Cardiac Stem Cell Hybrids Enhance Myocardial Repair

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Rationale: Dual cell transplantation of cardiac progenitor cells (CPCs) and mesenchymal stem cells (MSCs) after infarction improves myocardial repair and performance in large animal models relative to delivery of either cell population. Objective: To demonstrate that CardioChimeras (CCs) formed by fusion between CPCs and MSCs have enhanced reparative potential in a mouse model of myocardial infarction relative to individual stem cells or combined cell delivery. Methods and Results: Two distinct and clonally derived CCs, CC1 and CC2, were used for this study. CCs improved left ventricular anterior wall thickness at 4 weeks post injury, but only CC1 treatment preserved anterior wall thickness at 18 weeks. Ejection fraction was enhanced at 6 weeks in CCs, and functional improvements were maintained in CCs and CPC+MSC groups at 18 weeks. Infarct size was decreased in CCs, whereas CPC+MSC and CPC parent groups remained unchanged at 12 weeks. CCs exhibited increased persistence, engraftment, and expression of early commitment markers within the border zone relative to combinatorial and individual cell population–injected groups. CCs increased capillary density and preserved cardiomyocyte size in the infarcted regions suggesting CCs role in protective paracrine secretion. Conclusions: CCs merge the application of distinct cells into a single entity for cellular therapeutic intervention in the progression of heart failure. CCs are a novel cell therapy that improves on combinatorial cell approaches to support myocardial regeneration. (Circ Res. 2015;117:695-706. DOI: 10.1161/CIRCRESAHA.115.306838.)

Key Words: cell fusion • mesenchymal stromal cells • myocardial infarction • myocytes, cardiac • stem cells

Cell therapy for regeneration of the myocardium after myocardial infarction involves 2 concurrent processes: (1) stimulation of endogenous repair, and (2) exogenous cellular commitment. Regenerative medicine would benefit tremendously from identification of optimal stem cell population(s) that exert both direct and indirect mechanisms to mediate survival of existing cardiac myocytes, support proliferation and differentiation of endogenous stem cells, reduce inflammation, and prevent scar formation. Coupling intrinsic mechanisms of myocardial repair with the propensity of stem cells to undergo cardiomyogenesis should be carefully balanced and integrated with the existing heart scaffold. Delivery of single stem cell types promote relatively modest functional and structural recovery of the heart because of limited reparative capacity of donated cell populations derived from cardiac and bone marrow origin. Increasing cell numbers can enhance beneficial cellular properties, but excess reactive oxidative species and inflammation after acute damage contributes to elimination of >90% of delivered cells after 1 week. Although the genetic engineering of stem cells before delivery remains a promising alternative to enhance persistence and regeneration, potential additional benefits of combinatorial cell therapy remain largely unexplored.

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Resident c-kit+ cardiac progenitor cells (CPCs) are a desirable cell choice because of enhanced proliferative capacity and ability to form cardiac myocytes, vascular smooth muscle, and endothelial cells ex vivo. Endogenous c-kit+ cells have limited capacity toward cardiomyogenic commitment during development and after myocardial injury. Despite limited regenerative capability, clinical application of CPCs confers improvements in myocardial structure and function as highlighted in the Cardiac Stem Cell Infusion in Patients With Ischemic Cardiomyopathy (SCIPIO) patient trial. Bone marrow is the most popular source of adult-derived stem cells because of proven safety and efficacy after transplantation. In particular, mesenchymal stem cells (MSCs) are commonly...
used for autologous and allogeneic clinical therapies. MSCs are valued for paracrine-mediated effects such as reducing inflammation and promoting prosurvival and growth cascades to surrounding cells. MSC injection after infarction promotes robust recruitment of c-kit+ CPCs, induces cardiomyocyte cycling, and facilitates the outgrowth of stem cells from myocardial biopsies (ex vivo). Recently, combining these 2 distinct stem cells types, CPCs and MSCs, was investigated in a porcine model of myocardial damage. Functional recovery and detection of human derived cells in the myocardium was improved over injection of single cells alone, indicating synergism of combining 2 cell types. However, cellular mechanisms of myocardial recovery were not addressed and ratios of cell numbers were skewed toward increased MSC numbers to confer protective effects in vivo.

Cell fusion and creation of syncitia is an endogenous and homeostatic process coupled with differentiation and organ development. Although fusion is low at basal levels, fusion increases in acute and chronic settings of inflammation, DNA damage, and apoptotic events after bone marrow cell transplantation. Artificial cell fusion between the same and different cells types to produce heterokaryons can be accomplished with addition of polyethylene glycol, electric pulses, or viral fusogens. In rare events, mononucleated hybrids (syncytiotrophoblasts) from binucleated cell states occur, which is largely dependent on the ability of one cell type to undergo DNA synthesis after fusion. Bone marrow cells and MSCs have been observed to readily fuse to more mature cells, allowing successful transfer of mitochondria and phenotypic traits, such as increased survival and proliferation. Spontaneous in vivo cell fusion as a mechanism to support regenerative therapy has been underlying leading to the conclusion that cell fusion alone is not a major contributor to heart regeneration.

This article, we present the creation and characterization of CPC and MSC hybrids, referred to as CardioChimeras (CCs), generated by ex vivo viral cell fusion. CCs exhibit enhanced molecular and phenotypic traits relative to individual stem cells, and these distinct hybrids were evaluated for in vivo therapeutic effects after myocardial damage in a mouse model. Recovery of anterior wall thickness (AWT) and ejection fraction (EF) were markedly improved, concomitant with increased engraftment and expression of early cardiomyogenic lineage markers in CC-treated hearts. CCs represent a novel therapeutic that complements the paracrine effects of MSCs to orchestrate endogenous repair with direct cell contributions from CPCs in promotion of de novo cellular regeneration.

Methods
Full Materials and Methods are available in the Online Data Supplement.

Cell Fusion and Creation of CCs
Cell fusion was conducted using the GenomONE-CF EX Sendai virus (Hemagglutinating Virus of Japan or HVJ) Envelope Cell Fusion Kit (Cosmo Bio.). According to the manufacturer’s protocol, we subjected MSCs and CPCs to the plating method of cell fusion. Here, 100,000 MSCs expressing GFP in a 100-mm dish were incubated in CPC media for 24 hours. Next day, 100,000 CPCs expressing mcherry were suspended in 20 μL of cell fusion buffer and 10 μL of Sendai virus and plated on ice for 5 minutes for absorption of the virus on the cell membrane. Media from the MSC plate was removed and washed once with cell fusion buffer, and CPCs plus Sendai virus was added. The plate was then centrifuged (10 minutes, 550 g at 4°C) to force cell-to-cell contact. Cells were plated at 37°C for a total of 15 minutes to induce cell fusion. Nonfused cells were removed, and media was added back to the plate. The next day, media was changed, and within 48 hours, cells were trypsinized and subjected to fluorescence-activated cell sorting to place 1 cell per well of a 96-well microplate to allow for clonal expansion of double fluorescence cell populations.

