Bone-Derived Stem Cells Repair the Heart After Myocardial Infarction Through Transdifferentiation and Paracrine Signaling Mechanisms

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Rationale: Autologous bone marrow–derived or cardiac-derived stem cell therapy for heart disease has demonstrated safety and efficacy in clinical trials, but functional improvements have been limited. Finding the optimal stem cell type best suited for cardiac regeneration is the key toward improving clinical outcomes.

Objective: To determine the mechanism by which novel bone-derived stem cells support the injured heart.

Methods and Results: Cortical bone–derived stem cells (CBSCs) and cardiac-derived stem cells were isolated from enhanced green fluorescent protein (EGFP+) transgenic mice and were shown to express c-kit and Sca-1 as well as 8 paracrine factors involved in cardioprotection, angiogenesis, and stem cell function. Wild-type C57BL/6 mice underwent sham operation (n=21) or myocardial infarction with injection of CBSCs (n=67), cardiac-derived stem cells (n=36), or saline (n=60). Cardiac function was monitored using echocardiography. Only 2/8 paracrine factors were detected in EGFP+ CBSCs in vivo (basic fibroblast growth factor and vascular endothelial growth factor), and this expression was associated with increased neovascularization of the infarct border zone. CBSC therapy improved survival, cardiac function, regional strain, attenuated remodeling, and decreased infarct size relative to cardiac-derived stem cells– or saline-treated myocardial infarction controls. By 6 weeks, EGFP+ cardiomyocytes, vascular smooth muscle, and endothelial cells could be identified in CBSC-treated, but not in cardiac-derived stem cells–treated, animals. EGFP+ CBSC-derived isolated myocytes were smaller and more frequently mononucleated, but were functionally indistinguishable from EGFP− myocytes.

Conclusions: CBSCs improve survival, cardiac function, and attenuate remodeling through the following 2 mechanisms: (1) secretion of proangiogenic factors that stimulate endogenous neovascularization, and (2) differentiation into functional adult myocytes and vascular cells. (Circ. Res. 2013;113:539-552.)

Key Words: differentiation ■ myocardial infarction ■ neovascularization ■ paracrine communication ■ stem cells

Ischemic injury of the heart, including myocardial infarction (MI), is a major health problem that leads to structural and functional remodeling and often culminates in heart failure. Novel therapies to repair or replace damaged cardiac tissue are needed to improve the prognosis of patients with MI. Stem cell therapy has the potential to repair hearts after ischemic injury. A variety of adult stem cell types that might repair the injured heart have been tested in animal models. These studies have shown that transplantation of autologous cardiac- or bone marrow–derived stem cells induced pluripotent stem cells, and direct reprogramming of endogenous nonstem cells into cardiogenic phenotypes can improve cardiac function after injury. Early stage clinical trials have largely focused on autologous stem cells because of their ease of isolation and lack of immunogenicity. These trials have demonstrated the ability of both bone marrow– and cardiac-derived cells to offer moderate functional benefits when transplanted after cardiac injury. Although the outcomes of these trials continue to improve, the overall beneficial effects of autologous stem cell therapies are still relatively modest, and the fundamental mechanisms of stem cell–mediated repair are largely unknown and controversial.

In This Issue, see p 479
Editorial, see p 480

The mechanisms of stem cell–mediated cardiac repair are critical unanswered questions in the field. Many preclinical
studies in animal models have shown that differentiation of injected cells into new cardiac myocytes is one potential mechanism of this repair. Secretion of paracrine factors that enhance cardioprotection of the endogenous myocardium, neovascularization, and recruitment of endogenous stem cells that promote repair are other major mechanisms. The goal of the present study was to define the contributions of differentiation of transplanted bone–derived stem cells into new cardiac tissue (cardiac myocytes and blood vessels) versus stem cell–mediated induction of endogenous cardiac repair via secretion of paracrine factors. Well-characterized sources of stem cells shown to enhance cardiac repair of the diseased heart should lead to effective cell therapies. Certain stem cell types may have a greater capacity to transdifferentiate, whereas others may produce more paracrine factors and have a greater potential to stimulate neovascularization and other endogenous repair mechanisms. In this study, we examined the following 8 specific paracrine factors that have been previously suggested to play some role in stem cell–mediated repair of the heart: angiopoietin-1 (Ang-1), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), stem cell factor (SCF), stromal-derived factor-1 (SDF-1), and vascular endothelial growth factor (VEGF). HGF and IGF-1 are thought to be cardioprotective: HGF has cytoprotective, antiapoptotic, and proangiogenic effects, whereas IGF-1 can inhibit apoptosis and may stimulate growth and proliferation of stem cells. SCF and SDF-1 are thought to stimulate stem cell function: SCF, the ligand for the c-kit receptor, may stimulate stem cell homing, whereas SDF-1 is a chemotactic ligand that induces stem cell proliferation and homing to the site of injury. Ang-1, bFGF, PDGF, and VEGF all promote angiogenesis: Ang-1 induces vascular cell migration and enhances stability of newly formed vasculature. bFGF induces proliferation of endothelial and smooth muscle cells. PDGF stimulates smooth muscle cell proliferation, and VEGF induces endothelial cell proliferation and tube formation. Although a solid consensus about stem cell repair mechanisms is lacking, most cardiac stem cell researchers currently think that bone marrow–derived stem cells act primarily through paracrine stimulation of new blood vessel formation or through differentiation into vascular cells rather than transforming to myocytes, whereas cardiac-derived stem cells (CDCs) may differentiate into adult myocytes. Although some studies have demonstrated that bone marrow–derived stem cells can regenerate adult myocytes, others have suggested that these new myocytes are mostly formed through stimulation of endogenous cardiac stem cells rather than through direct differentiation of the transplanted cells. Our goal was to define whether and by what mechanisms, bone-derived stem cells induce repair of the injured heart. It has long been hypothesized that both the heart and bone marrow contain stem cell niches where cells with the capability to differentiate down the cardiac or mesenchymal lineage exist in a dedifferentiated state. In these environments, the pluripotent state of the stem cell is thought to be supported by complex signaling interactions with surrounding cells, such as those found in the stromal lining of the bone marrow cavity. Investigators looking for a source of multipotent stem cells developed a new isolation technique using cortical bone tissue, rather than the bone marrow, to isolate cells that might be in a more primitive state. These cells, which can be easily obtained through routine bone biopsy procedures, were negative for most markers of the hematopoietic lineage and expressed the pluripotency marker Sca-1. Under specific culture conditions, these cells differentiate in vitro into osteoblasts, chondrocytes, and adipocytes; however, no one has yet tested their cardiogenic potential in the injured heart. In this study, we report for the first time that injection of cortical bone–derived stem cells (CBSCs) into the heart after MI improved survival and cardiac function, and CBSCs demonstrated greater improvements across all parameters compared with the more widely studied CDCs. CBSCs differentiated into mature cardiac tissue, whereas CDCs did not, and CBSCs demonstrated a greater capacity for paracrine-mediated endogenous repair to produce these effects. Our study suggests that CBSCs are a source of stem cells that are more abundant and more easily isolated than CDCs, and that CBSCs have a greater capacity to repair hearts damaged by ischemic injury.

Methods
Please refer to the Supplemental Methods section for more detailed experimental methods.

Results
Cortical Bone Stem Cell Characterization
CBSCs or CDCs were analyzed for expression of c-kit and Sca-1 mRNA abundance using quantitative real-time polymerase chain reaction (Online Figure I). C-kit and Sca-1 protein expression was detected using both immunostaining (Online Figure II) and flow cytometry (Online Figure III). CBSCs expressed greater levels of both transcripts than did CDCs: more than 3-fold higher levels of c-kit and 2-fold higher levels of Sca-1 (Online Figure I). Both stem cells types demonstrated positive membrane immunostaining for c-kit and Sca-1 (Online Figure II). Flow cytometry analysis demonstrated that the majority of CBSCs and CDCs expressed c-kit (CD117), Sca-1, and β1-Integrin (CD29). Additionally both cell types lacked expression of
the hematopoietic stem cell marker CD34, the common leukocyte antigen CD45, and other common markers of the hematopoietic lineage that can be detected by a cocktail of antibodies against CD5, CD11b, CD45R, antigen 7/4, Gr-1, Ly6G/C, and Terr-119 (Online Figure III).

