2†</td>
</tr>
</tbody>
</table>

Data are means±SEM. CPC indicates cardiac progenitor cell; and LV, left ventricular.

\* \( P<0.05 \).

† \( P<0.01 \) vs vehicle-treated.
versus 62.2±2.5%; \( P<0.01 \), and 12 months of follow-up (70.8±2.2% versus 62.9±2.8%; \( P<0.01 \)). Taken together, the echocardiographic data indicate that treatment with CPCs markedly attenuated LV dilation and regional and global dysfunction throughout the duration of the study.

Consistent with the echocardiographic data, hemodynamic studies (performed before euthanasia using a Millar conductance catheter) demonstrated that LV function was significantly improved by the administration of CPCs (Figure 4C). At 12 months after MI, compared with control rats, CPC-treated rats exhibited a significant decrease in LV end-diastolic pressure and a significant increase in both load-dependent parameters of LV performance, such as LV ejection fraction and maximal and minimal LV dP/dt, and load-independent parameters, such as increased preload recruitable stroke work and end-systolic elastance (Figure 4C).

**Analysis of BrdU Positivity**

To delineate the proliferative response of the heart to MI and CPC transplantation, in phase B, we infused BrdC for 30 days
during the 3rd, 7th, and 12th month post MI. In the risk region, at all 3 time periods examined (3rd, 7th, and 12th month), the total number of BrdU<sup>POS</sup> cells was markedly increased compared with the posterior wall (noninfarcted region) in the same hearts and compared with normal (noninfarcted), age-matched rats that received BrdC at an age equivalent to the 12th month after MI in the infarcted rats (Figure 5A and 5B), indicating that MI promotes sustained cell proliferation in the infarcted region that lasts at least 12 months. The total number of BrdU<sup>POS</sup> cells was highest in the third month post MI and then declined progressively, but even in the 12th month, it was still much higher (almost 10-fold) than in normal (noninfarcted) rats (Figure 5B). In the posterior wall (noninfarcted region), the proliferative response was less pronounced and more short lived, both in CPC-treated and control rats: in both groups, the total number of BrdU<sup>POS</sup> cells was higher than in the normal (noninfarcted) rats during the 3rd month post MI but not during the 7th or 12th month (Figure 5B), indicating that the cellular proliferation induced by MI in noninfarcted LV regions subsided between 3 and 7 months. There was no significant difference in the total number of BrdU<sup>POS</sup> cells between the control and CPC-treated groups at any time point, either in the risk region or in the noninfarcted region (Figure 5B). Thus, total cellular proliferation was not affected by CPC administration.

Costaining of tissue with BrdU and α-SA antibodies revealed that, compared with normal (noninfarcted) rats, the number of double positive (BrdU<sup>POS</sup>/α-SA<sup>POS</sup>) cells was significantly increased in the risk region in the 3rd, 7th, and 12th month and in the noninfarcted region in the 3rd and 7th month post MI, suggesting increased formation of new cells that expressed cardiomyocytic proteins (Figure 5C). This increase occurred in both CPC-treated and vehicle-treated (control) rats (Figure 5C). However, in contrast to the total number of BrdU<sup>POS</sup> cells, there were significant differences between the 2 groups: compared with vehicle-treated rats, CPC-treated rats exhibited a greater number of double positive (BrdU<sup>POS</sup>/α-SA<sup>POS</sup>) cells in the risk region in the 3rd month (+105%, P<0.05) and 7th month (+122%, P<0.05) and in the noninfarcted region in the 3rd month (+66%; P<0.05; Figure 5C). These data suggest that transplantation of CPCs promotes formation of new cells expressing cardiomyocyte markers for at least 7 months in the risk region and for at least 3 months in the noninfarcted region. It is important to point out, however, that even at 1 year the double positive (BrdU<sup>POS</sup>/α-SA<sup>POS</sup>) cells did not exhibit the phenotype of mature cardiomyocytes; they were small and lacked organized sarcomeric structure (Figure 5A). In fact, very few, if any, BrdU<sup>POS</sup> mature cardiomyocytes were observed at any time point (3rd, 7th, and 12th month) in either treatment group. Therefore, the double positive cells were not mature cardiomyocytes.
positive (BrdUPOS/α-SAPOS) cells cannot be considered cycling myocytes or myocyte precursors.