Results
Phenotypic Characterization of CCs
CCs were created after fusion of fluorescently labeled CPCs (mcherry) and MSCs (eGFP) with an inactivated RNA Sendai virus (Figure 1A). After fusion, dual fluorescent hybrids were purified by fluorescence-activated cell sorting and allowed to undergo clonal expansion (Figure 1A; Online Figure IIA). Eighteen mononucleated hybrids were successfully expanded 1 month after initial sorting. Additional information about the analysis and selection criteria of the 2 CCs from the 18 clones is described in the Online Data Supplement (Online Figure I; Online Table I). CC1 and CC2 were chosen from the 18 clones because of enhanced proliferation relative to the majority of clones, optimal cell survival, and the ability to provide progrowth and survival factors when coimmunized with cardiac myocytes (Online Figure IA–IE; Online Table I). CC2 exhibits a proliferative rate similar to CPCs, whereas CC1 shows modest proliferation, and all cells had increased proliferation over MSCs based on a fluorescent-dependent cell proliferation assay and cell doubling time (Figure 1B and 1C). CCs are not increasingly susceptible to cell death compared with parent cells (Figure 1D) and do not exhibit elevated expression of cell cycle arrest or senescence markers based on mRNA for p16 or p53 (Figure 1E and 1F). CC1 has increased cell size and is morphologically similar to MSCs (Figure 1G, 1I, and 1J). CC2 displays a slight increase in cell size but is not significantly different from CPCs (Figure 1G, 1H, and 1K).

Mononucleated CC1 and CC2 exhibit increased nuclear size and centromere intensity relative to parent cells after nuclear hybridization (Online Figure IIB–IIIF). Collectively, CCs represent a novel stem cell population where increased DNA content does not negatively impact on survival or proliferation after induced cell fusion.
CCs Exhibit Increased Basal Level Expression of Cardiomyogenic Commitment Markers

MSCs and CC1 are low to negative for the stem/progenitor cells marker c-kit*, whereas CC2 and CPCs maintain 20% and 50% c-kit positivity, respectively (Online Figure IIIA). Gap junction marker connexin43 and platelet endothelial cell adhesion molecule (PECAM or CD31) mRNA are modestly upregulated in CC2 at basal levels (Online Figure IIIB and IIIC). MSCs express high levels of endothelial and smooth muscle markers as indicated by CD31 and smooth muscle 22 (SM22) gene expression (Online Figure IIIC and IIID).20 Although SM22 was not upregulated in CCs, coinubation of CPCs with MSCs at a 1:1 ratio increased mRNA expression of SM22 (Online Figure IIID). Interestingly, CC1 has increased...
mRNA for cardiac troponin T (cTNT or tnnt3; Online Figure IIIIE). Analysis of basal cardiomyogenic activity further confirmed the identification of CC1 and CC2 after cell fusion. CC1 has increased cardiogenic potential based on expression of cTNT, which corresponds to the lack of c-kit expression. CC2 retains low levels of c-kit expression but has increased expression of endothelial markers, a phenotype that has previously been reported to improve the regenerative capacity of CPCs.2

CCs Promote Cardiomyocyte Growth After In Vitro Coculture
To test the beneficial effects mediated by CCs and parental cells before in vivo cell transfer, neonatal rat cardiac myocytes (NRCMs) were cocultivated with stem cell groups (CPC, MSCs, CPC+MSC, CC1, and CC2) at a ratio of 1:10 in serum-depleted conditions. NRCMs maintained in low serum conditions (0.5%) resulted in smaller cardiac myocytes relative to NRCMs maintained in high-serum conditions (10%; Figure 2A, 2B, and 2G). Addition of MSCs, CPC+MSC, CC1, or CC2 to low serum–treated NRCMs significantly increased cardiomyocyte size within 24 hours (Figure 2C–2E and 2G), but CPCs could not induce significant growth of NRCMs (Figure 2F and 2G). Slow twitch β-myosin heavy chain (mhy7) over fast twitch α-myosin heavy chain (mhy6) gene expression was not significantly elevated in cardiac myocytes after 24 hours coinoculation with stem cell groups but is highly expressed in low serum conditions indicating that the addition of stem cells does not induce a maladaptive hypertrophic response in cardiac myocytes (Figure 2H). Regardless of the stem cell population added to cardiac myocytes, NRCMs were protected from cell death based on flow cytometric analysis of apoptotic and necrotic markers (Figure 2I). With the addition of CC1 and CC2, NRCMs had increased mRNA for stromal derived factor-1 (sdf-1; Figure 2J), a cardioprotective cytokine and homing ligand for C-X-C chemokine receptor type-4 positive stem cells.21

CCs Have Increased Gene Expression of Commitment and Paracrine Markers After In Vitro Coculture With Cardiac Myocytes
After coculture with cardiac myocytes, sm22 was not significantly upregulated in CC groups (Figure 2K). However, CPC+MSC and CC2 displayed the largest induction of endothelial marker expression pecam, whereas CC2 induced cTNT gene expression after 7 days of coculture with NRCMs (Figure 2L and 2M). Paracrine factors are routinely touted as a mechanism for cardioprotection22; therefore, we analyzed our stem cells for expression of growth and immunomodulatory factors. Gene and protein expression for interleukin-6 (IL-6) is upregulated in CC2 after 24-hour incubation with serum starved NRCMs (Figure 2N and 2O). Early release of immunomodulatory factors, such as IL-6, after acute cardiac damage has been shown to have antiapoptotic properties.23 In summary, CC1 shows increased cellular size and expression of early cardiac commitment markers without impairment in cell proliferation. CC2 has similar morphological features to CPCs in addition to having a higher proliferative status relative to CC1. In fact, CC2 was most responsive to differentiation as evidenced by the upregulation of endothelial and cardiac markers in addition to increased expression of the immunomodulatory factor, IL-6. This preliminary data further validate the in vivo application of these 2 distinct cell hybrids.

CCs Improve Left Ventricular Structure and Cardiac Function After Myocardial Injury
To establish the therapeutic efficacy of CCs relative to parental cells or parental cells combined, we injected a total of 100,000 cells in the border zone region of an acutely damaged mouse heart. At 1 week post injury (WPI), all groups had similar reductions in AWT and EF (Figure 3A and 3D; Online Table II). CC1 and CC2 exhibited increased AWT at 4 WPI, but only CC1-treated hearts preserved AWT ≤18 WPI (Figure 3A). Heart weight/body weight ratio at 12 and 18 weeks did not increase in CC-treated hearts indicating that hypertrophy was not a contributing factor for increasing AWT (Figure 3B and 3C). Rather, CC1 hearts had significantly reduced heart weight/body weight ratio relative to vehicle control group (PBS; Figure 3C). EF was increased in CC1 and CPC+MSC hearts starting at 3 WPI, and CC1 and CC2 had increased EF over PBS at 6 WPI (Figure 3D). CC- and CPC+MSC-treated groups exhibited improved EF starting at 12 WPI, whereas the CPC treatment was beneficial for cardiac function only at 18 WPI (Figure 3D). Heart rates and structural/functional data are detailed in Online Table II. Correlating with improved EF, CC1 treatment significantly improved positive developed pressure over time (dP/dt; Figure 3E) and negative dP/dt (Figure 3F). CC1, CC2, CPC+MSC, and CPC hearts had smaller infarct sizes relative to PBS (Figure 3G), MSC groups exhibited increased infarct size when measuring scar between 4 and 12 WPI, CPC and CPC+MSC hearts remain unchanged, and CC1 and CC2 treatment reduced infarct size as represented by Masson’s Trichrome staining (Figure 3G–3N).