Next, we studied whether the stem cells, cultured in vitro, expressed or secreted paracrine factors that are thought to be involved in cardioprotection, neovascularization, or recruitment of endogenous cardiac stem cells. The following 8 specific factors produced by CBSCs and CDCs were analyzed: Ang-1, bFGF, HGF, IGF-1, PDGF, SCF, SDF-1, and VEGF. Protein expression of all 8 factors was detected by Western analysis performed on CBSC or CDC lysates collected in vitro (Figure 1A). Positive expression of these factors in vitro was confirmed by immunostaining (Figure 1B). ELISA of stem cell–conditioned media demonstrated that IGF-1, VEGF, and SDF-1 were all secreted by proliferating CBSC and CDCs in culture (Figure 1C), and no significant difference between the amount of paracrine factors secreted by each cell type could be detected. Neither HGF nor SCF was secreted in detectable amounts by either stem cell type, although both factors were seen at the protein level by both Western analysis and immunostaining. These results show that both CBSCs and CDCs produce factors known to be associated with beneficial cardiac remodeling after MI.

**CBSCs Differentiate In Vitro in Coculture With Neonatal Rat Ventricular Myocytes**

Both bone marrow–derived and CDCs have previously been shown to differentiate in vitro in coculture with neonatal rat ventricular myocytes. To quantify the rate of in vitro differentiation into cells expressing cardiac myocyte proteins, CBSCs cocultured with neonatal rat ventricular myocytes were fixed and stained for α-sarcomeric actin (Online Figure IVA and IVB) or connexin43 (Online Figure IVC and IVD). Staining revealed that 11.5±0.9% (n=5) of enhanced green fluorescent protein positive (EGFP+) cells expressed α-sarcomeric actin and 7.5±1.7% (n=5) appeared coupled via

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**Figure 1. In vitro characterization of stem cells.** A, Cortical bone–derived stem cell (CBSC) or cardiac-derived stem cells (CDC) lysates were analyzed by Western analysis. Positive controls include mouse endothelial fibroblasts (MEF), liver, bone marrow (BM), and B lymphocytes (B cell). Myocyte (MYO) lysates were used as negative controls for all samples. B, CBSCs (green) were fixed in vitro and immunostained against each paracrine factor (red). Nuclei are labeled with 4',6-diamidino-2-phenylindole (blue) and scale bars, 20 μm. C, CBSCs or CDCs were allowed to proliferate for 72 hours, and their culture media was analyzed by ELISA for the presence of soluble hepatocyte growth factor (HGF), insulin-like growth factor (IGF), stem cell factor (SCF), stromal-derived factor-1 (SDF-1), and vascular endothelial growth factor (VEGF). Samples were analyzed in triplicate, and background signal was subtracted using unconditioned media blanks. Ang-1 indicates angiopoietin-1; bFGF, basic fibroblast growth factor; GADPH, glyceraldehyde 3-phosphate dehydrogenase; and PDGF, platelet-derived growth factor. NS, no significant difference (P>0.05).
connexin43 gap junctions to their neighboring cells. A very small fraction of EGFP+ cells (<1%) were observed to contract spontaneously after 72 hours in coculture. Thus, like bone marrow–derived or CDCs, CBSCs, in vitro, demonstrate some capacity to differentiate into cells expressing cardiac specific proteins in vitro.

CBSC Transplants Improved Survival and Cardiac Function, Attenuated Adverse Left Ventricular Remodeling, and Reduced Infarct Size

The effects of CBSCs versus CDCs on post-MI structural and functional remodeling were studied. The MI procedure reduced 6-week survival to 50.4% in animals receiving sham saline injections. Animals receiving MI and CBSC therapy demonstrated a 76.5% 6-week survival, which was greater than CDC-treated animals and significantly greater than the saline-treated controls (Figure 2). Animals receiving CDC therapy did demonstrate an improvement in survival relative to saline-treated MI controls (66.4%), but this improvement was not statistically significant. Both stem cell therapies improved cardiac function and attenuated adverse cardiac remodeling characteristic of MIs in this model system (Figure 3), and these changes were most pronounced in the MI+CBSC group. Animals receiving MI+CBSC had significantly improved ejection fraction and fractional shortening (FS) compared with the MI+saline control animals as early as 1 week post-MI, and these changes were sustained through 6 weeks post-MI. Improvements in ejection fraction and FS were greater in CBSC-treated animals versus CDC-treated animals. Both stem cell treatment groups had an initial decline in stroke volume,
but stroke volume returned to normal by 2 weeks post-MI and remained significantly improved relative to MI + saline control animals. Animals in the MI + saline group demonstrated significant increases in end-diastolic and systolic volumes with thinning of the infarct-affected anterior wall (Figure 3). By contrast, MI animals treated with CBSCs or CDCs had significantly smaller post-MI diastolic and systolic volumes at all time points studied, and they demonstrated attenuated thinning of the anterior wall relative to saline-injected controls, with CBSC-treated animals demonstrating the most profound attenuation across all parameters (Figure 3).

Five mice were randomly selected from each group (MI + Saline, MI + CDC, or MI + CBSC) to undergo acute infarct size analysis at 24 hours post-MI, and no significant difference between the area at risk or ischemic area was detected between animals in any group (Online Figure V). These results demonstrate that acute infarct size was similar in all study animals. Therefore, the structural and functional improvements in the MI + CBSC and MI + CDC groups can be attributed to the effects of cell transplantation alone and are not the result of a stem cell–mediated reduction in initial infarct size. After 6 weeks post-MI, chronic infarct size was analyzed by measuring the area of infarcted tissues relative to total myocardial area on hematoxylin and eosin–stained cross-sections from saline-, CDC-, or CBSC-injected hearts. Animals receiving either CDC or CBSC therapy had significantly smaller infarcted areas relative to saline-treated controls, with CBSC-treated animals demonstrating the most significantly reduced infarct sizes, suggesting that infarct size or expansion was reduced 6 weeks after CBSC therapy.

**Contractile Myocardium Was Detected at CBSC Injection Sites**

Strain analysis was performed on long-axis B-mode images to determine whether regions injected with CBSCs developed contractile activity. Figure 4A shows representative 3-dimensional

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Left ventricular (LV) endomyocardial strain. **A**, Three-dimensional regional wall velocity diagrams showing contraction (orange/positive values) or relaxation (blue/negative values) of 3 consecutive cardiac cycles. **B**, Vector diagrams showing the direction and magnitude of endocardial contraction at midsystole. **C**, Global averages of strain and strain rate measured in the radial or longitudinal axes across the LV endocardium. AW indicates anterior wall; CBSC, cortical bone–derived stem cell; CDC, cardiac-derived stem cells; MI, myocardial infarction; and PW, posterior wall. */ **/**/** (**blue)**, CBSC vs saline; */ **/**/** (**red)**, CBSC vs CDC.
(3D) wall velocity diagrams for 3 consecutive cardiac cycles taken from animals at baseline, 6 weeks post-MI+Saline, and 6 weeks post-MI+CBSC. Online Figure VI outlines how 3D wall velocity diagrams are constructed relative to the acquired parasternal long-axis B-mode echocardiogram of the left ventricle (LV). Points along the LV endocardial surface are plotted along the x-axis, from the base of the posterior wall to the apex to the base of the anterior wall. At baseline, all hearts demonstrated uniform and synchronous contraction and relaxation across the LV endocardium (Figure 4A). In the MI+saline control group after 6 weeks, there was a dramatic reduction in wall velocity across the endocardium of the infarct-related anterior wall (Figure 4A). CDC-treated animals demonstrated some improvements in wall velocity at the infarct border zone, whereas CBSC-treated MI animals demonstrated even greater improvements in wall velocity at the infarct border zone segments where CBSCs were injected (Figure 4A).

Figure 4B shows vector diagrams taken from long-axis B-mode echocardiograms, and Online Videos I through IV show live video loops of 3 consecutive cardiac cycles from each heart. The normal hearts demonstrate uniform, synchronous contractions, and relaxations throughout the cardiac cycle (Online Movie I). Animals with MI injury showed pronounced LV chamber dilation, wall thinning, and hypokinesis of the infarcted wall, as evidenced by the lack of vector activity in this region (Online Movie IV). In animals receiving stem cell therapy, the LV chamber was less dilated, with attenuated wall thinning and increased contractile activity in the border zone segments that received cell therapy. Although CDC-treated animals did show some improvements in wall velocity and strain at the border zone segments (Online Movie III), CBSC-treated animals had much greater wall velocities and contraction vectors nearly equal in magnitude to baseline control hearts at the infarct border zone segments where CBSCs were injected (Online Movie II).

Global averages of LV endocardial strain and strain rate are plotted in Figure 4C. Strain, which measures change in length relative to the initial length (strain=final length [L]/initial length [L₀]), was measured either in the radial (from the center of the ventricle cavity outward) or longitudinal axis (from the apex to the base). The rate of change in strain (strain rate=strain/time) was also calculated. In both axes, strain and strain rate were significantly reduced after MI. Significant improvements in both strain and strain rate in both the radial and longitudinal axes were observed in MI animals receiving CBSC or CDC therapy relative to MI+saline controls, and CBSC-treated animals showed improved strain relative to CDC animals across all parameters at all time points. These data demonstrate that CBSC-treated hearts had significantly improved global and regional contractility by 6 weeks post-MI.