**Analysis of Y-ChromosomePOS Cells**
To assess the fate of transplanted male CPCs, the number of Y-chromosomePOS cells was measured by fluorescence in situ hybridization at 1 year after transplantation. The advantage of using the Y-chromosome to track transplanted cells in long-term studies is that, unlike enhanced green fluorescent protein or β-galactosidase, its presence is not affected by changes in gene expression.24–26 Y-chromosomePOS cells were still detectable at 1 year after transplantation; they were more abundant in the risk region (7.7±1.2% of total nuclei) than in the noninfarcted region (3.8±0.9%; Figure 6A through 6E and 6G). Similarly, double positive (Y-chromosomePOS/α-SAPOS) cells were found predominantly in the risk region (4.8±0.9% of the nuclei versus 0.5±0.2% in the noninfarcted region; Figure 6A through 6E and 6H). The ratio of Y-chromosomePOS/α-SAPOS cells to total Y-chromosomePOS cells in the risk region was 62.2±2.1% (Figure 6I), suggesting that most surviving CPCs (or their progeny) expressed cardiomyocytic proteins. However, in absolute terms, the number of double positive (Y-chromosomePOS/α-SAPOS) cells was low, as indicated above (4.8±0.9% of nuclei in the risk region and 0.5±0.2% in the noninfarcted region; Figure 6H). Furthermore, these cells did not resemble mature myocytes: most were mononucleated and small, without a sarcomeric structure, resembling late fetal/neonatal cells; mature, large Y-chromosomePOS myocytes were found only rarely in the risk region (white arrows in Figure 6B and 6D). In summary, these data indicate that some transplanted CPCs (or their progeny) persisted at 1 year after intracoronary delivery. In the risk region, most of these cells expressed cardiac proteins (α-SA) but, even after 1 year, failed to acquire a mature myocyte phenotype; furthermore, their number (<5% of nuclei) was not sufficient to account for the functional improvement observed.

**Analysis of Newly Formed CPCs**
The failure of transplanted CPCs to differentiate into mature myocytes implies that these cells improved LV function via
paracrine mechanisms. One such potential mechanism is activation of endogenous CPCs. To test this hypothesis, we measured the number of c-kit^{POS} cells at 1 year after transplantation, separating those of hematopoietic origin (which are c-kit^{POS}/CD45^{POS}) from CPCs (which are c-kit^{POS}/CD45^{NEG}; Figure 7A through 7G and Online Figure VI, Online Data Supplement). The total number of c-kit^{POS} cells in both the risk and noninfarcted regions was similar in CPC-treated and control rats (Figure 7H). When the number of CPCs (c-kit^{POS}/CD45^{NEG} cells) was expressed as a percent of total c-kit^{POS} cells, it was significantly higher in CPC-treated animals (47.6±7.0% of total c-kit^{POS} cells versus 27.9±4.1% in the risk region; P<0.05; 61.9±14.0% versus 38.7±14.6% in the noninfarcted region; Figure 7I). However, when the number of CPCs (c-kit^{POS}/CD45^{NEG} cells) was calculated as a percent of total nuclei, it did not differ significantly in the 2 groups although it was higher in CPC-treated animals (Figure 7J). Thus, calculation of static CPC numbers did not reveal a significant effect of CPC transplantation on the abundance of CPCs in the myocardium 1 year later.

Analysis of CPC turnover, however, yielded different results. The total number of new c-kit^{POS} cells (c-kit^{POS}/BrdU^{POS} cells) formed during the 12th month after MI and c-kit^{POS}/CD45^{NEG}/BrdU^{POS} cells (new CPCs formed during the 12th month after MI) at 1 year after CPC transplantation. Data are means±SEM. The region at risk comprises both the border zones and the scarred region. Bar is 10 µm. BrdU indicates 5-bromo-2′-deoxyuridine, CPC, cardiac progenitor cell; MI, myocardial infarction; NR, noninfarcted region; and RR, risk region.
Long-Term Effects of Cardiac Progenitor Cells

(c-kitPOS/CD45NEG cells) than in control rats: 47.4±4.8% versus 21.9±5.2% in the risk region, P<0.01; 54.8±7.8% versus 36.4±16.0% in the noninfarcted region (Figure 7M). Taken together, these data indicate that, at 1 year after CPC administration, the formation of new CPCs was increased in CPC-treated hearts compared with vehicle-treated hearts, particularly in the risk region.