Cellular Engraftment of CCs 4 Weeks After Damage
Scar size measured at 4 WPI was not significantly different among infarcted heart groups (Online Figure IVA and IVB–IVE). Next, we were interested in determining cell persistence at this time point and were able to detect CPCs labeled with mcherry in CPC alone and CPC+MSC-treated hearts (Online Figure IVF and IVG). Interestingly, mcherry+ CPCs were detected near small c-kit+/cTNT+ cardiac myocytes in the infarct area (Online Figure IVH). CC1 detected by both GFP and mcherry expression did not display evidence of commitment at this early time point (Online Figure IVI–IVK).

CCs Have Increased Engraftment, Expression of Cardiomyogenic Markers, and Support the Increased Presence of C-Kit Positive Cells in the Myocardium 12 Weeks After Damage
C-kit+ cell recruitment in damaged regions supports endogenous differentiation and myocardial repair.25 Although infarction sizes were similar at the 4-week time point, induction of endogenous c-kit cells in the infarcted area was increased in MSC-, CPC+MSC-, and CC1-treated hearts (Figure 4A–4C). At 12 WPI, a high number of c-kit+ cells were observed in PBS- and MSC-treated hearts, yet c-kit+ cells remained visually present in CPC+MSC-, CC1-, and CC2-treated hearts.
Figure 2. CardioChimeras (CCs) promote cell growth and have increased commitment and paracrine gene expression after in vitro coculture with cardiac myocytes. A, Neonatal rat cardiac myocytes (NRCMs) in low serum. B, NRCMS in high serum. C, NRCMs in low serum and after the addition of mesenchymal stem cells (MSCs). D, CC1, E, CC2, or F, cardiac progenitor cells (CPCs) for 24 hours. Cardiac myocytes were visualized by staining with sarcomeric actinin. TO-PRO-3 iodide was used to visualize nuclei. G, Quantification of cardiomyocyte size. H, Gene expression of mhy7 over mhy6 represented as fold change relative to high serum. I, Cardiomyocyte cell death. Values are represented as fold change of Annexin V+ and Sytox Blue+ cells relative to high serum. J, sdf-1 gene expression in cardiac myocytes alone after the addition of stem cells. K–M, Gene expression in stem cells after a 7-day coculture with NRCMs. K, sm22, L, pecam, and M, tnt3 gene expression. N, Il-6 gene expression analyzed in stem cells after a 24-hour coculture with NRCMs. O, IL-6 expression confirmed by ELISA. G–J, Statistical values were determined by 1-way ANOVA compared with low serum controls. Scale bar is 40 μm. IL indicates interleukin; NS, non-significant; pecam, platelet endothelial cell adhesion molecule; sm22, smooth muscle 22; sdf-1, stromal derived factor-1; and tnt3, cardiac troponin T. *P<0.05, **P<0.01, and ***P<0.001.
surrounding mcherry+ cells in the border zone regions (Figure 4D–4G). The percentage of cell engraftment was increased in CC1 and CC2 hearts at 1.9% and 1.1%, respectively, relative to 0.21% and 0.29% in CPC and CPC+MSC hearts (Figure 4H and 4K–4O). MSCs were detected at a much lower level or 0.04% of the total left ventricular free wall (Figure 4H–4J). CPCs discovered in the border zone areas coexpressed c-kit and mcherry in CPC hearts and expressed mcherry alone in CPC+MSC hearts (Figure 4K and 4L). CC1 and CC2 had increased levels of engraftment, expressed cTNT, and were surrounded by endogenous c-kit+ cells (Figure 4M–4O).

Figure 3. CardioChimeras (CCs) improve left ventricular wall structure and cardiac function after myocardial injury. A, Longitudinal assessment of anterior wall thickness during systole (mm) >18 weeks. B, Heart weight/body weight ratio (mg/g) at 12 week post injury (WPI) and (C) 18 WPI. Sample sizes of 3 to 5 mice per group. D, Longitudinal assessment of ejection fraction (%). E, Positive and (F) negative developed pressure over time represented as mm Hg/s at 4, 12, and 18 WPI. G, Change in infarct size between 4- and 12-week time points. P values were determined by 1-way ANOVA compared with PBS-treated controls. H–N, Masson’s Trichrome staining and representative images of infarct size and fibrosis in (H) sham, (I) PBS, (J) mesenchymal stem cell (MSC), (K) cardiac progenitor cell (CPC), (L) CPC+MSC, (M) CC1, and (N) CC2. Sample sizes are specified in the Online Table II. All statistical values were determined by 2-way ANOVA compared with PBS-treated hearts. Colors of asterisk(s) correspond to heart group. Scale bar is 250 μm. *P<0.05, **P<0.01, and ***P<0.001.
CCs Increase Capillary Density in the Infarct Area

Capillary density was measured in the border zone and infarcted areas at 12 WPI. Shams, noninjured controls, are included as a standard for capillary density compared with injured hearts (Figure 5A–5C). Parent cells, individual or combined, or CC treatment did not significantly increase...
capillary density in the border zone regions relative to PBS (Figure 5A and 5C–5I). MSC, CPC, or CPC+MSC-treated hearts similarly did not affect the number of capillaries discovered in the infarct zone (Figure 5B and 5J–5M). Notably, CC1- and CC2-treated hearts had significant increases in isolectin+ structures in the infarct regions at 12 WPI (Figure 78B and 78N–78O).

**CPC, MSC, and CC Treatment Antagonizes Cardiomyocyte Hypertrophy in the Remote Region and Preserves Cardiomyocyte Size in the Infarcted Regions**

Cellular treatment and long-term engraftment of cells are reported to induce compensatory hypertrophy in areas of damage preventing progression of heart failure after myocardial infarction.24 MSC- and vehicle-treated hearts showed increased cardiomyocyte size in the remote area relative to sham (Figure 6A and 6C–6E). CPC+MSC-, CC1-, and CC2-treated hearts maintained cardiomyocyte size in the remote region similar to noninjured controls (Figure 6A, 6C, and 6G–6I). Although stem cell treatments could not modify border zone cardiomyocyte size (Online Figure V A–VG), injection of CPC, CPC+MSC, and both CCs increased cardiomyocyte size in the infarcted regions relative to PBS- and MSC-treated hearts ≤12 WPI (Figure 6B and 6J–6O). These data indicate that improved engraftment of stem cells correlates with the presence of microvascular structures and preservation of cardiomyocyte size in the remote and infarct regions relative to failing and severely damaged hearts.

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**Figure 5.** CardioChimeras (CCs) increase capillary density in the infarct area. A, Capillary density in the border zone and (B) infarcted heart regions. Sample sizes are 3 to 4 mice per group. Sham controls (dashed line) are represented as control for baseline density of isolectin+ structures per mm². C–I, Representative border zone images to visualize isolectin+ structures. J–O, Representative infarct zone images to visualize and quantify isolectin+ structures. Green=isolectin B4, white=cardiac troponin T, and blue=DAPI to stain for nuclei. Scale bar is 25 μm. CPC indicates cardiac progenitor cell; DAPI, 4′,6-diamidino-2-phenylindole; and MPC, mesenchymal stem cell. ***P<0.001. N’ and O’ represent additional views of capillaries in the infarct area of CC1- and CC2-treated hearts.
Discussion