Expression of Proangiogenic Paracrine Factors In Vivo by Stem Cells Induces New Blood Vessel Formation

The environment in the infarct border zone is dramatically different from that in the tissue culture dish. Therefore, paracrine factor expression and production are likely to change after transplantation of CBSCs into the infarcted heart. At 24 hours post-MI, hearts receiving CBSC transplants were immunostained for the 8 paracrine factors studied in vitro. Injection sites containing EGFP+ CBSCs were identified and found to express bFGF and VEGF, but Ang-1, IGF-1, HGF, or PDGF were not detected at any injection sites examined (Figure 5). Injection sites containing EGFP+ CDCs were found to express Ang-1 in addition to bFGF and VEGF, but we could not detect the 5 other factors. These studies suggest that, when injected into the MI border zone, CBSCs express only bFGF and VEGF (and CDCs express only Ang-1, bFGF, and VEGF). Despite expressing an additional paracrine factor after 24 hours, expression of all paracrine factors by CDCs was not sustained (Online Table II). No amounts of Ang-1, bFGF, or VEGF could be detected in any CDC injection sites after 24 hours post-MI (data not shown).

VEGF expression was detected in CBSC injection sites as late as 2 weeks post-MI (Online Figure VII). The fluorescence intensity tracings of each color channel (green: EGFP, red: VEGF, blue: 4',6-diamidino-2-phenylindole) across a confocal line scan of the cell selected in Online Figure VIIIA demonstrates the colocalization of the VEGF and EGFP signals (Online Figure VIIIB). A scatterplot of pixel intensities (Online Figure VIIC) in the red channel versus the green channel was generated, and the diagonal deflection documents colocalized red/green pixels (56.2% of green and red pixels in this line scan were colocalized). A control scatterplot of the blue (4',6-diamidino-2-phenylindole) versus green channel (EGFP) shows only 13.6% of blue/green pixels were colocalized (Online Figure VIIID). Paracrine factors involved in cardioprotection or homing and recruitment of endogenous stem cells (HGF, IGF-1, SCF, and SDF-1) were not detected at any time point in vivo. An overview of all in vitro and in vivo paracrine factor analyses for CBSCs can be found in Online Table I, while a summary of CDC paracrine factor analyses is shown in Online Table II.

To determine whether the in vivo expression of the CBSC-secreted proangiogenic factors bFGF and VEGF (and Ang-1 secreted by CDCs) resulted in increased neovascularization, blood vessel density was measured in the infarct zones from MI animals treated with saline, CDCs, or CBSCs (Online Figure VIII). MI animals receiving CBSC treatment had 15.13±3.04 von Willebrand factor–positive blood vessels per field, whereas saline-treated controls had only 4.95±1.30 von Willebrand factor–positive vessels per field. These data support the idea that secretion of proangiogenic paracrine factors in vivo by both stem cell types, and especially by CBSCs, induced increased neovascularization. Increased blood supply to the injured heart could have contributed to the functional benefits we observed after CBSC transplant. The majority of these blood vessels did not contain EGFP+ cells, suggesting that they were derived via endogenous repair rather than from the injected cells.

EGFP+ CBSCs Expand Over Time as Cells Proliferate and CBSCs Transdifferentiate Into 5 Distinct Phenotypes

CBSC injection sites were examined 1, 2, and 6 weeks after MI (Figure 6). Injection sites were identified after immunostaining against α-sarcomeric actin (red) and EGFP (green).
Histological samples from CBSC- and saline-injected hearts were stained simultaneously, and the absence of EGFP signal in the saline-injected controls was confirmed, documenting a very low level of autofluorescence under the conditions used. All tissue sections were first examined under low magnification to identify injection sites, and then high magnification confocal images were analyzed. At 1 week post-MI, the majority of EGFP+ cells were found in discrete groups of small, round, poorly differentiated cells. By 2 weeks post-MI, the area of the heart with EGFP+ cells had expanded, and the majority of these EGFP+ cells were enlarged and had an elongated appearance. By 6 weeks post-MI, injected stem cells had spread throughout the infarct zone and into the infarct border zone and discrete injection sites were no longer visible. Six weeks after injection, the EGFP+ stem cells were spread throughout the border zone and infarct zone.

Time-related changes in CBSC phenotype were studied. After 1 week and most EGFP+ cells were still small (10–20 μm) and round in shape (similar to the morphology of the injected cells), but the majority had begun to express unorganized cytosolic α-sarcomeric actin (Figure 7A). A small number of cells that were in close contact with viable endogenous myocytes began to elongate and align in the axis of contraction, as defined by the sarcomeric organization of surviving myocytes in the region. By 2 weeks post-MI, as the injection sites had begun to expand, the majority of cells were elongated, aligned along the axis of contraction, and were expressing additional cytosolic α-sarcomeric actin (Figure 7B). At 2 weeks post-MI, EGFP+ cells that had engrafted closest to viable endogenous myocytes appeared the largest and most mature, with even greater amounts of α-sarcomeric actin visible in their cytoplasm. Although actin expression seemed to be increased by 2 weeks post-MI, it was still relatively unorganized, without the characteristic sarcomeric striations of adult myocytes. Individual color channel images for Figure 7A and 7B and staining controls for 1 and 2 weeks post-MI+saline are shown in Online Figure IX.

By 6 weeks Post-MI, newly formed EGFP+ myocardium was visible at the border zone, and the following 5 distinct EGFP+ stem cell phenotypes were identified: (1) cardiac myocytes with α-sarcomeric actin in organized sarcomeres were found to be coupled to neighboring cells via connexin43+ gap junctions (Figure 7C), (2) cells that express unorganized α-sarcomeric actin (Figure 7C), (3) vascular smooth muscle cells that stain positive for α-smooth muscle actin (Figure 7D), (4) vascular endothelial cells that stain positive for von Willebrand factor (Figure 7D), and (5) EGFP+ stem cells that stain negative for HGF, IGF-1, PDGF, SCF, and SDF-1 (Figure 7D). EGFP+ stem cells that stain positive for bFGF, Ang-1, and VEGF (shown in red) but negative for HGF, IGF-1, PDGF, SCF, and SDF-1. Nuclei are labeled with DAPI (blue), and injected CBSCs are green. Ang-1 indicates angiopoietin-1; bFGF, basic fibroblast growth factor; CBSC, cortical bone–derived stem cell; CDC, cardiac-derived stem cell; DAPI, 4',6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; MI, myocardial infarction; PDGF, platelet-derived growth factor; SCF, stem cell factor; SDF-1, stromal-derived factor-1; and VEGF, vascular endothelial growth factor.
cells that are smaller (<20 μm) and lack any expression of adult cardiomyocyte or vascular cell proteins (Figure 7D). Online Figure X shows an additional low-magnification image of the CBSC-treated border zone at 6 weeks post-MI in which a newly formed region of EGFP+ myocardium can be seen adjacent to a region EGFP− endogenous myocardium. Online Figure XI shows a low magnification view of the blood vessel shown in Figure 7D, in which the EGFP+ vessel is clearly surrounded by regions of EGFP− myocardium. Individual confocal color channel images and staining controls for Figure 7C are shown in Online Figure XII. Individual color channel images and staining controls for Figure 7D are shown in Online Figure XIII. These results support the idea that CBSCs can differentiate into the major cell types of the adult heart.

Cardiac-Derived Stem Cells Expand and Proliferate Over Time But Adopt a Less Mature Adult Myocyte Phenotype

Online Figure XIV shows images from the hearts of CDC-treated MI animals studied at 1, 2, and 6 weeks post-MI. EGFP+ CDC injection sites were identified and stained for α-sarcomeric actin (red) in Online Figure XIV A, or for α-sarcomeric actin (white) and connexin43 (red) in Online Figure XIV B. Similar to what we observed with CBSCs, by 1 week post-MI CDCs initially appear small (10–20 μm) and round, and they had...
begun to express unorganized \(\alpha\)-sarcomeric actin. By 2 weeks, the cells have begun to align and elongate like CBSCs, and they continue to express unorganized \(\alpha\)-sarcomeric actin. By 6 weeks post-MI, however, no EGFP+ cells with organized sarcomeres were found. Cells continue to grow larger and are still aligned, and some had even begun to express connexin43 gap junctions (Online Figure S-XIVB, right panel), but cytosolic \(\alpha\)-sarcomeric actin was not yet striated and mature gap junctional plaques were not identified. These data suggest that under our conditions, CDCs adopted a less mature cardiomyocyte phenotype during the 6-week course of this study.