Next, we sought to determine whether the newly formed CPCs derived from exogenous (Y-chromosome POS) or endogenous (Y-chromosome NEG) cells. To this end, we examined the presence of Y-chromosome in c-kitPOS/BrdUPOS cells (Figure 8A and Online Figure VIIA to VIIC, Online Data Supplement). We found that proliferation of Y-chromosome POS cells was still robust at 12 months after transplantation because newly formed Y-chromosome POS cells (Y-chromosome POS/BrdUPOS cells) constituted 29.0±3.2% and 19.2±6.5% of total Y-chromosome POS nuclei in the risk and noninfarcted region, respectively (Figure 8B). The number of newly formed Y-chromosome POS cells was significantly higher in the risk region than in the noninfarcted region (2.5±0.3% of total nuclei versus 1.2±0.4%, respectively; Figure 8F).

With regard to c-kit expression, at 1 year after transplantation, only a small fraction of Y-chromosome POS cells were c-kitPOS (14.0±2.5% in the risk region and 6.7±3.7% in the noninfarcted region; Figure 8D), indicating that most transplanted cells and their progeny had lost c-kit positivity, presumably as a result of differentiation into more mature phenotypes. Importantly, Y-chromosome POS/c-kitPOS cells constituted only 1.0±0.2% of total nuclei in the risk region and 0.5±0.3% in the noninfarcted region (Figure 8E); these percentages were much lower than the percentages of CPCs (c-kitPOS/CD45NEG cells; 6.6±1.0% and 5.3±1.4% of total nuclei in the risk and noninfarcted regions, respectively; Figure 7J). Similarly, the number of new Y-chromosome POS/c-kitPOS cells formed during the 12th month after MI (Y-chromosome POS/c-kitPOS/BrdUPOS cells) was much lower than the number of new CPCs (c-kitPOS/CD45NEG/BrdUPOS cells) formed during the 12th month after MI in the risk region (0.9±0.2% of total nuclei [Figure 8C] versus 3.9±0.6% of total nuclei, Figure 7L) as well as in the noninfarcted region (0.5±0.3% of total nuclei [Figure 8C] versus 3.5±0.8% of total nuclei [Figure 7L]). Thus, even if all of the Y-chromosome POS/c-kitPOS cells were CPCs (which is unlikely), they would be a small fraction (≤20% or less) of...
both total CPCs and newly formed CPCs, implying that neither the majority of CPCs nor the majority of newly formed CPCs were derived from the transplanted (Y-chromosome<sup>POS</sup>) cells. These data indicate that the increased proliferation of CPCs observed 1 year after CPC transplantation cannot be accounted for by proliferation of transplanted CPCs and therefore must involve primarily endogenous cells.

**Discussion**

Although the ability of CPCs to alleviate LV dysfunction and remodeling has been demonstrated by several laboratories,<sup>2,4,8,10</sup> no previous study has examined the long-term outcome of CPC therapy—an issue that is obviously fundamental from a translational standpoint. Thus, at the preclinical level, the duration of the salubrious effects of CPC therapy and its potential tumorigenic effects remain unknown. Furthermore, no information is available regarding the long-term fate of transplanted CPCs, which hinders elucidation of their mechanism(s) of action.