The restorative impact of cell therapy to advance regenerative medicine remains to be fully realized and continues to be the focus of intense investigation. Increased knowledge of stem cell biology emerges from the use and application of a variety of adult stem cells. Unfortunately, ideal cellular properties are compromised by massive cellular death on introduction into damaged myocardium. In this report, we demonstrate a novel approach using cell fusion to enhance delivery of novel and unique stem cell properties created within a single cell. Cardiac-derived CPCs and bone marrow–derived MSCs were chosen for this study as both of these cell types have established roles in the heart: CPCs contribute to direct cardiomyogenic differentiation, whereas MSCs provide for protective immunomodulatory and growth factor paracrine secretion. CCs injected into the acutely damaged heart improved structural integrity and reduced infarct size (Figure 3). Furthermore, functional improvements were observed in CC-treated hearts, and increased engraftment was apparent in the border zones after 12 WPI (Figures 3 and 4). Specifically, CC1 significantly improved myocardial wall structure compared with control groups, and both CC1 and CC2 showed increased cellular engraftment in the border zone regions corresponding to a reduced infarct size and preservation of vascular structures in the neighboring infarct (Figures 3–5). Notably, CCs improved EF earlier in the assessment (6 WPI) relative to combined and single cell injections (Figure 3). At 12 WPI, CC therapy promoted increases in cardiac function, induction of endogenous c-kit cells, and maintenance of cardiomyocyte size that were comparable with mixed cell injections (Figures 3, 4 and 6). Initial improvements in cardiac function are most likely mediated by the combination of increased cell persistence and growth factor secretions conferred by CCs supporting long-term vascular stability and mitigation of adverse scar remodeling that is improved over combined therapy of CPCs and MSCs (Figures 3 and 5).

Bone marrow cells, the most common stem cell for cardiac therapy, apparently undergo engraftment through a combination of cell fusion and to a lesser degree by direct transdifferentiation events. Membrane fusion is dependent on signaling mechanisms involving paxillin-induced focal adhesions and recycling of integrins as demonstrated between macrophages and myoblasts. In the heart, cell fusion is increased between exogenous stem cells and apoptotic cardiac myocytes similar to enhanced myoblast fusion in the presence of phosphatidylserine presenting cells. Altered DNA content has been raised as an issue following fusion events as genomic instability leads to cellular aging. Somatic cells
exhibiting chromosomal mosaicism such as through the loss or deletions of chromosomes do not significantly affect stem cell properties or cell fate. As a result, CCs do not appear transformed but rather retain properties of CPCs and MSCs to support enhanced myocardial repair. To this effect, we were interested in correlating the in vitro properties of CC1 and CC2 to the observed effects in the myocardium. Although, both CC1 and CC2 were responsive to coculture with cardiac myocytes (Figure 2), CC-treated hearts showed only a modest upregulation of cTNT in vivo (Figure 4). CC1 in culture did not undergo significant cardiomyogenic commitment or secrete IL-6, yet CC1 hearts had stabilized AWT (Figure 3). We hypothesize that the larger cell body of CC1 contributed to higher rates of engraftment contributing to the improvement in myocardial structure without significant evidence of cardiogenic commitment (Figures 1 and 4). Before injection, CC2 exhibited a predominately CPC phenotype and supported in vivo effects, such as enhanced persistence and increased cardiac function, similar to CC1- and CPC+MSC-treated hearts. We propose that the high proliferative capacity of CC2 and expression of immunomodulatory factor IL-6 contributed to structural and functional benefits but through the contribution of distinct phenotypic characteristics from CC1 and CC2, respectively.

Increased basal expression of cardiomyogenic factors was observed in CCs (Online Figure III). Precommitted cells, but not fully mature stem cell–derived cardiac myocytes, improve exogenous cell coupling and formation of gap junction proteins. CCs display coordinated phenotypic properties of commitment and increased paracrine abilities to promote cardiomyocyte health much like the MSC parent and CPC+MSC-treated hearts. We hypothesize that the large cell body of CC1 contributed to higher rates of engraftment contributing to the improvement in myocardial structure without significant evidence of cardiogenic commitment (Figures 1 and 4). Before injection, CC2 exhibited a predominately CPC phenotype and supported in vivo effects, such as enhanced persistence and increased cardiac function, similar to CC1- and CPC+MSC-treated hearts. We propose that the high proliferative capacity of CC2 and expression of immunomodulatory factor IL-6 contributed to structural and functional benefits but through the contribution of distinct phenotypic characteristics from CC1 and CC2, respectively.

Therapeutic delivery of MSCs improves cardiac function and structure mainly through paracrine-mediated effects. Secretion of factors, such as stromal-derived factor-1 and insulin growth factor-1, support endogenous recruitment of c-kit+ progenitor cells and further facilitate cardiomyocyte cell cycle entry and survival. Immunomodulatory functions of MSCs to inhibit excess scar formation is an attractive therapy for several disease states. In this study, MSC treatment was unable to prevent increases in scar size or decreases in cardiac function ≤18 weeks similar to the deteriorating PBS-treated hearts. Although MSC addition did maintain size and survival of the responding cardiac myocytes, these beneficial effects were not recapitulated in vivo after MSC transfer (Figures 2 and 3). Apoptosis and slow proliferation rate are likely contributing factors to the disappearance of MSCs at later time points (Figures 1 and 4). Instead, MSC- and PBS-treated hearts sustained increases in c-kit+ cells, which are most likely increased through chronic inflammation and recruitment of hematopoietic-derived c-kit+ mast cells (Figure 4).

The optimal cell number chosen for therapy is a critical aspect to promote structural and functional recovery after myocardial infarction. Delivery of human CPC+MSC in a pig model of ischemia resulted in positive remodeling and engraftment using 200-fold more MSCs relative to CPCs. For our study, we placed CPCs/MSCs at a one-to-one ratio as the appropriate control compared with our CCs. The engraftment efficiency of MSCs could have been greatly limited from the beginning of the experiment because of reduced MSC numbers (Figure 4). Benefits of coculture of CPCs with MSCs are consistent with previous findings as MSC coinulation with CPCs at equal ratios increased basal differentiation markers, such as sm22, which was not observed in CCs (Online Figure III). Furthermore, during coculture with NRCCMs, CPC+MSC groups exhibited increased cardiomyogenic markers sm22, pcam, and cTNT (Figure 2). It remains unclear if differentiation resulted from CPCs alone in culture with MSCs, although significant cell death of MSCs alone was observed when cocultured with NRCCMs for 7 days. The comparatively modest therapeutic benefit of unmodified CPCs has been previously shown from our laboratory. Clearly, pinpointing the mechanistic contribution of MSCs to support CPCs in our CPC+MSC-treated hearts is an important unanswered question to be resolved in future investigations.

