Stem Cell Factor Gene Transfer Promotes Cardiac Repair After Myocardial Infarction via In Situ Recruitment and Expansion of c-kit+ Cells

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Rationale: There is growing evidence that the myocardium responds to injury by recruiting c-kit+ cardiac progenitor cells to the damage tissue. Even though the ability of exogenously introducing c-kit+ cells to injured myocardium has been established, the capability of recruiting these cells through modulation of local signaling pathways by gene transfer has not been tested.

Objective: To determine whether stem cell factor gene transfer mediates cardiac regeneration in a rat myocardial infarction model, through survival and recruitment of c-kit+ progenitors and cell-cycle activation in cardiomyocytes, and explore the mechanisms involved.

Methods and Results: Infarct size, cardiac function, cardiac progenitor cells recruitment, fibrosis, and cardiomyocyte cell-cycle activation were measured at different time points in controls (n=10) and upon stem cell factor gene transfer (n=13) after myocardial infarction. We found a regenerative response because of stem cell factor overexpression characterized by an enhancement in cardiac hemodynamic function: an improvement in survival; a reduction in fibrosis, infarct size and apoptosis; an increase in cardiac c-kit+ progenitor cells recruitment to the injured area; an increase in cardiomyocyte cell-cycle activation; and Wnt/β-catenin pathway induction.

Conclusions: Stem cell factor gene transfer induces c-kit+ stem/progenitor cell expansion in situ and cardiomyocyte proliferation, which may represent a new therapeutic strategy to reverse adverse remodeling after myocardial infarction. (Circ Res. 2012;111:1434-1445.)

Key Words: cardiac myocyte regeneration ■ gene transfer ■ myocardial infarction ■ stem cell factor

The recent demonstration of adult human cardiac renewal1 and identification and extensive characterization of c-kit+ cardiac stem and progenitor cells suggests that the heart is not terminally differentiated but an organ with regenerative potential. These results provide hope for development of therapeutic strategies to augment the limited regenerative process for the failing heart. We sought to enhance the limited endogenous repair process of the myocardium after injury using methods easily translated into clinical practice, by locally expanding c-kit+ cells2 using SCF (stem cell factor) gene transfer. Despite the fact that c-kit+ cells have been used extensively as a cell surface marker and much studied with respect to cells homing to infarcted myocardium, together with its ligand, SCF, the tyrosine kinase receptor c-kit is a key proliferation-controlling protein, driving not only the recruitment but the expansion of a number of stem cell types, including hematopoietic, neuronal, germ, and cardiac.4,6 SCF binds induce c-kit dimerization, activation of its intrinsic tyrosine kinase, and autophosphorylation leading to downstream signaling,7 including the Wnt-β-catenin pathway.8 Increased expression of SCF occurs naturally in response to myocardial infarction (MI), which has been shown to mediate migration of c-kit+ cardiac and bone marrow (BM) cells9 via activation of p38 mitogen-activated protein kinase,9 driven by infiltrating macrophages.10 Genetically mutant mice deficient in c-kit signaling (W/Wv) fare worse after MI, and transgenic mice overexpressing SCF in a cardiac-specific manner fare better after MI than their wild-type littersmates.5,11,12 SCF has been implicated in promoting the reverse remodeling observed after left ventricular assist device implantation.13

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Recently, dramatic improvements were reported in patients with ischemic cardiomyopathy, after intracoronary infusion of autologous c-kit+ cardiac stem cells (CSCs).14 This trial underscored the importance of c-kit+ cells in cardiac reparation. More
recently cardiosphere (which contain a significant amount of c-kit+ cells) injection in patients with heart failure was also shown to improve clinical parameters in the injected patients.\textsuperscript{15}

We therefore undertook an alternative strategy consisting of SCF adenoviral gene transfer into the infarcted myocardium in rats, to test the potential of SCF to recruit c-kit+ cells from cardiac and BM origin. In addition, we tested whether this strategy would lead to enhanced cardiac repair, function, and survival and finally to define whether SCF has an effect on cardiomyocyte proliferation and cell-cycle reentry.