This study is unique because of its long (1 year) follow-up after CPC administration. Our results provide several new findings that are important from both a conceptual and a therapeutic standpoint: i) the beneficial effects of CPCs on regional and global LV function after acute MI are sustained for at least 1 year and thus are likely to be permanent; ii) CPC therapy also affords a sustained (1 year) improvement in LV remodeling, as evidenced by a reduction in LV dilatation, LV hypertrophy, myocardial fibrosis in the risk region, and LV expansion index; iii) transplantation of CPCs is not associated with any evidence of tumor formation over the ensuing 12 months; iv) at 12 months after CPC administration, the risk region exhibits an increase in viable myocardium and myocyte density, both of which suggest (but do not prove) myocyte regeneration; v) some transplanted CPCs (or their progeny) engraft and persist at 1 year after transplantation, but they fail to acquire a mature cardiomyocyte phenotype, and their number (4–8% of nuclei) is insufficient to account for the functional and structural benefits of CPC therapy, implying that the major mode of action of CPCs is via paracrine actions; vi) transplantation of exogenous CPCs triggers a robust and surprisingly sustained proliferative response of endogenous cells that is still present 12 months later; vii) a major component of this proliferative response is the formation of new cells that express cardiomyocytic proteins (α-SA) but do not have a mature cardiomyocyte phenotype; viii) an additional component is the increased formation of new vessels in the risk region which, however, is not associated with increased vascular density; ix) the CPC-induced proliferative response includes also increased formation of new CPCs, which is still evident at 12 months after CPC administration; and x) although the transplanted (Y-chromosome<sup>POS</sup>) cells continue to proliferate briskly at 12 months, the increased formation of new CPCs observed at this time cannot be accounted for by proliferation of exogenous (Y-chromosome<sup>POS</sup>) CPCs and thus must result from activation of endogenous (Y-chromosome<sup>NEG</sup>) cells. Taken together, these results offer new important insights into the long-term effects and mechanism of action of CPC therapy and have important implications for therapeutic translation.

Every effort was made to ensure that the conclusions of this study were supported by robust data. Sample sizes were relatively large. LV function was carefully addressed with 2 independent techniques. The results were analyzed by investigators who were blind to treatment. Furthermore, LV function was assessed using multiple parameters, including measures of regional and global function and load-dependent and independent indices. An exhaustive immunohistological analysis was performed to quantify the frequency of selected antigens. In each heart, an average of 1702 nuclei was counted and 25 confocal microscopic fields were examined for measurements of BrdU, wheat germ agglutinin, IB4, α-SA, c-kit, CD45, and Y-chromosome positivity.

An important goal of this study was to evaluate, in a careful and systematic manner, the possible tumorigenic actions of exogenous CPCs. To accomplish this goal, we studied 50 rats that received CPCs and were followed for 1 year. At postmortem examination, we found no evidence of tumor formation in any of these animals, or in any of the other animals used for the functional and structural analyses, indicating that transplantation of CPCs, at least when done within the first 5 passages (as in this study), is not tumorigenic.

One of the most striking results of this investigation is the magnitude of the salubrious effects produced by a single administration of CPCs. There was a significant improvement in global LV systolic function, as documented by 2 independent methods (echocardiographic and hemodynamic studies) and by a multitude of parameters, both load dependent and load independent (Figure 4). Regional function in the infarcted region was also ameliorated (Figure 4B), presumably as a result of the greater proportion of viable tissue in this region and the increased wall thickness (Figure 3). Another important benefit of CPC therapy was the reduction in LV hypertrophy (Table 1), which probably resulted from the functional improvement effected by CPCs. The reduction in collagen content observed in the risk region (Online Figure II, Online Data Supplement) may be the consequence of both increased myocyte regeneration (as suggested by the increased amount of viable tissue in the risk region [Figure 3A and 3B]) and favorable effects of CPCs on the extracellular matrix. A decrease in collagen is not only a marker of reduced cardiac injury but also a potential mechanism for improved cardiac performance.

Perhaps, the most important question regarding the benefits imparted by CPCs pertains to their mechanism of action. Previous studies from our laboratory have shown that when CPCs were transplanted into injured hearts, they gave rise to cells that expressed myocyte-specific proteins (such as α-SA) but were small and did not resemble adult myocytes.<sup>2,3,9,11,13</sup> Furthermore, the number of cells derived from exogenous CPCs was insufficient to explain the improvement in cardiac function.<sup>2,3,9,11,13</sup> For example, <3% of transplanted CPCs could be found in the mouse heart at 35 days after adoptive transfer (a time when cardiac performance was enhanced)<sup>12,27</sup> and intracoronary infusion of CPCs promoted myocardial repair in rat and porcine models of chronic ischemic cardiomyopathy despite the persistence of a low number of transplanted cells.<sup>9,11</sup> In other studies, however, particularly in models of acute MI, the magnitude of CPC differentiation.
into adult myocytes has been reported to be substantial.14,28 As a consequence of these discrepant results, 2 different schools of thought have emerged: one that asserts that the beneficial effects of CPCs are underlain by the differentiation of transplanted cells into new vascular cells and mature myocytes29,30 and another that asserts that transplanted CPCs produce their salubrious effects via paracrine mechanisms because they do not differentiate into adult myocytes and most of them disappear shortly after adoptive transfer.2,3