Although engraftment efficiency of CPCs cocinjected with MSCs was not significantly improved relative to CPC hearts alone, function was improved in CPC+MSC hearts at a much earlier time point. We can hypothesize that MSCs in the acute stages of damage (<4 weeks) facilitated protective endogenous cell reprogramming without long-term persistence, which was not sufficient to impact on exogenous CPC proliferation and engraftment, consistent with reports from other groups. From the numerous cell types touted to be efficacious for cardiac clinical therapy, CPCs and MSCs are particularly promising because of established protocols for cell isolation and expansion in clinical settings. Although MSCs show much lower rates of persistence in the damaged heart than CPCs, cell therapeutic practices could benefit from investigation of how to enhance immunomodulatory effects of MSCs. Currently, off-the-shelf allogeneic cellular options include cardiosphere-derived cells and MSCs that may exert beneficial effects after myocardial infarction but had poor persistence after delivery. In comparison, embryonic stem cells and induced pluripotent stem cells exhibit extended proliferation and are less prone to immune rejection and cell senescence after transplantation. However, embryonic stem cells have reduced capacity for integrative cardiomyogenesis as demonstrated by arrhythmogenic events in large animal models. Our cell fusion approach aims to capitalize on adult
stem cells that have validated cardiac therapeutic effects to create an exceptional composite hybrid with anti-inflammatory functions arising from the inclusion of allogeneic MSCs. Transplanted MSCs have suggested immunomodulatory functions by regulation of immune cells in the damaged setting. Mechanistically, MSCs have the potential to balance the inhibition of T-cell proliferation by secretion of indoleamine and promotion of dendritic cell differentiation into T-regulatory cells by secretion of IL-6 and IL-10, making this cellular source an essential component of future cardiac stem cell hybrids. In addition, fusion of aged stem cells with more youthful cells could confer cell rejuvenation and reverse signs of cellular aging. In the era of human cord blood banking, the isolation of immunoprivileged stromal cells from the same patient can be easily fused with stem cells harboring tissue-specific regenerative capacity, resulting in a novel cell type that is resistant to rejection in addition to having desired cellular effects, such as proliferation and direct tissue commitment. From a translational perspective, cell fusion is an adaptable genetic engineering strategy that qualitatively enhances adult specific regenerative capacity, resulting in a novel cell type resistant to rejection in addition to having desired cell-specific regenerative capacity, resulting in a novel cell type that is resistant to rejection in addition to having desired cellular effects, such as proliferation and direct tissue commitment.

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**Disclosures**

M.A. Sussman is a founder and co-owner of CardioCreate Inc. The other authors report no conflicts.

**References**


CardioChimeras exhibit sustained engraftment concomitant with in vivo functions such as direct cardiomyogenic differentiation or paracrine growth factor secretion, respectively. Delivery of CPCs or MSCs individually reveals contrasting but complementary functions of distinct cells to mitigate cardiac damage. In this study, we are the first to use novel cardiac stem cell hybrids created by cell fusion between CPCs and MSCs, also known as CardioChimeras, to support and enhance combinatorial cell delivery approaches. By inheriting properties of CPCs and MSCs, CardioChimeras exhibit optimal properties, such as cardiac commitment and enhanced paracrine secretion. CardioChimeras have increased engraftment in the left ventricle compared with groups treated with CPCs or MSCs individually or combined. Mechanistically, CardioChimeras promote an increase in capillary density and preserve cardiomyocyte size in the infarct area 12 weeks after damage. CardioChimeras represent an efficient fused product with beneficial and cardioprotective properties for effective cardiac repair.

Novelty and Significance

What New Information Does this Article Contribute?

- Cell fusion to create cardiac stem cell hybrids or CardioChimeras between CPCs and MSCs combines optimal traits, such as proliferation, survival, paracrine secretion, and cardiomyogenic differentiation ability, in a single cell type.
- Adaptive transfer of CardioChimeras after acute myocardial infarction promotes long-term improvements in anterior wall thickness and cardiac function.
- CardioChimeras exhibit sustained engraftment concomitant with increased vascular stability and prevention of maladaptive cardiomyocyte hypertrophy.

Cardiac stem cell–based therapy for the treatment of ischemic damage is popularized by the application of diverse cell types that exhibit distinct phenotypic traits. Specifically, delivery of CPCs or MSCs individually reveals contrasting but complementary in vivo functions such as direct cardiomyogenic differentiation or paracrine growth factor secretion, respectively. Dual cell delivery has emerged as a unique strategy to combine desirable functions of distinct cells to mitigate cardiac damage. In this study, we are the first to use novel cardiac stem cell hybrids created by cell fusion between CPCs and MSCs, also known as CardioChimeras, to support and enhance combinatorial cell delivery approaches. By inheriting properties of CPCs and MSCs, CardioChimeras exhibit optimal properties, such as cardiac commitment and enhanced paracrine secretion. CardioChimeras have increased engraftment in the left ventricle compared with groups treated with CPCs or MSCs individually or combined. Mechanistically, CardioChimeras promote an increase in capillary density and preserve cardiomyocyte size in the infarct area 12 weeks after damage. CardioChimeras represent an efficient fused product with beneficial and cardioprotective properties for effective cardiac repair.

What Is Known?

- Adult stem cell therapy leads to modest reparative effects because of low proliferation and survival of delivered cells in the damaged myocardium.
- Combined delivery of cardiac progenitor cells (CPCs) and bone marrow–derived mesenchymal stem cells (MSCs) support enhanced cellular engraftment and reduction in scar size after acute myocardial infarction.
Cardiac Stem Cell Hybrids Enhance Myocardial Repair
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Supplemental Material for

Cardiac stem cell hybrids enhance myocardial repair

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Supplemental Methods

Study design

*In vitro* studies were designed to predict the reparative potential of CPCs, MSCs, CPC + MSC and CardioChimeras (CCs) in a mouse model of injury. Cell-to-cell fusion yielded 18 CC clones from a total of 192 wells. For ease in naming clones, we identified CCs based on well number followed by A (1st 96-well plate) or B (2nd 96-well plate). Selected CCs were analyzed for proliferation, survival and paracrine-mediated effects on cardiomyocytes and/or expression of cardiomyogenic commitment markers (Online Figure III). The 18 CCs exhibited variable proliferative capabilities relative to individual CPCs and MSCs and were classified as slow, slow-medium, medium-fast and fast growing clones based on a 1-2 fold, 2-4 fold, 4-6 fold or >6 fold change in fluorescence relative to day of plating respectively (Online Figure IA). A number of slow-growing CCs were excluded due to a low expansion rate (Online Figure IA and Online Table I). We further characterized two-three clones per proliferative status for analysis of cell death after oxidative stress and morphological features such as cell size. With the addition of 40µM hydrogen peroxide, 4 CCs were excluded D.6 (A), B.10 (A), D.10 (A), E.10 (A) due to poor cell survival (Online Figure IB and Online Table I). Although cell size was not a defining reason for exclusion, cell morphological assessments correlated CC phenotype to individual parent cells. CC1 (F.7 (A)) and A.3 (A) exhibited a similar cell surface area to MSCs, whereas the majority of CCs including CC2 (E.2(A)) had a similar surface area to CPCs (Online Figure IC). Furthermore, co-culturing CCs with NRCMs mediated the identification of clones that could facilitate survival and growth of cardiomyocytes as compared to parent MSCs. Neonatal rat cardiomyocytes (NRCMs) were maintained in low serum (0.5% Fetal Bovine Serum) for 24 hours followed by the addition of CCs, CPCs, MSCs or CPCs and MSCs combined for an additional 24 hours and cardiomyocyte size and cell death was measured (Online Figure I, D and E). CCs were considered based on the ability to promote cardiomyocyte growth and prevent cardiomyocyte cell death following co-culture (Online Table I). Of the four candidates for adoptive transfer studies, F.9(B) and G.7(B) were eliminated due to a lower proliferative status (Online Table I). These initial analyses facilitated the identification of CCs that are suitable for cardiac therapy during acute damage by identification of clones with optimal proliferation and survival properties in addition to displaying the potential to secrete protective factors to preserve cardiomyocyte health.