**Methods**

SCF adenoviruses were injected in rats to enhance cardiac repair after MI. Molecular and functional approaches were performed to assess cardiac regeneration after SCF therapy. Detailed methods are provided in the Online Data Supplement.

**Results**

**SCF Gene Transfer Decreases Infarct Size After LAD-Ligation**

SCF alternative splicing leads to 2 isoforms, a soluble and a membrane-bound form. Stimulation with the soluble protein leads to rapid and transient activation, autophosphorylation, and fast degradation of SCF receptor, c-kit; whereas stimulation with the membrane-associated form leads to more sustained activation by preventing receptor–ligand complex internalization.\textsuperscript{7} Our strategy consisted of using adenoviral-mediated gene transfer of SCF as a therapy to promote cardiac regenerative mechanisms in rats undergoing MI. Myocardial regeneration and function was assessed 1, 2, 4, and 12 weeks post-MI (Figure 1A). Therefore, to maximize the proliferative signal for c-kit+ cells, we generated recombinant adenoviruses expressing SCF membrane-bound form and green fluorescent protein (Ad.SCF). Similarly, we generated adenoviruses containing β-gal and green fluorescent protein, used as controls (Ad.β-gal; Figure 1B). By permanent ligation of the left anterior descending coronary artery (LAD), we induced the MI and later, Ad.SCF and Ad.β-gal adenoviruses were delivered into the peri-infarct left ventricle (LV). Gene transfer was confirmed 1 week post-MI by β-galactosidase, c-kit, and green fluorescent protein expression in controls and SCF-treated rats (Figure 1C–1F; Online Figure I). After SCF overexpression a

**Figure 1. Study design.** A, Experimental timeline. Our strategy consisted of using stem cell factor (SCF) as a therapy in cardiac regeneration in rats undergoing myocardial infarction (MI) after delivering adenoviruses expressing SCF membrane-bound form and green fluorescent protein (Ad.SCF) or adenoviruses containing β-gal and green fluorescent protein (Ad.β-gal) recombinant adenoviruses in the peri-infarct area. Myocardial regeneration and function was assessed at 1, 2, 4, and 12 weeks post-MI. B, Recombinant Ad.SCF and Ad.β-gal adenoviruses were generated after cloning SCF membrane isoform and β-gal gene sequences, respectively, into an adenoviral plasmid containing GFP as a reporter gene. C–E, Detection of gene transfer within the myocardium 1 week post-MI. Distribution of the viral infection was confirmed by GFP expression, imaged through digital photography (C) and by X-gal staining (blue) (D and E) in control rats (Ad.β-gal). F, Analysis of c-kit receptor expression by western blot (WB), in lysates from control and Ad.SCF-treated hearts. α-GAPDH was used as protein loading control. G and H, Infarct size (white arrows) (G) was measured by cardiac magnetic resonance imaging 2 weeks post-MI (15.3±1.4% Ad.β-gal vs 8.6±1.6% Ad.SCF; *P<0.05; H). LAD indicates left anterior descending coronary artery; IRES, internal ribosomal entry site.
reduction in the scar area was detected compared with controls (Figure 1G; Online Figure II). Cardiac magnetic resonance imaging was used to measure cardiac morphology and function at baseline (2 days) and 2 and 4 weeks post-MI. A significant reduction in infarct size, normalized to total LV, was detected after SCF therapy 2 weeks post-MI (15.3%±1.4% in Ad.β-gal versus 8.6%±1.6% in Ad.SCF; P<0.05) and 4 weeks post-MI (17.7%±0.3% in Ad.β-gal versus 7.6%±1.2% in Ad.SCF; P<0.01) (Figure 1H; Online Figure IIIA and IIIB). Thus, after SCF gene transfer, no significant differences in ejection fraction (EF) were found at baseline (2 days post-MI) between controls and SCF-treated rats (62.0%±0.5% in Ad.β-gal versus 63.5%±1.2% in Ad.SCF; P=0.31). Although a nonsignificant EF improvement was detected after SCF therapy 2 weeks post-MI (48.7%±0.8% Ad.β-gal versus 56.5%±2.9% Ad.SCF; P=0.06), we showed a significant improvement 4 weeks post-MI (48.3%±0.4% Ad.β-gal versus 58.4%±0.6% Ad.SCF; P<0.01; Online Figure III C). These preliminary observations indicate that SCF might be playing a role in enhancing cardiac protection after MI.