This study provides important new information that shreds light on this issue. In phase A, we found that, after administration of Y-chromosomePOS CPCs, robust structural and functional benefits persisted for 1 year despite the fact that only 7.7±1.2% of nuclei were Y-chromosomePOS in the risk region and 3.8±1.0% in the noninfarcted region at 1 year (Figure 6G). The number of Y-chromosomePOS cells expressing cardiac proteins (α-SAPOS cells) was even lower (Figure 6I). Furthermore, Y-chromosomePOS cells with a morphology resembling adult myocytes were rarely observed, if ever (Figure 6A through 6D). These findings demonstrate that, with possible rare exceptions, exogenous CPCs do not differentiate into mature myocytes, even at 1 year after transplantation. Consequently, the remarkable improvement in LV function and remodeling observed at 1 year (Figures 3 and 4) cannot be accounted for by differentiation of exogenous CPCs into new functional myocytes; the major mechanism of action must involve paracrine mechanisms on the host cells.

Nevertheless, the finding of persistent Y-chromosomePOS cells is noteworthy. Contrary to previous proposals,31,32 our data show that transplanted CPCs (or their progeny) are not completely cleared from the heart, even after 1 year (Figures 6 and 8) and continue to proliferate robustly at this time (Figure 8B). This rather surprising observation raises the intriguing possibility that the paracrine actions of transplanted cells may be sustained for very prolonged periods of time after a single administration. The nature and function of engrafted Y-chromosomePOS cells are unclear. At 1 year, most of these cells (>85%) have lost c-kit expression (Figure 8D); 62.2±2.1% express cardiac proteins (α-SA) in the risk region and 14.0±6.4% in the noninfarcted region (Figure 6I) although they fail to acquire a mature myocyte phenotype (Figure 6A through 6D). Further studies will be necessary to determine whether the engraftment of Y-chromosomePOS cells is an epiphenomenon or is necessary for the LV functional improvement to occur.

It is unlikely that the Y-chromosomePOS cells were the result of fusion of transplanted CPCs with endogenous cells, such as myocytes, because fusion would be expected to produce binucleated cells of size comparable with the original cells and fusion events are rare (<1×10⁵–10⁶ cells33,34). In contrast, the Y-chromosomePOS cells we observed were small, mononucleated, and relatively frequent (Figure 6A through 6D). Fusion has never been reported for CPCs.Engulfment of transplanted CPCs by macrophages is also unlikely because the Y-chromosome of engulfed cells would not be localized to the nucleus and, in any case, would disappear as a result of DNA degradation, particularly after 1 year.

One potential paracrine action of transplanted CPCs would be activation of endogenous cells. To determine whether infusion of exogenous CPCs elicits proliferation of endogenous cells and, if so, what the time-course is, in phase B of the study, we administered BrdC for 1 month during the 3rd, 7th, or 12th month after MI. These experiments led to the remarkable finding that, in CPC-treated rats, a single infusion of cells resulted in a prolonged proliferative response within the host myocardium, which persisted for at least 12 months in the risk region and 3 months in the noninfarcted region and was manifested in the formation of new cells that expressed cardiac-specific proteins, such as α-SA (BrdUPOS/α-SAPOS cells; Figure 5). The nature of these cells remains unclear; although the expression of α-SA would suggest differentiation toward a cardiomyocyte lineage, the cells were small and did not acquire the phenotype of mature myocytes, even after 12 months (Figure 5). Indeed, although we examined all 3 subgroups of rats that received BrdC (infusion of BrdC in the 3rd, 7th, and 12th month after MI), we observed few, if any, BrdUPOS mature myocytes in any of these subgroups. Therefore, the double positive (BrdUPOS/α-SAPOS) cells that we found (Figure 5) cannot be regarded as cycling myocytes or myocyte precursors. Further studies will be necessary to ascertain their nature. As was the case for the rest of the proliferative response, the CPC-induced proliferation of BrdUPOS/α-SAPOS cells involved both the infarcted and the noninfarcted regions but was more robust in the former (Figure 5). The CPC-induced proliferation also resulted in increased formation of endothelial cells in the risk region; vessel density was not augmented, however, suggesting increased turnover of endothelial cells (Online Figure III, Online Data Supplement).