CBSC-Derived EGFP+ Myocytes Isolated 6 Weeks After Injection Demonstrated Mature Contractile Properties

Some animals that received MI and CBSC therapy were euthanized 6 weeks after injury and myocytes from their left ventricles were isolated and studied in vitro. Isolated myocytes were also immunostained for \(\alpha\)-sarcomeric actin (red), EGFP (green), and nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI; blue) to analyze myocyte size and the number of nuclei per cell (Figure 8). Of all the myocytes in the left ventricle, an estimated 0.84% expressed EGFP, suggesting...
that these cells had been derived from transplanted CBSCs. To estimate how many new myocytes were formed from EGFP+ CBSCs (assuming that 0.84% of isolated myocytes were EGFP+), the total number and volume of myocytes in the heart was approximated. Our laboratory has previously estimated the total number of ventricular myocytes in the feline heart by measuring the average volume of the heart and the average volume of a myocyte. The mean weight of the mouse ventricles at 6 weeks post-MI+CBSC=205.3±10.8 mg, and the average volume of a mouse myocyte can be approximated using average myocyte dimensions (100×20×10 μm=20000 μm³=2×10⁻⁸ mL). If the density of myocardial tissues=1.06 mg/mm³, then the average volume of hearts at 6 weeks post-MI+CBSC=205.3/1.06=193.7 mm³=0.1937 mL. Myocytes are known to make up ≈80% of the heart by volume, so the volume of the heart constituting myocytes=0.8×0.1937=0.1550 mL. Thus, the total number of new EGFP+ myocytes in the average mouse heart at 6 weeks post-MI+CBSC=0.1550 mL/2×10⁻⁸ mL=7.75×10⁶ total myocytes/heart. If 0.84% of myocytes analyzed were positive for EGFP, then 0.0084×7.75×10⁶=65100 new EGFP+ myocytes were formed per heart. At the time of surgery, 40000 cells were initially injected. To form this number of new myocytes, the injected CBSCs must have proliferated and committed to the cardiac lineage. As shown in Figure 7, only a fraction of the EGFP+/α-sarcomeric actin+ cells were striated.

Figure 8. Isolated myocyte size, number of nuclei, and cell physiology were analyzed 6 weeks post-MI+CBSC. A, Myocytes were immunostained against α-sarcomeric actin (red), EGFP (green), and DAPI (blue). B, Average surface area of EGFP+ vs EGFP− myocytes and percentage of total EGFP+ or EGFP− cells that were mono-, bi-, or tetranucleated. C, Representative fractional shortening and Ca²⁺ transients of EGFP+ myocytes with 1 or 2 nuclei, or EGFP− myocytes with 1, 2, or 4 nuclei. D, Mean fractional shortening, peak Ca²⁺ F/F₀, and the time constant of decay (τ) of Ca²⁺ transients for all EGFP+ vs EGFP− myocytes. DAPI indicates 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; and MI, myocardial infarction.
In Online Figure SXV, we measured the relative numbers of striated (mature) and nonstriated/unorganized (less mature) EGFP+α-actin+ cells in 6 weeks post-MI CBSC hearts. This histological analysis showed that there were approximately equal numbers of striated and nonstriated EGFP+α-actin+ new myocytes. Collectively, these analyses suggest that the number of CBSC-derived new myocytes (about 130,000) is ≈3x greater than the number of cells originally injected (40,000).

EGFP+ isolated myocytes were identified, and the size and number of nuclei per cell were analyzed and compared with EGFP− myocytes isolated from the same hearts. Figure 8A shows a representative EGFP+ myocyte with 2 nuclei. When compared with EGFP− controls, EGFP+ myocytes had smaller average cross-sectional surface area (Figure 8B). More than 90% of EGFP− myocytes were binucleated, whereas only 5.4% of EGFP− cells were mononucleated, and 4.1% were tetraneucleated. In contrast, there were significantly more mononucleated and significantly fewer binucleated EGFP+ myocytes, with 1/3 EGFP+ myocytes having only 1 nucleus (Figure 8B). There was no significant difference in tetraneucleation between EGFP+ and EGFP− myocytes.

Isolated EGFP+ and EGFP− myocyte FS and Ca2+ transients were measured. Figure 8C shows representative EGFP+ and EGFP− myocytes under fluorescence or bright field histology with their corresponding FS or Ca2+ transients. Mean FS, peak Ca2+ (F/F0), and time constant of decay of the Ca2+ transients were measured (Figure 8D). EGFP+ myocytes isolated 6 weeks after MI had contractions and Ca2+ transients that were indistinguishable from EGFP− controls, documenting that they had assumed a mature adult phenotype.

Discussion
This study explored the potential beneficial effects of transplanting a novel population of c-kit+/Sca-1+ cells from the stem cell niches within the bone, rather than from bone marrow, into the border zone of a myocardial infarction, and the effects of this novel cell type were compared with a widely studied population of c-kit+/Sca-1+ CDCs. Our results show that CBSCs have beneficial effects on the structure and function of the heart after MI. Animals with MI that received a CBSC transplant had improved 6-week survival, cardiac function, and attenuation of adverse left ventricular remodeling compared with saline-injected MI controls and CDC-treated animals. Strain analysis demonstrated that CBSC-treated animals not only had improved global function, but their hearts also had greater contractile function at the MI border zone relative to saline- and CDC-injected hearts. Strain analysis allows for precise imaging at the infarct border zone, where stem cells were specifically administered to achieve optimal effect.22 At border zone segments where new myocardium was detected using histological techniques, strain analysis of CBSC-treated mice showed greater contraction (Figure 4A-B), suggesting that the stem cells enhanced contractile function in the region where they were injected. Histological analysis showed that, whereas CDCs adopted less mature adult phenotypes, EGFP+ stem cell–derived adult cardiac myocytes with normally striated α-sarcomeric actin networks and connexin43+ gap junctions were present throughout the border zone in CBSC-treated mice (Figure 7C). CBSC-derived new cardiac myocytes that were isolated from hearts 6 weeks after MI had normal contractions and Ca2+ transients. These findings support the idea that newly formed cardiac muscle was derived from injected CBSCs, and these cells make a contribution to the improved contractile function of the injured heart.

CBSCs Secrete Proangiogenic Factors and Promote Neovascularization
A major goal of the present study was to explore paracrine effects of injected CBSCs. Many studies have looked at stem cell paracrine factors in vitro and drawn conclusions without studying the changes in expression of these factors after injection into the heart.21 Other groups have looked at changes in paracrine factor expression by stem cells after injection, but they have either studied total myocardial expression of these factors43 (rather than expression specifically by stem cells) or they have only examined specific stem cell expression of a single factor.26 Ours is the first study to both establish in vitro expression of multiple paracrine factors and examine the temporal changes in expression of all of these factors specifically by the stem cells after transplantation.21–24,27,28,30,44 We specifically explored neovascularization and the presence of paracrine factors in regions with injected CBSCs. Our studies showed that CBSCs produced the proangiogenic factors bFGF and VEGF (both in vitro and in vivo). These factors are known to be involved in vascular cell proliferation and induction of angiogenesis,22–25,28–30 and there was evidence of increased neovascularization in the infarct zone of hearts treated with stem cells in this study (Figure S-IV). Although CDCs initially produced Ang-1 in addition to bFGF and VEGF, these factors were not observed past 24 hours post-MI, and the neovascularization of the border zone in CDC-treated animals was less robust than CBSC-treated animals by 6 weeks post-MI. Additionally, our studies showed that, whereas CDCs did not transdifferentiate into mature cardiac myocytes or blood vessels by 6 weeks post-MI, CBSCs transdifferentiated into adult vascular cells, including smooth muscle and endothelial cells, although this was observed relatively infrequently. Although there was strong evidence of myocyte transdifferentiation (EGFP+ cardiac myocytes were identified in 5/8 hearts analyzed 6 weeks post-MI), and many cells from hearts at earlier time points could be identified in intermediate stages of myocyte differentiation (in 6/6 hearts analyzed after 1 week post-MI+CBSC and in 6/6 analyzed fixed at 2 weeks post-MI+CBSC), the same could not be said for cells of the vascular lineage (EGFP+ vascular cells were only identified in 2/8 hearts analyzed at 6 weeks post-MI, and no intermediate EGFP+ lumen or tube-like structures were found at 1 or 2 weeks post-MI). These findings suggest that paracrine-induced new blood vessel formation via endogenous repair seems to be the primary mechanism for the enhanced angiogenesis observed in CBSC-injected hearts. The in vivo paracrine factor analysis showed those factors involved in cardioprotection (HGF, IGF) or stimulation of endogenous stem cells (SCF, SDF–1) that were expressed in CBSCs in vitro were not found in CBSCs after they were injected into the MI border zone. These results suggest that part of the beneficial
CBSCs Differentiate Into New Cardiac Myocytes
In the present study, we used CBSCs and CDCs from an EGFP+ mouse so that we could easily trace the fate of the injected cells. Using time course in vivo histological analysis with this stably expressed fluorophore, we were able to demonstrate that CBSCs differentiated over time down the myocyte lineage by 6 weeks post-MI, whereas CDCs did not adopt a fully mature cardiac myocyte phenotype. As discussed above, we found evidence for enhanced revascularization of the MI border and infarct zone in CBSC-injected hearts. However, the new blood vessels rarely comprised EGFP+ cells, suggesting that CBSCs enhanced endogenous repair. Our experiments demonstrate that EGFP+ CBSCs express cardiac proteins soon after injection into the MI heart, and by 6 weeks post-MI, these cells have organized sarcomeres, are connected to their neighbors via gap junctions, and contract with Ca2+ transients that are similar to those of EGFP− myocytes isolated from the same hearts.