SCF Improves Survival and Cardiac Function

Previous studies show that human SCF transgene expression in mice induces c-kit receptor activation, promoting an improvement in survival and cardiac function after MI.12 Survival was monitored at 3 months postoperation in sham, control, and Ad.SCF-treated groups (n=5, n=10, and n=13, respectively). Sham groups survival was 100%, whereas MI led to an increase in mortality. SCF gene transfer resulted in increased survival post-MI compared with controls (90% Ad.SCF versus 65% Ad.β-gal; P=0.07) (Figure 2A). We next evaluated the impact that SCF overexpression had on cardiac structure, analyzing cardiac function and LV dimension by echocardiography. SCF administration enhanced LV function at 1 and 3 months post-MI (Figure 2B and 2C). LAD ligation led to a time-dependent decrease in the fraction of shortening and EF, as well as an increase in LV chamber dimensions. However, the reduction in fraction of shortening attributable to MI was ameliorated after SCF overexpression (n=13) was compared with controls (n=10) at 1 month post-MI (46±8% Ad.SCF versus 30±7% Ad.β-gal; P<0.001). Similarly, EF decrease attributable to MI was improved after Ad.SCF administration at 1 month post-MI (67±14% Ad.SCF versus 51±11% Ad.β-gal; P<0.001) (Figure 2D). SCF overexpression also caused an improvement in fraction of shortening (43±6% Ad.SCF versus 29±8 Ad.β-gal; P<0.0001) and EF (64±7% Ad.SCF versus 46±16% Ad.β-gal; P<0.0001) at 3 months post-MI (Figure 2E). Furthermore, LV chamber dimensions were also increased because of MI, although SCF overexpression attenuated the MI-induced increase in LV end-systolic and end-diastolic volume (Table). These results suggest that SCF administration continue to improve LV function 3 months after MI.

SCF Reduces Fibrosis in the Infarct Heart

The beneficial effects of SCF in improving cardiac function and survival after MI result from recruiting endothelial progenitor cells and decreasing cardiac remodeling.16 An important hallmark in fibrosis is the increase in collagen and other extracellular matrix components in the myocardium17 and the release of transforming growth factor-β (TGF-β) by active myofibroblasts.18,19 Consistent with the cardiac magnetic resonance imaging and echocardiography data, SCF therapy decreased LV wall thinning, increasing viable tissue within the risk region 1 month post-MI (Figure 3A). Thus, a reduction in fibrosis was detected after SCF overexpression. The collagenous area in the pericardial region was significantly decreased in SCF-treated rats (5.1%±0.2% Ad.β-gal versus 2.0%±0.1% Ad.SCF; P<0.0001). Similarly, the fibrotic area around the perivascular region was markedly reduced after SCF therapy (19.3%±1.2% Ad.β-gal versus 6.4%±0.7% Ad.SCF; P<0.001; Figure 3B–3E). Moreover, collagen content measured by Sircol Collagen Assay 3 months post-MI showed a significant decrease in the infarcted (44.8%±4.1% Ad.β-gal versus 27.8%±4.1% Ad.SCF; P<0.05) and remote regions (26.2%±1.8% Ad.β-gal versus 18.7%±2.0% Ad.SCF; P<0.05) after SCF overexpression (Online Figure IVA and IVB). Along with the progression of fibrosis, the expression of TGF-β was decreased in both remote and infarcted area after SCF therapy. In contrast, collagen III expression was only decreased in the remote area, and no significant differences were found in collagen I expression levels after SCF therapy (Figure 3F; Online Figure IVC and IVD). Together, these data suggest that SCF therapy might play a protective role against fibrosis replacement by reducing scar formation, restoring partially the structural integrity of the infarcted heart.