Having observed that infusion of exogenous CPCs induced a surprisingly prolonged proliferative response of small α-SAPOS cells and endothelial cells, we investigated whether this phenomenon was associated with activation and proliferation of CPCs (c-kitPOS/CD45NEG cells). We found that, at 1 year after transplantation, the total number of CPCs was not significantly different in vehicle- and CPC-treated rats (Figure 7H). However, the turnover of CPCs was significantly increased in CPC-treated rats because the frequency of c-kitPOS/CD45NEG cells that were BrdUPOS (ie, formed during the 12th month after MI) was greater than in control rats, both when they were expressed as a percentage of total nuclei (Figure 7L) and of total CPCs (Figure 7M). Previous studies have suggested that exosomes produced by various stem/progenitor cells activate endogenous CPCs.33 The fact that CPCs proliferated at a higher rate, yet the total number of these cells did not increase, suggests a shortened cell life span, possibly because of increased differentiation, increased cell death, or both.

What is the source of this proliferation? We were able to demonstrate that the vast majority of the proliferating (newly formed during the 12th month after MI) CPCs were endogenous because Y-chromosomePOS/c-kitPOS cells (Figure 8E) were <20% of the CPCs (Figure 7J) and newly formed Y-chromosomePOS/c-kitPOS cells (Y-chromosomePOS/c-kitPOS/BrdUPOS cells; Figure 8C) were <25% of total newly formed CPCs (c-kitPOS/CD45NEG/BrdUPOS cells; Figure 7L). Even if one assumes that all Y-chromosomePOS/c-kitPOS cells were CPCs.
(which is not plausible), the majority (>75%) of the CPCs and newly formed CPC at 12 months were not Y-chromosomePOS.

Beside the striking proliferative response, we found circumstantial evidence that CPC administration promoted myocyte regeneration in the risk region. Specifically, this region contained a greater percentage of viable tissue in CPC-treated versus vehicle-treated rats (65.8% versus 53.6%, respectively [Figure 3]) and was more densely populated with mature myocytes, as evidenced by an increase in myocyte density (Online Figure IV, Online Data Supplement)—2 observations that would be consistent with regeneration of myocytes although other explanations are also possible. If new mature myocytes were formed, their origin is unclear. Given that transplanted CPCs failed to acquire a mature cardiomyocyte phenotype, any new myocytes must be derived from endogenous cells, eg, from differentiation of endogenous CPCs or other precursor cells. If such differentiation took place, it must have occurred in the absence of steady proliferation of precursor cells because, as mentioned above, we found almost no BrdUPOS mature myocytes irrespective of when BrdC was administered (3rd, 7th, or 12th months after CPC therapy), indicating that the endogenous cells that gave rise to mature myocytes (myocyte precursors) did not proliferate during those 3 months. It is possible, however, that the myocyte precursors may have proliferated in other months, when BrdC was not infused.

In conclusion, this is the first long-term study of CPCs. Our results demonstrate that the salubrious effects of these cells on LV function and remodeling after MI persist for at least 1 year and, therefore, are likely to be permanent. This sustained improvement is not associated with tumor formation. The beneficial effects of CPCs cannot be explained by differentiation of transplanted cell into cardiac myocytes and, therefore, must reflect paracrine actions. Our finding that some transplanted cells are still present, and continue to proliferate, at 1 year (constituting 4–8% of the nuclei) raises the possibility that their paracrine actions may be much longer-lasting than previously thought. Surprisingly, we found that a single administration of CPCs causes a robust and sustained proliferative response leading to formation of endothelial cells and small cells that express cardiac proteins (aSA). The exact nature of these latter cells is unclear, but they do not resemble adult cardiomyocytes, even at 12 months after CPC transplantation and thus cannot be considered cycling myocytes or myocyte precursors. The formation of small aSAPOS cells is associated with increased proliferation of endogenous CPCs that is still demonstrable 12 months after CPC infusion. To our knowledge, this is the first evidence that transplantation of cells (of any type) in the heart induces a sustained proliferative response that lasts at least 12 months. Although the mechanism for this phenomenon remains to be elucidated, its occurrence offers a plausible explanation for the seemingly paradoxical observation that the salutary effects of cell therapy persist even after the transplanted cells have disappeared. The data are consistent with a paradigm in which exogenous CPCs activate endogenous CPCs and other cells (eg, endothelial cells and small cells expressing cardiac proteins), which proliferate and promote cardiac repair. Further research is warranted to elucidate the mechanism(s) whereby injection of CPCs triggers such a strikingly long-lasting proliferative response and the significance thereof. Meanwhile, the long-term safety and efficacy of CPCs observed in this study provide a strong rationale for clinical translation.