For the adoptive transfer analysis, we required approximately 16 animals per cell group to allow for analysis of at 3-5 mice per time point (4, 12 and 18 WPI) without impacting on statistical significance obtained during longitudinal assessment by echocardiography. This number was chosen based on an average of 65-75% survival rate immediately after injury. Mice with a measured EF between 35-50% one-week post infarction were included in the experiment. EF >50% or <35% were excluded from the experiment. Throughout the time course, mice were not subjected to echocardiography if mice were perceived to be in distress. The study was concluded after determining statistical significance in wall thickness recovery and EF at 18 WPI. The study was not randomized, but was blinded to the operator during echocardiographic acquisition and analysis.

CPC and MSC isolation

CPCs were isolated and maintained as previously described1. CPCs were used during passages 10-20. Mesenchymal stem cells were isolated from 12 week old female FVB mice by flushing the femur and tibiae with 5% Fetal Bovine Serum in PBS through a 40-µm filter and centrifuged (10 minutes, 600g,
Cells were resuspended in media consisting of α modified minimum essential media and 15% FBS. Cells were plated in 150mm dish and media was changed every two days to remove non-adherent cells. Adherent cells created colonies in approximately two weeks. MSCs were passaged using 0.25% Trypsin and used during passages 2-4 for experiments.

**Lentiviral constructs and stem cell transduction**

A third generation enhanced green fluorescent protein (eGFP) lentivirus with a phosphoglycerate kinase (PGK) and puromycin (puro) selection marker was purchased from Addgene. pLenti PGK GFP Puro was used as a backbone to sub clone mcherry in the place of eGFP and bleomycin (bleo) to replace the puro gene in order to create pLenti PGK mcherry Bleo. MSCs at passage 1 were lentivirally transduced with pLenti PGK GFP Puro at a multiplicity of infection (MOI) of 50 and maintained in puromycin supplemented MSC media for one week starting at 48 hours post-infection. CPCs at passage 10 were lentivirally transduced with pLenti PGK mcherry Bleo at a MOI of 10 and subjected to fluorescent activated cell sorting (FACS) to purify mcherry positive CPCs. Fluorescent protein expression in MSCs (MSC-GFP) and CPCs (CPC-mcherry) was confirmed by fluorescent light microscopy and flow cytometric analysis.

**Light microscopy and measurement of cell morphology**

Images of stem cells were obtained on a Leica DMIL microscope and cell outlines were traced using ImageJ software. Relative surface area was determined as previously described².

**Centromere labeling (fluorescence in situ hybridization)**

Cells were fixed on glass two chamber slides in 3:1 ethanol:acetic acid for 30 minutes and then passed through graded alcohol series 70, 90, 100% (2 minutes each step). Slides were baked at 65°C for 15 minutes and then transferred to acetone for 10 minutes. Slides were then incubated for 1h at 37°C in 2X SSC (NACl/NA Citrate) + RNase (100µg/ml). Cell were treated with pepsin, 10mM HCl mixed with 0.5µl of stock pepsin solution (1mg/ml) at room temperature for 2-3 minutes and then dehydrated through ethanol series. Denaturing cellular DNA was done by immersing slides in 70% formamide in 2X SSC at 70°C for 2 minutes and then placed in ice cold 70% ethanol for 2 minutes followed by passing through an ethanol series. Prior to hybridization the centromere probe, CENPB-Cy3 (PNA Bio; ATTCGTTGGAAACGGGA), was warmed to 37°C for 5 minutes. The probe was denatured for 10 minutes at 85°C then immediately chilled on ice before applying probe to the slides. The hybridization protocol required 16 hours at 37°C. Post hybridization washes for 5 minutes at 37°C in 2X SSC were followed by two washes in 50% formamide/2X SSC 37°C, for 5 min each time and final wash in 2X SSC, twice for 5 min each time. DAPI (Sigma-Aldrich) was added to the final wash. Cell nuclei were visualized using a Leica TCS SP8 confocal microscope and the Z-stacking feature. Measurements of nuclear size and centromere intensity were determined after outlining the nucleus and getting the area (µm²) and mean gray values (fluorescent intensity/µm²) after creating a projection of Z-Stack scans.

**Proliferation assay and cell doubling time**

Cell proliferation was determined using the CyQuant Direct Cell Proliferation Assay (Life Technologies) according the manufacturer’s instruction and as previously described². Population doubling times were calculated using the readings from CyQuant Direct Proliferation Assay and use of a population doubling time online calculator (http://www.doubling-time.com/compute.php).

**Cell death assay**

Stem cells were plated in a 6-well dish (80,000 cells per well) and incubated in starvation media (growth factor and FBS depleted media) with 1% PSG for 18 hours. The cells were then treated with either 40µM or 80µM hydrogen peroxide for 4 hours. Cells were resuspended with Sytox Blue (Life Technologies) to label necrotic cells. Data was acquired on a FACS Aria (BD Biosciences) and analyzed with FACS Diva.
software (BD Biosciences). Cell death was quantitated by measurement of Sytox Blue positive cells and represented as a fold change relative to cells in starvation media alone.

In co-culture conditions of stem cells with NRCMs, whole populations were analyzed and stained with Annexin V (BD Biosciences) and Sytox Blue and only the negative (non-fluorescent NRCM) population was analyzed for cell death. Cell death of NRCMs was represented as a fold change relative to cells in growth media (10% M199). NRCMs in 0.5% M199 and 0.5% plus add back of 10% M199 at the time of stem cell addition were maintained as positive and negative controls for cell death.

Neonatal rat cardiomyocyte (NRCM) co-culture with stem cells
NRCMs were isolated and plated as previously described. After enzymatic digestion, cells were plated in M199 media (Life Technologies) with 15% FBS (Omega Scientific Inc.) at a density of 260,000 cells per well of a 6-well culture dish pretreated with 1% gelatin (Sigma-Aldrich). Within 18 hours, myocyte cultures were washed with PBS and incubated with M199 with 10% fetal bovine serum for 24 hours. The next morning, the cells were subjected to serum starvation (0.5% FBS in M199) for 24 hours. After low serum conditions, stem cells were added to the plate at a ratio of 1:10 (CPCs, MSCs, CPC + MSC combined, CC1 and CC2) and allowed to incubate with NRCMs for an additional 24 hours in low serum conditions. Controls for NRCMs included leaving cells in 0.5% alone, adding back 10% M199 or maintaining NRCMS in 10% M199 for the duration of the experiment. NRCM size was measured after staining cardiomyocytes with sarcomeric actinin (1:100, Sigma-Aldrich) and nuclei with TO-PRO-3 iodide and as previously described. Separation of NRCM and stem cells was accomplished with fluorescent activated cell sorting (FACS) of negative cells (NRCMs) versus GFP+, mcherry+ or GFP+/mcherry+ stem cells. After sorting, cells were centrifuged and suspended in RNase buffer for isolation and quantitation of mRNA from NRCMs or stem cells.

Immunocytochemistry
Stem cells were placed at a density of 15,000 per well of a two-chamber permanox slide and stained according to previous studies. Before scanning, cells were washed in PBS containing TO-PRO-3 iodide (Life Technologies) to stain for nuclei. Slides were visualized using a Leica TCS SP2 confocal microscope. Primary and secondary antibodies used are listed in Table S2.

Flow cytometric analysis
Cells in suspension were counted (200,000 cells per sample) and stained with primary and secondary antibodies as indicated in the Online Table III. Samples were analyzed using a FACS Canto (BD Biosciences).

mRNA isolation, cDNA synthesis and quantitative RT-PCR
RNA was enriched using the Quick RNA Mini Prep kit from ZymoResearch according to the manufacturers instructions. Reverse transcriptase was performed using protocol for the iScript cDNA Synthesis Kit (BIORAD). qRT-PCR was read after incubation of cDNA, primers (100nM) and IQ SYBR Green Supermix (BIORAD). Data was analyzed using the ΔΔC(t). Primer sequences are listed in the Online Table IV.