SCF Gene Transfer Augments Cardiac Progenitor Cell Recruitment to the Ischemic Myocardium

The SCF receptor, c-kit, is used as a marker to identify several types of adult stem cells, including those in cardiac, hematopoietic, brain, liver pancreatic, and lung tissue, and its expression is the primary characteristic of cardiac lineage commitment.20-22 The membrane-associates SCF isoform induces more persistent c-kit receptor activation, promoting cell survival by recruiting c-kit+ stem cells.23 We then asked whether Ad.SCF therapy could enhance the exogenous and endogenous recruitment of cardiac progenitor cells (CPCs) and CSCs to the infarcted myocardium promoting the recovery of the injured heart. Our results confirmed our hypothesis, showing that Ad.SCF overexpression resulted in a 4-fold increase of c-kit+ cells compared with sham-operated (69±39 cells/mm3 in sham versus 307±11 cells/mm3 in Ad.SCF; P<0.05) or control (64±17 cells/mm3 in Ad.β-gal versus 307±11 cells/mm3 in Ad.SCF; P<0.05) 1 month post-MI (Figure 4A and 4B). To further confirm the faithful increase of CPCs, cardiac c-kit+ population was purified by fluorescence-activated cell sorting from the whole heart. SCF overexpression resulted in higher absolute numbers of c-kit+ cells within the heart (1:10000) compared with control (1:37000) or sham (1:55000) groups (Figure 4C). We next determined whether SCF expression was altering the hematopoietic c-kit+ population. The lack of lineage markers (Lin–) and c-kit+ expression was analyzed by fluorescence-activated cell sorting in hematopoietic stem cells (Lin–c-kit+) from blood and BM cells 1 week post-MI. No differences were found in circulating or BM-derived cells after SCF overexpression (Figure 4D).

CPCs have been characterized as a heterogeneous cell population in a state of differentiation and commitment to cardiac...
Figure 2. Cardiac function in the stem cell factor (SCF)-treated myocardium. A, Kaplan–Meier survival analysis in sham (n=5), adenoviruses containing β-gal and green fluorescent protein (Ad.β-gal)–treated group (n=10), and adenoviruses expressing SCF membrane-bound form and green fluorescent protein (Ad.SCF)–treated groups (n=13). Although there was no statistical significance, a tendency toward an increase is detected in survival after SCF treatment compared with controls 3 months postmyocardial infarction (MI; 90% Ad.SCF vs 65% Ad.β-gal; P=0.07 vs Ad.β-gal). B–E, Echocardiographic measurements performed 1 and 3 months post-MI. Representative parameters short-axis M-mode view, in Ad.SCF-treated and control (Ad.β-gal) rats at 1 (B) and 3 months post-MI (C). Fractional shortening (FS) and ejection fraction (EF) 1 month (D) and 3 months (E) after Ad.SCF overexpression.
lineage, scoring negative for α-sarcomeric actin but positive for GATA4, Nkx2.5, or MEF2 expression markers. Cardiac c-kit+ population was gated and examined for coexpression of transcription factors expressed at early and late stages of cardiomyocyte development. Despite this heterogeneity, an increase in MEF2, MEF2C, Nkx2.5, and GATA4 early cardiac markers was found to be significant in the periinfarct zone 1 week post-Ad.SCF therapy (Figure 5A). However, no demonstrable changes in cyclin D1, proliferating cell nuclear Ag, or P-H3 expression were found in the remote region (Figure 5C). The increase of P-H3 and Ki67-positive cardiomyocytes was detected in the infarcted remote region 1 week post-Ad.SCF therapy. CyclinD1, proliferating cell nuclear Ag, and bromodeoxyuridine-labeled cardiomyocytes (3.3±0.4% controls)24 together with c-kit+ cells' coexpression of the CD45+ hematopoietic lineage marker, suggesting an accumulation of immature c-kit+ population on SCF administration. These results confirm the heterogeneity of c-kit population, as well as its different stage of commitment to cardiac lineage.