Sources of Funding
This study was supported in part by NIH Grants HL-113530, HL-78825, HL-55757, HL-74351, and HL-91202.

Disclosures
None.

References


Novelty and Significance

What Is Known?

- c-kit+/Sca-1+ CPCs can be easily expanded from adult cardiac tissue.
- Short-term follow-up (<6 weeks) studies from multiple laboratories have consistently shown that administration of CPCs promotes cardiac repair, attenuates LV dysfunction, and improves LV remodeling in various animal models of both acute and chronic post-MI heart failure.
- However, the long-term effects of CPC therapy, in terms of safety (tumor formation), fate of transplanted cells, and efficacy, are unknown.

What New Information Does This Article Contribute?

- The beneficial effects of CPCs on regional and global LV function and on LV remodeling after acute MI are sustained for at least 1 year and thus are likely to be permanent.
- Transplantation of CPCs is not associated with any evidence of tumor formation over the ensuing 12 months.
- Some transplanted CPCs (or their progeny) engraf and persist at 1 year after transplantation, but they fail to acquire a mature cardiomyocyte phenotype, and their number is insufficient to account for the functional and structural benefits of CPC therapy, implying that the major mode of action of CPCs is via paracrine actions.
- The benefits of CPCs on regional and global LV function and on LV remodeling after acute MI are sustained for at least 1 year and thus are likely to be permanent.

This is the first long-term study of CPCs. Our finding that some transplanted cells persist, and continue to proliferate, at 1 year (constituting 4%–8% of the nuclei) raises the possibility that their paracrine actions may be much longer lasting than previously thought. CPC administration caused a robust and sustained (at least 12 months) proliferative response leading to formation of endogenous CPCs, endothelial cells, and small cells that express cardiac proteins (alpha-sarcomeric actin). The exact nature of these latter cells is unclear, but they do not resemble adult cardiomyocytes, even at 12 months after CPC transplantation, and these latter cells is unclear, but they do not resemble adult cardiomyocytes, even at 12 months after CPC transplantation, and thus cannot be considered cycling myocytes or myocyte precursors. This is the first evidence that transplantation of cells (of any type) in the heart induces a sustained proliferative response that lasts at least 12 months. Although the mechanism for this phenomenon remains to be elucidated, its occurrence offers a plausible explanation for the seemingly paradoxical observation that the salutary effects of cell therapy persist even after the transplanted cells have disappeared. The data are consistent with a paradigm in which exogenous CPCs activate endogenous CPCs and other cells, which proliferate and promote cardiac repair. The long-term safety and efficacy of CPCs observed in this study provide a strong rationale for clinical translation.
Long-Term Outcome of Administration of c-kit<sup>POS</sup> Cardiac Progenitor Cells After Acute Myocardial Infarction: Transplanted Cells Do not Become Cardiomyocytes, but Structural and Functional Improvement and Proliferation of Endogenous Cells Persist for at Least One Year

Xian-Liang Tang, Qianhong Li, Gregg Rokosh, Santosh K. Sanganalmath, Ning Chen, Qinghui Ou, Heather Stowers, Greg Hunt and Roberto Bolli

_Circ Res._ 2016;118:1091-1105; originally published online February 2, 2016; doi: 10.1161/CIRCRESAHA.115.307647

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/118/7/1091

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/02/02/CIRCRESAHA.115.307647.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Correction