Our studies suggest that the transition from an injected CBSC to a fully functional cardiac myocyte takes between 2 and 6 weeks under our conditions. After 1 week, most the EGFP+ cells had immature characteristics and expressed unorganized cardiac contractile proteins. Two weeks after MI, areas with EGFP+ cells were larger, suggesting cell proliferation, and the cells had begun to elongate along the axis of EGFP− myocytes that had survived the infarct. More EGFP+ cells expressed cardiac contractile proteins but no organized sarcomeres were found. By 6 weeks post-MI, there were many regions containing EGFP+ myocytes with organized sarcomeres, and these cells were well integrated in the myocardium via gap junctions. The contribution of these new myocytes to the improved cardiac contractile performance of the post-MI heart cannot be proven with the techniques used in these experiments. However, regional strain measurements from sites of CBSC injection documented increased contractile performance in the regions where we isolated EGFP+ myocytes with robust contractile activity. These findings support the idea that CBSC-mediated new myocyte formation is at least partially responsible for improvements in cardiac structure and function in MI hearts with CBSC treatments.

Our major evidence for CBSC-derived new myocyte formation is that we found EGFP+ cardiac myocytes in the MI border zone of hearts 6 weeks after CBSC injections. There is always concern that injected cells might have fused with existing myocytes, but little or no evidence for fusion in these types of experiments has been observed, and complex studies using multiple stem cell labeling strategies have largely contradicted the fusion hypothesis. We also have evidence that the EGFP+ myocytes we found were newly formed from smaller CBSC progeny. Our experiments showed that EGFP+ isolated myocytes were smaller, and a higher percentage of these cells were mononucleated, consistent with a maturing adult cardiac myocyte. We estimated the number of new, CBSC-derived EGFP+ myocytes by isolating myocytes from hearts 6 weeks after CBSC injection into the MI border zone. We estimated that the number of new myocytes significantly exceeds the number of injected CBSCs, consistent with proliferation of either CBSCs or newly formed myocyte progeny. Importantly, our approach may have significantly underestimated the number of new myocytes because it is more difficult to isolate viable myocytes from infarct zones with fibrotic regions than from undamaged regions of the same heart. The present data are also consistent with previously published reports from our laboratory in which newly formed myocytes were detected in the growing heart during adolescence. These results are also similar to observations made in several studies by the Anversa laboratory, in which newly formed isolated myocytes were observed to be smaller than spared endogenous myocytes that lacked the stem cell marker. Collectively, our results suggest that CBSCs survive in the MI border zone and within weeks they begin to form new cardiac tissue. The survival and differentiation of CBSCs was associated with improved cardiac structure and function and enhanced survival.

Other CBSC Cardioprotective Effects
Our experiments show improved cardiac structure and function in the first 2 weeks after MI, before injected cells had enhanced vascularity and local contractility. The mechanisms of these early beneficial effects are not clear. One possibility is that injected cells thicken and stabilize the infarcted wall, thereby reducing wall stress and slowing dilation. These results are consistent with early stage clinical trials with wall stabilizing agents. The cardiac functional data of MI animals treated with CDCs and CBSCs also provide some insight into the wall stabilizing effect of cell therapy. By 1 week post-MI, both the MI+CDC and MI+CBSC groups had similar cardiac functional parameters (ejection fraction and FS). However, with time the functional improvements in the MI+CBSC group were sustained, whereas the function in MI+CDC animals continued to decline. The initial functional benefit may be attributed to a wall stabilizing effect that would likely be the same between both cell types, whereas the sustained functional improvement seen only in the MI+CBSC group could be the result of enhanced paracrine-mediated revascularization and maturation of CBSCs into new cardiac tissue.

CBSCs Versus CDCs and Other Stem Cell Types
Overall our data suggest that a bone-derived c-kit+/Sca-1+ stem cell population can support the injured heart through direct transdifferentiation into adult cardiomyocytes and vascular cells as well as through secretion of proangiogenic paracrine factors. CBSCs proved somewhat more effective at repairing the injured heart than CDCs, in part because CBSCs showed the capacity to transdifferentiate and form cells with a mature adult phenotypes in this animal model. Although we did not observe full differentiation of CDCs within the 6-week time frame of this study, it is possible that they could differentiate if allowed more time (their phenotypic changes during 6 weeks did mimic those of CBSCs in the first 2 weeks post-MI). Difficulty of maintaining the undifferentiated state of CDCs in vitro during cell expansion also likely contributed to the less robust cardiogenic potential of the CDCs used in the present experiments. We think that cortical bone provides an easy-to-access, more abundant source of stem cells that are potentially more pluripotent than has previously been isolated from the bone marrow. Additionally, these stem cells can be
isolated from cortical bone in high numbers without the need for arduous sorting processes, such as fluorescence-activated cell sorting or magnetic bead sorting that are required to isolate the relatively rare populations of c-kit+ cells from the bone marrow or myocardium. We are cautious when making comparisons with previous studies with other cell types because the effectiveness of different cell therapies is likely to be influenced by a host of factors, including the isolation approach, methods of cell expansion and purification, and the animal model in which the cells are tested.

Previously, there has been much skepticism that cells from outside the heart are capable of cardiomyogenesis, and many researchers have argued that new myocytes in stem cell–treated hearts are derived from paracrine stimulation of endogenous stem cells. Our study shows that bone-derived stem cells can directly form new myocytes, independently of endogenous CDCs, and these new myocytes can be isolated and their contractile properties and Ca2+ transients are indistinguishable from endogenous myocytes. Transplanted CDCs in our model failed to produce myocytes with a mature adult phenotype. Additionally, our stem cells have shown a wide potential to support the injured heart through several mechanisms without the need of modifications that have been used in other studies.

In summary, our studies show that CBSCs can survive the hostile environment of the post-MI heart without modification, secrete factors that enhance endogenous angiogenesis-mediated repair, and differentiate into new cardiac tissue. These beneficial effects culminate in a heart with less structural remodeling and improved cardiac pump function.

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Disclosures
None.

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Bone-Derived Stem Cells Repair the Heart After Myocardial Infarction Through Transdifferentiation and Paracrine Signaling Mechanisms

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Supplemental Material

Materials and Methods

Animals and Anesthesia

All animal procedures were approved by the Temple University School of Medicine Institutional Animal Care and Use Committee. For all procedures (bone isolation, myocardial infarction, echocardiography and cardiectomy) anesthesia was induced using 3% isoflurane and maintained using 1% isoflurane (Butler Shein Animal Health; Dublin, Ohio). Adequate induction of anesthesia was confirmed prior to any intervention by observation of a negative paw- or tail-pinch reflex. For procedures involving a thoracotomy (i.e. myocardial infarction surgeries) animals were intubated after induction of anesthesia and ventilated at a rate of 180-190 breaths per minute and a tidal volume of 250-300 μL (ventilation parameters were adjusted accordingly depending on the size of each animal). For all other procedures, maintenance anesthesia was delivered via nose cone. All animals used for in vivo studies were 12-week-old male C57BL/6 mice (The Jackson Laboratory; Bar Harbor, ME).