Enzyme-Linked Immuno Assay (ELISA)
The ELISA assay was performed in NRCMs alone (0.5%, 0.5% + 10% rescue, and 10% M199 treated cells), NRCMs incubated with stem cell groups and stem cells alone in normal growth media. Briefly, after 24 hour incubation with serum or stem cells, the 96-well microplate was centrifuged for 5 minutes at 1200rpm and 100µL of media supernatant was removed and used for IL-6 Mouse ELISA Kit (Life Technologies) performed according the company’s instructions.

Myocardial infarction and intramyocardial cell injections
Myocardial infarctions were carried out in eleven-week old female FVB mice under 2-3% isoflurane anesthesia and by tying off the left anterior descending artery (LAD) using a modified protocol. After ligation, injections with either PBS (5µL per injection, 5 injections total per mouse), parents (CPCs or MSCs), parents combined (CPC + MSC) or CCs CC1 (20,000 cells per 5µL injection, 5 injections making a total of 100,000 cells injected per mouse) were introduced to the pre-ischemic border. Placing the heart out of the chest and placing it back in the chest without ligation of the LAD was considered a sham surgery. The review board of the Institutional Animal Care and Use Committee at San Diego State University approved all animal protocols and studies.

Retroperfusion
Mice were sacrificed under chloral hydrate sedation before removing hearts from mice and as previously described. After retroperfusion, hearts were processed for paraffin embedding.

Immunohistochemistry
Heart sections were deparaffinized, and incubated with primary and secondary antibodies as previously described. Subsequent tyramide amplification was performed as necessary. Slides were incubated in DAPI (Sigma-Aldrich) for 10 minutes to stain for nuclei. Primary and secondary antibodies used are listed in the Online Table III.

Echocardiography and hemodynamics
Echocardiography was used to evaluate cardiac function after MI and injections using the Vevo 2100 (Visual Sonics) and as previously described. Closed-chest hemodynamic assessment was performed after insertion of a microtip pressure transducer (FT111B, Scisense) and as previously described. Cardiac function assessed by echocardiography 2 days post-infarction was not statistically different between infarcted/injected groups. The review board of the Institutional Animal Care and Use Committee at San Diego State University approved all animal protocols and studies.

Quantitation of c-kit cells, infarct size and cellular engraftment
Paraffin sections were probed with primary antibodies for proteins cardiac troponin T, c-kit, GFP and mcherry and visualized on a Leica TCS SP8 Confocal Microscope. Nuclei were visualized after DAPI staining. For infarct size, cTNT was probed to visualize live myocardium and DAPI to determine nuclei distribution and area of infarction. Area of live versus dead myocardium was measured using the drawing tool in the Leica Software and normalized to the total area of the left ventricular free wall and converted to percentage. In this area, c-kit+ cells were counted. For engraftment, area of mcherry (CPCs, CPCs in CPC + MSC group, CC1 and CC2) or GFP (MSCs alone and MSCs in CPC + MSC group) was measured and normalized to total area. 4 and 12 week sections had an N=3-4 hearts per group.

Isolectin staining and measurement of capillary dimensions
Paraffin sections were probed with Isolecin B4-488 (Life Technologies) in combination with cTNT and DAPI. Scans consisted of border zone and infarct regions for each heart analyzed. The analysis software on the Leica SP8, quantitated the number of positive cells in each field of view. The area of the field of view was measured and used to normalize capillary numbers per mm². An N=3-4 hearts per group was measured.

Measurement of cardiomyocyte hypertrophy
Paraffin sections were stained for cTNT to visualize live myocardium, wheat germ agglutinin-555 (Life Technologies) to outline cardiomyocyte membrane and DAPI to visualize nuclei and area of infarction. Myocytes were measured in the border zone of the infarct or in this infarct. Cross-sectional views of cardiomyocytes were considered and measured using the drawing tool to determine area using the SP8 TCS Leica Software. An N=3-4 hearts per group was measured.
**Masson’s Trichrome**
Trichrome (Masson) kit was used to stain for collagen deposition in infarcted hearts according to manufacturer’s protocol and based on previous reports. Staining was visualized using a Leica DMIL microscope.

**Statistical analyses**
All data are expressed as mean +/- SEM. Statistical analyses was done using paired or unpaired Student’s t-test, one-way ANOVA or two-way ANOVA with a Dunnett post-test to compare groups to a control group using Graph Pad Prism v5.0. A value of p<0.05 was considered statistically significant.
Online Figure I. Phenotypic Characterization of CardioChimera clones.

(A) Proliferation data for the 18 CardioChimera clones relative to day of plating using a direct-fluorescent based assay (CyQuant Assay). CardioChimeras are categorized as slow (blue), slow-medium (orange), medium-fast (red) and fast (green) growing. Experimental control groups (CPC, MSC and CPC + MSC) cell lines are represented as dashed bold lines. CardioChimera 1 and 2 are represented as solid bold lines.

(B) CardioChimera death after treatment with hydrogen peroxide stimulus. Values are represented as a fold change of Annexin V⁺ and Sytox Blue⁺ compared to cells in growth media alone.

(C) Neonatal rat cardiomyocytes incubated in high or low serum or with the addition of parent cells, parent cells combined or CardioChimeras.

(D) Cell death was quantitated by measuring a fold change of Annexin V⁺ and Sytox Blue⁺ cardiomyocytes relative to cardiomyocytes in high serum.

(E) Cardiomyocyte size was quantitated in high serum or with the addition of parent cells, parent cells combined or CardioChimeras.
Online Figure II. CardioChimeras have increased nuclear size and DNA content. (A) Detailed protocol for the fusion and clonal expansion of CardioChimeras. Briefly, mouse CPCs were co-incubated with mouse MSCs at a 1:1 ratio with addition of Sendai virus. Cells were centrifuged to force cell contact and single cell sorted based on fluorescent expression of mcherry and GFP. Clones were confirmed by flow cytometric analysis. (B) Measurement of nuclear size and (C) Centromere intensity in parent MSCs, CC2 and CC1. (D) Representative images of nuclei in parent (E) CC2 and (F) CC1. Blue represents DAPI staining of DNA content and red represents centromere probe binding. Scale bar is 20 µm. * p<0.05, ** p<0.01, *** p<0.001.
Online Figure III. CardioChimeras have increased expression of cardiomyogenic commitment markers at basal levels. (A) C-kit protein expression as analyzed by flow cytometry. C-kit purified bone marrow cells were utilized as a positive control. (B) connexin 43, (C) pecam (cd31), (D) sm22 and (E) cTNT (tnnt3) gene expression was analyzed by qRT-PCR in CPC, MSC, CPC + MSC, CC1 and CC2 after normalization to ribosomal 18s. Values are represented as a fold change relative to CPCs.
Online Figure IV. Cellular Engraftment of CardioChimeras 4 weeks after damage. (A) Infarct size was not significantly different between infarcted groups (mean=18.23%). N=2-4 per group. (B-E) Masson’s Trichrome staining and representative images of (B) PBS, (C) CPC, (D) CPC + MSC and (E) CC1 hearts to visualize scar size and fibrosis. Scale bar is 250µm. (F) Mcherry+ CPCs detected in the infarct area in CPC treated hearts. (G) Mcherry+ CPCs detected in the infarct area in CPC + MSC treated hearts. (H) Mcherry+ CPCs adjacent to c-kit+/cTNT+ cardiomyocytes in CPC + MSC treated hearts. (I) and (J) CC1 expressing eGFP and mcherry in the infarcted area. (K) 2x zoom of CC1. Scale bar is 25µm for confocal images. Scale bar is 50µm in (G).
Online Figure V. Cardiomyocyte size is unaffected in the border zone region after treatment. (A) Mean cardiomyocyte size in the border zone regions. Sample size is 3-4 mice per group. (B-G) Representative images of border zone area cardiomyocytes. Red=Wheat germ agglutinin, White=cardiac troponin T and Blue=DAPI to stain for nuclei. Scale bar is 25µm.* p<0.05, ** p<0.01, *** p<0.001.
Online Table I. Phenotypic characterization of the 18 CardioChimeras. Individual clones were analyzed for phenotypic properties such as proliferation, cell death and cell surface area and potential for paracrine mediated effects on cardiomyocytes (Cardiomyocyte growth and Cardiomyocyte Death). The last panel specifies exclusion reason(s).