Tang et al. Long-Term Outcome of Administration of c-kitPOS Cardiac Progenitor Cells After Acute Myocardial Infarction: Transplanted Cells Do Not Become Cardiomyocytes, but Structural and Functional Improvement and Proliferation of Endogenous Cells Persist for At Least One Year Circ Res. 2016; first published on February 2, 2016 as doi:10.1161/CIRCRESAHA.115.307647

The original version of this article, published Online First on February 2, 2016, inadvertently omitted the Novelty & Significance section. The article was corrected and re-posted on February 16, 2016.
Supplementary Fig. I

The figure shows the percent survival over time after MI, with two treatment groups: Vehicle-treated (red line) and CPC-treated (blue line). The x-axis represents time after MI, with markers for Baseline, 1 week (1 wk), 1 month (1 mon), 2 months (2 mon), 3 months (3 mon), 4 months (4 mon), 5 months (5 mon), 6 months (6 mon), 7 months (7 mon), 8 months (8 mon), 9 months (9 mon), 10 months (10 mon), 11 months (11 mon), and 12 months (12 mon). The y-axis represents percent survival, ranging from 0% to 100%. The graph indicates a decrease in percent survival over time, with CPC-treated subjects generally showing a higher percent survival compared to Vehicle-treated subjects.
Supplementary Fig. II

A  Vehicle-treated  CPC-treated

B

C

Risk Region  Posterior Wall

Collagen Content (% of total area)

Vehicle  n=12  CPC  n=14  Vehicle  n=12  CPC  n=14

P<0.05
**Supplementary Fig. IIIA-F**

**C** Vessel Density

<table>
<thead>
<tr>
<th></th>
<th>Risk Region</th>
<th>Remote Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n/mm²</strong></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td></td>
</tr>
</tbody>
</table>

**D** BrdU<sup>pos</sup> Vessel Density

<table>
<thead>
<tr>
<th></th>
<th>Risk Region</th>
<th>Remote Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n/mm²</strong></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>250</td>
</tr>
</tbody>
</table>

**E** BrdU<sup>pos</sup> Vessels

<table>
<thead>
<tr>
<th></th>
<th>Risk Region</th>
<th>Remote Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% of Total Vessels</strong></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

**F** BrdU<sup>pos</sup> Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>Risk Region</th>
<th>Remote Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% of Total Endothelial Cells</strong></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

*IB4, α-SMA, BrdU, DAPI*
Supplementary Fig. III G-H

**Risk Region**
- Vehicle-treated (1,290 vessels)
- CPC-treated (1,280 vessels)

**Noninfarcted Region**
- Vehicle-treated (329 vessels)
- CPC-treated (269 vessels)
Supplementary Fig. IIII-K

I

Risk Region

Diameter Range

Vehicle (1,290 vessels): 0.2 µm – 25.4 µm
CPC (1,280 vessels): 0.2 µm – 52.5 µm

n=6/group

J

Noninfarcted Region

Diameter Range

Vehicle (329 vessels): 0.2 µm – 20.1 µm
CPC (269 vessels): 0.4 µm – 34.3 µm

n=6/group

K

P<0.01

Vessel Diameter (µm)

Risk Region

Vehicle-treated (n=6)
CPC-treated (n=6)

1,290
1,280

Noninfarcted Region

329
269 Vessel Numbers
**Myocyte Nuclear Density**

- **Vehicle-treated (n=6)**
- **CPC-treated (n=6)**

- **P<0.01**

<table>
<thead>
<tr>
<th>Region</th>
<th>Vehicle-treated</th>
<th>CPC-treated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk Region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote Region</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Myocyte Density**

- **Risk Region**
- **Remote Region**

- **P<0.01**
- **P<0.01**

Supplementary Fig. IV
Supplementary Fig. V

**BrdU^{pos}/a-SA^{pos} Cells**

Risk region

% of Total Nucleated a-SA^{pos} Cells

<table>
<thead>
<tr>
<th></th>
<th>3rd</th>
<th>7th</th>
<th>12th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated (n=5-6)</td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
</tr>
<tr>
<td>CPC-treated (n=5-6)</td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
<td><img src="image" alt="Bar Graph" /></td>
</tr>
</tbody>
</table>

Month after MI during which BrdC was infused

- P < 0.01
- P < 0.01
- P < 0.01
Supplemental Fig. VIIA
Supplemental Fig. VIIIB
Supplemental Fig. VII C