Isolation and culture of cortical bone-derived stem cells

Cortical bone stem cells (CBSCs) were isolated using previously published techniques.1 Femurs and tibias were isolated from transgenic 12-week-old male C57BL/6-Tg(CAG-EGFP)1Osb/J mice (The Jackson Laboratory; Bar Harbor, ME), which constitutively express enhanced green fluorescent protein (EGFP) off of the β-actin promoter in most cells of the body. The epiphyses of the bones were removed, and the marrow cavity was flushed three times with phosphate-buffered saline (PBS) and the marrow was discarded. The remaining cortical bone was crushed using a sterilized mortar and pestle, and bone fragments were further digested using collagenase II. Bone chunks were then plated in CBSC culture media: DMEM/F12 Media (Lonza/Biowhittaker; Basel, Switzerland) + 10% fetal bovine serum (Gibco Life Technologies; Grand Island, NY), 1% Penicillin/Streptomycin/L-glutamine (Gibco Life Technologies; Grand Island, NY), 0.2% insulin-transferrin-selenium (Lonza; Basel, Switzerland), 0.02% basic-fibroblast growth factor (Peprotech; Rock Hill, NJ), 0.02% epidermal growth factor (Sigma; St. Louis, MO), and 0.01% leukemia inhibitory factor (Millipore; Billerica, MA). Over the first week in culture, fibroblast-like stem cells began to grow out from the bone chunks, and after 1 week in culture, the remaining chunks of bone were washed away and the adherent population of CBSCs could be passaged for expansion. Expanded cells could be resuspended in CBSC culture media + 10% DMSO to be frozen and stored long-term in liquid nitrogen.

Isolation of cardiac-derived stem cells

Cardiectomy was performed on transgenic 12 week-old male C57BL/6-Tg(CAG-EGFP)1Osb/J mice (The Jackson Laboratory; Bar Harbor, ME) under general anesthesia. Hearts were cannulated and perfusion digested to dissociate stem cells from the left ventricle, and cardiac-derived stem cells (CDCs) underwent sorting for c-kit using magnetic beads (Miltenyi Biotec; Cologne, Germany) following a previously described protocol.2,3 Cells were cultured and stored long term under identical conditions to CBSCs as previously described.

Isolation and Culture of Mouse Left Ventricular Myocytes

Left ventricular myocytes were isolated from mice receiving MI and CBSC therapy 6 weeks after injection for myocyte staining and cell physiology experiments. Cardiectomy was performed under general anesthesia then hearts were cannulated and perfusion digested with collagenase-containing Tyrodes solution on a constant-flow Langendorff apparatus, and left ventricular myocytes were isolated
and cultured as previously described. All myocytes isolated from the left ventricle of each MI+CBSC mouse were plated on laminin-coated 18mm round glass coverslips. Some coverslips were used to measure fractional shortening and calcium transients, others were fixed and immunostained for cell counts, surface area analysis, and nuclei counts.

To estimate the percentage of all LV myocytes that were EGFP+ (and thus derived from injected CBSCs), an average number of 15.67±3.67 EGFP+ myocytes were counted on each coverslip. The total number of myocytes on each coverslip was estimated by counting the myocytes in ten random 10X visual fields, and an average of 12.4±1.51 total myocytes/field were counted. A 10X visual field has a surface area of 1.30² mm², and an 18mm coverslip has a surface area of 81π mm², so there are 81π/1.30² = 150.18 10X visual fields/coverslip. Thus there were an average of 12.4 * 150.18 = 1862.26 total myocytes/coverslip. So by 6 weeks post-MI, an estimated 15.67/1862.26 = 0.84% of myocytes isolated from CBSC-injected animals were EGFP+.

**Fractional Shortening and Calcium Transients**

Mouse left ventricular myocytes isolated from CBSC-injected animals 6 weeks post-MI were simultaneously measured for fractional shortening and calcium transients as has been previously described.

**Flow Cytometry**

For flow cytometry, 5x10⁶ CBSCs or CDCs were incubated for 15 minutes at 4°C under gentle agitation in the appropriate antibody diluted 1:11 in wash buffer (PBS+ 0.5% bovine serum albumin + 2 mM EDTA, pH = 7.3). After incubation, cells were washed with 2 mL wash buffer, centrifuged at 300 Xg for 5 min, and supernatants were aspirated and discarded. Stained cells were resuspended in PBS for flow cytometry. A second sample of CBSCs or CDCs was stained in each condition with APC-conjugated Rat IgG2b, which was used as a negative isotype control. The following conjugated antibodies or pairs of primary and secondary antibodies along with their corresponding nonspecific negative isotype controls were used:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primary Antibody</th>
<th>Negative Isotype Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sca-1</td>
<td>Rat IgG2a anti-Sca-1-APC (Miltenyi Biotec; Cologne, Germany)</td>
<td>Rat IgG2a-APC Invitrogen; Cambridge, MA</td>
</tr>
<tr>
<td>CD29</td>
<td>Rat IgG anti-CD29-APC (eBioscience; San Diego, CA)</td>
<td>Rat IgG-APC (eBioscience; San Diego, CA)</td>
</tr>
<tr>
<td>CD34</td>
<td>Rat IgG2a anti-CD34-Alexa Fluor 647 (AbD Serotec; Kidlington, UK)</td>
<td>Rat IgG2a-APC (AbD Serotec; Kidlington, UK)</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat IgG2b anti-CD45-Alexa Fluor 647 (AbD Serotec; Kidlington, UK)</td>
<td>Rat IgG2b-APC (AbD Serotec; Kidlington, UK)</td>
</tr>
<tr>
<td>C-kit</td>
<td>1° goat IgG anti-SCF-R (R&amp;D Systems; Minneapolis, MN) 2° anti-Goat IgG Rhodamine Red-X (Jackson ImmunoResearch Labs; West Grove, PA)</td>
<td>1° goat IgG (R&amp;D Systems; Minneapolis, MN) 2° anti-Goat IgG Rhodamine Red-X (Jackson ImmunoResearch Labs; West Grove, PA)</td>
</tr>
<tr>
<td>Lineage Cocktail</td>
<td>1° Biotin-Conjugated Lineage Cocktail (Miltenyi Biotec; Cologne, Germany) 2° anti-Biotin-APC (Miltenyi Biotec; Cologne, Germany)</td>
<td>2° anti-Biotin-APC only (Miltenyi Biotec; Cologne, Germany)</td>
</tr>
</tbody>
</table>
RNA Isolation and PCR Analysis

CDCs or CBSCs were resuspended in QIAzol Lysis Reagent, and mRNA was isolated using an RNeasy Mini Kit. DNA was eliminated from the samples using RNase-free DNase I, and then cDNA was generated using RT² First Strand Kit. RT² qPCR Primer Assays for mouse Kit (c-kit), mouse Ly-6A (Sca-1), and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used with RT² SYBR Green qPCR mastermix to detect c-kit, Sca-1, or GAPDH mRNA expression, respectively. The amount of GAPDH mRNA in each cell type was determined using a 6-point standard curve with a 1:10 serial dilution of each transcript run on each primer set. The amount of transcript detected for c-kit or Sca-1 was normalized to detected levels of GAPDH and these data are presented as normalized arbitrary units. All qPCR reagents were purchased from Qiagen (Valencia, CA).

Protein Isolation and Western Analysis

CBSC or CDC lysates were prepared and analyzed using Western analysis as previously described.5,6 The following primary antibodies purchased from Abcam (Cambridge, MA), Cell Signaling (Danvers, MA) or AbD Serotec (Kidlington, UK) were used to detect target antigens: insulin-like growth factor-1 (Abcam ab106836), angiopoietin-1 (Abcam ab95230), basic-fibroblast growth factor (Abcam, ab8880), hepatocyte growth factor (Abcam ab83760), platelet-derived growth factor (Abcam ab125268), stem cell factor (Abcam ab9753), stromal-derived factor-1 (Cell Signaling #3740S), vascular endothelial growth factor (Abcam ab46154) and Glyceraldehyde 3-phosphate dehydrogenase (AbD Serotec). The following secondary antibodies were used: rabbit-HRP (GE#NA934V) and mouse-HRP (GE#NA931V) purchased from GE Healthcare (Little Chalfont, UK) and goat-HRP (sc-2020) purchased from Santa Cruz Biotechnology (Dallas, TX).

Enzyme-Linked Immunosorbent Assays

CBSCs or CDCs were plated at a low density of 25,000 cells/well in complete CBSC media or serum free media in a 6 well plate and allowed to proliferate to 90% confluency over 72 hours. Serum samples were collected every 24 hours and frozen at -20°C. Samples were analyzed using mouse DuoSet ELISA Kits for hepatocyte growth factor, insulin-like growth factor, stem cell factor, stromal-derived factor-1 and vascular endothelial growth factor. The presence of serum in the cell cultures did not affect cytokine production. All ELISA kits were purchased from R&D Systems (Minneapolis, MN). The data in each case is presented as a mean of 3 samples, and for each ELISA, background signal was subtracted using the mean of 3 samples containing unconditioned media only. A student’s T test was used to detect any significant difference in production of each paracrine factor between CBSCs and CDCs.