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<th>Growth Rate</th>
<th>Proliferation</th>
<th>Cell Death</th>
<th>Cell Surface Area/Morphology</th>
<th>Cardiomyocyte Growth</th>
<th>Cardiomyocyte Death</th>
<th>Exclusion Reason</th>
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<td>MSC</td>
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<td>MSC</td>
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## Anterior Wall Thickness; Systole (mm)

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## Posterior Wall Thickness; Systole (mm)

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## Left Anterior Ventricle Volume (mL)

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<td>14.58</td>
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</tbody>
</table>

## Anterior Myocardial Blood Flow (mL/min)

<table>
<thead>
<tr>
<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
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<tbody>
<tr>
<td>80.65</td>
<td>2.12</td>
<td>79.27</td>
<td>1.43</td>
<td>79.27</td>
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<tr>
<td>83.83</td>
<td>3.04</td>
<td>30.89</td>
<td>13.31</td>
<td>30.89</td>
<td>13.31</td>
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<tr>
<td>32.71</td>
<td>3.60</td>
<td>29.91</td>
<td>27.93</td>
<td>29.91</td>
<td>27.93</td>
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## Left Ventricle Ejection Fraction (%)

<table>
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<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
<th>CPC</th>
<th>SEM</th>
<th>PBS</th>
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</thead>
<tbody>
<tr>
<td>42.89</td>
<td>2.31</td>
<td>25.71</td>
<td>3.04</td>
<td>25.71</td>
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<tr>
<td>44.23</td>
<td>2.03</td>
<td>45.15</td>
<td>4.39</td>
<td>45.15</td>
<td>4.39</td>
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<tr>
<td>39.77</td>
<td>4.39</td>
<td>40.19</td>
<td>2.32</td>
<td>40.19</td>
<td>2.32</td>
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</tbody>
</table>
Online Table II. Heart rate and echocardiographic data. Echocardiographic data represented as mean ± SEM. Heart rate, anterior wall thickness, posterior wall thickness, left ventricular volume, ejection fraction and fractional shortening were measured at specified times after MI. (N) indicates the number of mice used in each group at the given time point.
<table>
<thead>
<tr>
<th>Use: Flow Cytometry</th>
<th>Company</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>Tyramide Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-CD117 (c-kit)</td>
<td>R&amp;D Systems</td>
<td>1:40</td>
<td>Donkey anti-goat 647 (1:400)</td>
<td>N/A</td>
</tr>
<tr>
<td>Goat anti-IgG</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>1:40</td>
<td>Donkey anti-goat 647 (1:400)</td>
<td>N/A</td>
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<tr>
<td>Annexin V-APC</td>
<td>BD Biosciences</td>
<td>1:40</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Sytox Blue</td>
<td>Life Technologies</td>
<td>1:2000</td>
<td>N/A</td>
<td>N/A</td>
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<table>
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<th>Company</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>Tyramide Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-mcherry</td>
<td>Life Technologies</td>
<td>1:100</td>
<td>Donkey anti-goat 555 (1:100)</td>
<td>N/A</td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>Life Technologies</td>
<td>1:100</td>
<td>Donkey anti-goat 488 (1:100)</td>
<td>N/A</td>
</tr>
<tr>
<td>TO-PRO-3 iodide</td>
<td>Life Technologies</td>
<td>1:10000</td>
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<td>N/A</td>
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<table>
<thead>
<tr>
<th>Use: Immunohistochemistry</th>
<th>Company</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>Tyramide Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-CD117 (c-kit)</td>
<td>R&amp;D Systems</td>
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<td>Bovin anti-goat HRP (1:200)</td>
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</tr>
<tr>
<td>Rat anti-mcherry</td>
<td>Life Technologies</td>
<td>1:200</td>
<td>Donkey anti-rat biotin (1:4500); Streptavidin HRP (1:100)</td>
<td>Yes</td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>Life Technologies</td>
<td>1:200</td>
<td>Donkey anti-rabbit FITC (1:200); Sheep anti-FITC-HRP (1:400)</td>
<td>Yes</td>
</tr>
<tr>
<td>Mouse anti-cardiac troponin T</td>
<td>Thermo Scientific</td>
<td>1:100</td>
<td>Donkey anti-mouse 647 (1:100)</td>
<td>No</td>
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<tr>
<td>Isolectin-B4-488</td>
<td>Life Technologies</td>
<td>1:100</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>Life Technologies</td>
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</tbody>
</table>

**Online Table III. Antibody list.**
<table>
<thead>
<tr>
<th>mRNA Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac troponin T</td>
<td>5’- ACCCTCAGGCTCAGGTTCA-3’</td>
<td>5’- GTGTGCAGTCCCTGTTCAGA-3’</td>
</tr>
<tr>
<td>Connexin-43</td>
<td>5’- GGACCTTGTCCAGCTGTTCA-3’</td>
<td>5’- TCCAAGGAGTTCCAACACT-3’</td>
</tr>
<tr>
<td>Smooth muscle 22</td>
<td>5’-GACTGCACTTCTCGGCTCAT-3’</td>
<td>5’-CCGAAGCTACTCTCCATCCA-3’</td>
</tr>
<tr>
<td>Platelet endothelial cell adhesion molecule</td>
<td>5’- TGCTCTCGAAGCCCATGATT-3’</td>
<td>5’- TGTGAATGTTGCTGGTCAT-3’</td>
</tr>
<tr>
<td>p53</td>
<td>5’- GCAGGGCTCACTCCAGTACCT-3’</td>
<td>5’- GTCAGTCTGAGTCAACGCCAATACT-3’</td>
</tr>
<tr>
<td>p16</td>
<td>5’- CGTACCCCAGTCTGATG-3’</td>
<td>5’- CGGCGGGAGAGGTAGT-3’</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>5’- ATCCAGTTGCTTTTGACGACT-3’</td>
<td>5’- TAAGCCTCGACTTGTGAGTGT-3’</td>
</tr>
<tr>
<td>18s</td>
<td>5’- CGAGCCGCTGGATACC-3’</td>
<td>5’- CATGGCCTCAGTTCGAAAA-3’</td>
</tr>
</tbody>
</table>

Online Table IV. qRT-PCR primer list.
Supplemental References