In vitro Differentiation Co-cultures

Neonatal rat ventricular myocytes were isolated following the Simpson and Savion protocol7 with minor modifications that we have previously described.8,9 Cells were plated overnight on gelatin-coated 18mm glass coverslips in a 12-well dish. Stem cells were added the following day at low densities (1000-5000 cells/well) on top of the neonatal rat myocytes. Cells were allowed to differentiate in co-culture for 72 hours. During this time some EGFP+ cells were observed to beat. After 72 hours, coverslips were fixed and stained for α-sarcomeric actin or connexin43, and the percentage of cells expressing either marker was determined after counting cells in 10 visual fields on 5 individual coverslips.

Mouse Myocardial Infarction and Intramyocardial Stem Cell Transplantation

Permanent occlusion myocardial infarction (MI) surgery was performed by ligating the left anterior descending coronary artery following a widely cited protocol.10 Immediately after MI, 40,000
CBSCs (n=67) or CDCs (n=36) suspended in normal saline were injected intramyocardially into the infarct border zone in four x 5 uL injections. MI control animals (n=60) received saline injection only, and sham control animals (n=21) received all surgical procedures except for ligation of the coronary artery and intramyocardial injection.

Each group of animals that underwent surgery was assigned a sacrifice date prior to surgery. This prevented selection bias at the time of sacrifice, so our Kaplan-Meier analysis was designed to measure 6-week survival from the time of myocardial infarction surgery. We designed our Kaplan-Meier analysis to detect “death” as event occurrence (if the animal died prior to its assigned sacrifice date). Animals that survived until their assigned sacrifice date (either at 1, 2, or 6 weeks post-MI, depending on the group) were censored in the Kaplan-Meier analysis.

Animals selected for 24-hour sacrifice were not included in Kaplan-Meier analysis. For the MI+Saline group, 5 were sacrificed at 24 hours post-MI for infarct size analysis. For the MI+CDC or MI+CBSC groups, a total of 10 animals were sacrificed at 24 hours post-MI (5 animals for infarct size analysis and 5 animals for histology). Animals assigned to 6-week sacrifice groups underwent serial echocardiography and strain analysis using the Vevo2100 ultrasound system and these data are presented in Figures 3 and 4.

Sham (n=21): 5 animals were sham operated for 1 week sacrifice, 5 animals were operated for 2 week sacrifice, and 11 animals were operated for 6 week sacrifice for a total of 21 animals. No animals in the sham-operated group died prior to their assigned sacrifice date (100% 6 week survival). All hearts were fixed for histology at each time point.

MI+Saline (n=60): 5 animals were sacrificed 24 hours post-MI for infarct size analysis and were not included in the Kaplan-Meier analysis. The remaining 55 animals were assigned to sacrifice groups at 1, 2, or 6 weeks post-MI and were included in Kaplan-Meier analysis. Of these 55 animals, 8 animals were operated for 1-week sacrifice and 2 died prior to sacrifice (the remaining 6 were fixed for histology). 23 animals were operated for 2-week sacrifice and 10 died prior to sacrifice (the remaining 13 hearts were fixed for histology). 24 animals were operated for 6-week sacrifice and 11 died prior to sacrifice (the remaining 13 hearts were fixed for histology). Thus, for Kaplan-Meier analysis, 55 animals were included and a total of 23 died prior to sacrifice and were counted as deaths.

MI+CBSC (n=67): 10 animals were sacrificed 24 hours post-MI for infarct size analysis (n=5) or histology (n=5) and were not included in the Kaplan-Meier analysis. The remaining 57 animals were assigned to sacrifice groups at 1, 2, or 6 weeks post-MI and were included in Kaplan-Meier analysis. Of these animals, 20 were operated for 1-week sacrifice and 6 died prior to sacrifice (the remaining 14 hearts were fixed for histology). 21 animals were operated for 2-week sacrifice and 3 died prior to sacrifice (the remaining 18 hearts were fixed for histology). 16 animals were operated for 6-week sacrifice and 3 died prior to sacrifice. Of the 13 animals sacrificed at 6 weeks post-MI, 5 were digested for myocyte isolation and 8 were perfusion fixed for histology. Thus, for Kaplan-Meier analysis, 57 animals were included and a total of 12 died prior to sacrifice and were counted as deaths.

MI+CDC (n=36): 10 animals were sacrificed 24 hours post-MI for infarct size analysis (n=5) or histology (n=5) and were not included in the Kaplan-Meier analysis. The remaining 26 animals were assigned to sacrifice groups at 1, 2, or 6 weeks post-MI and were included in Kaplan-Meier analysis. Of these animals, 10 were operated for 1-week sacrifice and 3 died prior to sacrifice (the remaining 7 hearts were fixed for histology). 11 animals were operated for 2-week sacrifice and 2 died prior to sacrifice (the remaining 9 hearts were fixed for histology). 5 animals were operated for 6-week sacrifice and 2 died prior to sacrifice. Of the 3 animals sacrificed at 6 weeks post-MI, 1 was digested for myocyte isolation and 2 were perfusion fixed for histology. Thus, for Kaplan-Meier analysis, 26 animals were included and a total of 12 died prior to sacrifice and were counted as deaths.

For histological analysis, 14 MI+CBSC hearts were fixed at 1-week post-MI, 18 were fixed at 2 weeks post-MI, and 8 were fixed at 6 weeks post-MI. When we analyze stem cell-injected hearts, we section each heart completely into 5 μm-thick sections and stain every single slide. Because such comprehensive histological analysis is necessary, we randomly selected 6 hearts fixed at 1 week post-MI and 6 hearts fixed at 2 weeks post-MI for analysis. All hearts fixed at 6 weeks post-MI were completely sectioned, stained, and analyzed.
histology (n=5) and were not included in the Kaplan-Meier analysis. The remaining 26 animals were assigned to sacrifice groups at 1, 2, or 6 weeks post-MI and were included in Kaplan-Meier analysis. Of these animals, 8 were operated for 1-week sacrifice and all animals survived (all 8 hearts were fixed for histology). 8 animals were operated for 2-week sacrifice and 4 died prior to sacrifice (the remaining 4 hearts were fixed for histology). 10 animals were operated for 6-week sacrifice and 4 died prior to sacrifice (the remaining 6 hearts were fixed for histology). Thus, for Kaplan-Meier analysis, 26 animals were included and a total of 8 died prior to sacrifice and were counted as deaths.

For histological analysis, all hearts were completely sectioned and analyzed on histology (8 at 1 week post-MI, 4 at 2 weeks post-MI and 6 at 6 weeks post-MI).

Infarct Size Analysis

After 24 hours post-MI, five animals from each study group (MI+CBSC, MI+CDC, or MI+Saline) were randomly selected to undergo acute infarct size analysis. Cardiectomy was performed under general anesthesia, and the hearts were perfused with 2% Evan’s Blue dye in PBS to stain the area at risk (AAR), which accounts for all areas of the myocardium except for those perfused by the ligated coronary artery. The heart was then flash-frozen in liquid nitrogen so it could be cut into 6-8 short-axis cross sections. The sections were washed in PBS to remove excess Evan’s Blue dye then incubated in 2% triphenyltetrazolium chloride in PBS for 10 minutes at 37°C to stain ischemic tissues white (ischemic area, IA) and viable tissues red. Samples were washed again in PBS then photographed under a top-lit dissecting scope. AAR and IA were measured on each photograph using NIH image J software, and a mean AAR and IA for each heart was calculated as a percentage of total ventricular area.

For chronic infarct size analysis, paraffin-embedded short-axis heart sections from MI+CBSC, MI+CDC, or MI+Saline sacrificed 6 weeks post-MI were stained with hematoxylin and eosin (H&E). Brightfield photographs were acquired on a dissecting microscope using a DS-Fi1 color camera and NIS Elements software (all from Nikon Inc.; Melville, NY). Pathologically infarcted regions of the myocardium were identified and their surface area was quantified using NIH Image J software. Infarct area was calculated as a percentage of total ventricular surface area for 3-4 cross-sections.

Two-Dimensional Echocardiography and Strain Analysis

Anesthetized mice underwent transthoracic echocardiography using a Vevo2100 ultrasound system (VisualSonics; Toronto, Canada). Repeated measurements were performed as previously described4,11,12 at baseline and at 1, 2, 4, and 6 weeks post-MI. Images were acquired by JMD in the short-axis B-mode and M-mode for analysis of cardiac function and dimensions. Long-axis B-mode images were recorded for longitudinal and radial strain analysis using the VevoStrain software following a recently published protocol.13 After echocardiograms were recorded, image series were randomly ordered and renumbered by CAM. All images were analyzed under their coded numbers in a blinded fashion by JMD, then the code was broken by CAM and animal data was sorted by treatment group then analyzed.

Perfusion Fixation

Cardiectomy was performed under general anesthesia and the heart was rinsed and weighed. The aorta was then cannuulated and the coronary arteries were cleared by perfusion with 1 mL cold Krebs-Henseleit Buffer. The heart was then arrested in diastole by perfusion with 1 mL of 100 mM cadmium chloride/1 M potassium chloride solution. The hearts were then gravity perfused with 30 mL 10% formalin at mean arterial pressure (100 mmHg). Fixed hearts were immersed overnight in 10% formalin and then stored in 70% ethanol for up to 1 week before being processed and embedded in paraffin wax blocks.
**Immunohistochemistry**

For *in vitro* staining, cells were plated on gelatin-coated coverslips overnight and then fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS (Fluka/Sigma-Aldrich; St. Louis, MO) and stained for the following proteins: c-kit and goat IgG isotype control for c-kit (AF1356 and AB-108-C, R&D Systems; Minneapolis, MN), Rat anti-Sca-1-biotin (130-093-421, Miltenyi Biotec; Auburn, CA) and rat IgG2a-biotin isotype control for Sca-1 (IC006B, R&D Systems; Minneapolis, MN), insulin-like growth factor-1 (SC-9013, Santa Cruz Biotechnology; Dallas, TX), angiopoietin-1 (ab95230), basic-fibroblast growth factor (ab8880), hepatocyte growth factor (ab83760), platelet-derived growth factor (ab61219), stem cell factor (ab64677), stromal-derived factor-1 (ab64677), and vascular endothelial growth factor (ab46154) all purchased from Abcam (Cambridge, MA). For neonatal rat ventricular myocyte co-cultures, cells were stained for α-sarcomeric actin (A2172, Sigma; St. Louis, MO) or connexin43 (AB1728, Millipore; Billerica, MA). The following secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and used for detection of primary antibodies as follows: Rhodamine Red-X donkey anti-goat IgG (705-295-147) was used to detect c-kit; Rhodamine Red-X donkey anti-biotin (200-292-211) was used to detect Sca-1; rhodamine red-X donkey anti-rabbit IgG (711-295-152) was used to detect Ang-1, bFGF, HGF, PDGF, SCF, SDF-1, VEGF, and connexin43; and rhodamine red-X donkey anti-Mouse IgM (715-295-020) was used to detect α-sarcomeric actin.

For fixed tissues, wax blocks were cut into 5 μm thick sections that were mounted on glass slides for staining. Slides were deparaffinized and underwent antigen retrieval in hot citric acid buffer. Stains were conducted against the following proteins: α-sarcomeric actin and α-smooth muscle actin (A2172 and A2547, Sigma; St. Louis, MO), EGFP and von Willebrand factor (ab111258 and ab6994, Abcam; Cambridge, MA), and connexin43 (AB1728 Millipore; Billerica, MA). The following secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and used for detection of primary antibodies as follows: rhodamine red-X donkey anti-Mouse IgM (715-295-020) or Cy5 donkey anti-Mouse IgG (715-175-140) were used to detect α-sarcomeric actin; rhodamine red-X donkey anti-Mouse IgG (715-295-151) or Cy5 donkey anti-Mouse IgG (715-175-151) were used to detect α-smooth muscle actin; FITC donkey anti-goat IgG (705-095-147) was used to detect EGFP; and rhodamine red-X donkey anti-rabbit IgG (711-295-152) or Cy5 donkey anti-rabbit IgG (711-175-152) was used to detect von Willebrand factor and connexin43. Nuclei in both cells and embedded tissues were stained with 4',6-diamidino-2-phenylindole (DAPI, Millipore; Billerica, MA).

Confocal micrographs of all immunostains were acquired using a Nikon Eclipse T1 confocal microscope (Nikon Inc.; Melville, NY).

**Statistical Analysis**

All statistical analyses were overseen by the Fox Chase Cancer Center Biostatistics and Bioinformatics Facility. Survival analysis is presented using a Kaplan-Meier regression and statistical significance was determined using the log-rank test. For ELISA, infarct size analysis, blood vessel counts and isolated myocyte measurements (where discrete measurements were compared at a single time point), a two-way T test was used. For follow-up parameters with repeated measures (echocardiography and strain analysis), growth curve models with cubic splines were used. All growth curve coefficients were fitted as random effects to allow deviation of individual growth from the mean of the treatment group. Interaction term with the treatment was also included to compare the mean growth rates by treatment. For all statistical tests, a p-value < 0.05 was considered statistically significant.
References for Supplemental Materials and Methods


Online Figure Legends

Online Figure I: Characterization of stem cells using quantitative real-time PCR (qPCR). Detected levels of c-kit and Sca-1 mRNA from cortical bone stem cells (CBSCs) or cardiac-derived stem cells (CDCs) were normalized to levels of GAPDH mRNA transcripts expressed by each cell type. Results are presented as normalized arbitrary units.

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Online Figure VII: Characterization of paracrine factors secreted by cortical bone stem cells in vivo 2 weeks after MI. Animals receiving MI+CBSCs were sacrificed 2 weeks post-MI and EGFP+ CBSC injection sites were identified and immunostained for VEGF (red). Nuclei are labeled with DAPI (blue). A) Immunostain showing an EGFP+/VEGF+ cell that was selected for fluorophore colocalization analysis by confocal line scan. B) Intensity of red, green, and blue phlorophores across the line scan of the cell selected in Figure A. From these data, scatterplots were constructed depicting colocalization of C) red vs. green channel or D) blue vs. green channel (control). E) Magnified image depicting the cell in Figure A along with single color channel images.
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Online Figure IX: Individual channel images and staining controls from Figure 7A and B. Scale bars = 50 μm.

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Online Figure XIII: Individual color channel images and staining controls for Figure 7D. Scale bars = 10 μm.

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Online Video I: Vector diagram video loop showing the direction and magnitude of endocardial strain in a mouse heart at baseline.

Online Video II: Vector diagram video loop showing the direction and magnitude of endocardial strain in a mouse heart at 6 weeks after MI+CBSC injection.

Online Video III: Vector diagram video loops showing the direction and magnitude of endocardial strain in a mouse heart at 6 weeks after MI+CDC injection.

Online Video IV: Vector diagram video loops showing the direction and magnitude of endocardial strain in a mouse heart at 6 weeks after MI+Saline injection.

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<table>
<thead>
<tr>
<th></th>
<th>Western</th>
<th>ELISA</th>
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<th>2 week</th>
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<th>2 week</th>
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<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cortical Bone Stem Cells</th>
<th>Cardiac-derived Stem Cells</th>
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<td>c-kit</td>
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<td>Sca-1</td>
<td>93.4%</td>
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<td>CD29</td>
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<td>CD45</td>
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</tr>
<tr>
<td>Lin</td>
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**Hematopoietic (Lin) markers:**

- **APC/AlexaFluor647**: 100K, 200K, 40K
- **CD45**: 0.00%, 200K
- **CD34**: 0.35%, 200K
- **Sca-1**: 0.78%, 200K
- **c-kit**: 0.89%, 200K

**Isotype Controls:**

- **Cortical Bone Stem Cells**: 0.00%, 0.22%, 1.46%
- **Cardiac-derived Stem Cells**: 98.7%, 0.00%, 2.70%
Online Figure IV: Bone-derived stem cells differentiate in vitro. CBSCs were cocultured with neonatal rat ventricular myocytes for 3 days. Cells were fixed and stained red for α-sarcomeric actin (A and B) or connexin43 (C and D). Nuclei are labeled with DAPI (blue). EGFP+ CBSCs are green.
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**A** 24 hours Post-MI

**B** 6 weeks Post-MI

![Graphs showing infarct size analysis results]

- MI+Saline
- MI+CDC
- MI+CBSC

* = p < 0.05, ** = p < 0.01, NS = Not Significant (p > 0.05)
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Online Figure IX: Individual channel images and staining controls from Figure 7A and B. Scale bars = 50 μm.

<table>
<thead>
<tr>
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<table>
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<tr>
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<td><strong>6 weeks Post-MI+Saline</strong></td>
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<td>Connexin43</td>
<td>Connexin43</td>
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<tr>
<td>DAPI</td>
<td>DAPI</td>
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</table>

Scale bars = 20 μm.
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- **α-SA-** (n=153): 58.6 ± 3.8%
- **Unorganized α-SA+** (n=60): 21.5 ± 6.2%
- **Striated α-SA+** (n=52): 20.0 ± 9.3%