**SCF Induces Proliferation of Cardiomyocytes in the Ischemic Heart**

SCF is a growth factor that promotes the proliferation of different stem cells populations, including cardiac, hematopoietic, neuronal, and germ cells, together with c-kit+ cells' recruitment to injured myocardium. The accumulation of c-kit+CPCs led us to investigate the role that SCF was playing in cardiomyocyte cell-cycle activation by evaluating their potential to divide and undergo DNA synthesis at 1 week post-MI. Cardiomyocyte potential to divide was assessed by colocalization of phosphohistone H3 (P-H3) and Ki67 together with α-sarcomeric actin marker. As expected, a global increase of P-H3 and Ki67-positive cardiomyocytes was detected in the periinfarct zone of the Ad.SCF-treated hearts at 1 week (Figure 5A; Online Figure V). Similarly, cardiomyocyte cytokinesis was assessed by colocalization of Aurora B kinase together with α-sarcomeric actin marker. Upon SCF therapy, Aurora B kinase–positive cardiomyocytes were detected in the periinfarct zone 1 week post-MI (Online Figure VI). As expected from these data, an increase in cardiomyocytes undergoing DNA synthesis at the time of bromodeoxyuridine labeling was detected in SCF-treated hearts compared with controls. The number of total bromodeoxyuridine-labeled nuclei (9.5±1.3% Ad.SCF versus 3.8±0.3% Ad.β-gal; P<0.001) and bromodeoxyuridine-labeled cardiomyocytes (3.3±0.4% Ad.SCF versus 1.3±0.3% Ad.β-gal; P=0.002) were significantly greater after SCF overexpression (Figure 5A and 5B; Online Figure V). Thus, an enhancement in the expression levels of proliferative markers was detected after SCF treatment by Western blot analysis. CyclinD1, proliferating cell nuclear Ag, and P-H3 expression were upregulated in the infarcted region 1 week post-Ad.SCF therapy. However, no demonstrable changes in cyclin D1, proliferating cell nuclear Ag, or P-H3 expression were found in the remote region (Figure 5C). The enhancement in ventricular function suggests that SCF could be inducing a protective response, enhancing cell survival. Using terminal deoxynucleotidyl transferase dUTP nick end labeling staining apoptotic cells were 1 week post-MI. The density of apoptotic cells per area in the border zone was significantly lower after SCF overexpression compared with controls (7.0±0.5% in Ad.β-gal versus 3.5±2.1% in Ad.SCF; P<0.0001; Figure 5D and 5E). Thus, caspase 3 activity was decreased upon SCF therapy in the infarcted area (Figure 5F).

Together, these data suggest a protective role of SCF in preventing apoptosis.

### Table. Effects of Myocardial Regeneration on Ad.SCF-Treated Rats

<table>
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<th>Parameter, unit</th>
<th>Sham 1 mo</th>
<th>Ad.β-gal 1 mo</th>
<th>Ad.SCF 1 mo</th>
<th>Sham 3 mo</th>
<th>Ad.β-gal 3 mo</th>
<th>Ad.SCF 3 mo</th>
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<td>IVSd, mm</td>
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<td>IVSs, mm</td>
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<td>2.6±0.9</td>
<td>3.5±0.9</td>
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<td>LVIDd, mm</td>
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<td>8.0±0.8</td>
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<tr>
<td>LVIDs, mm</td>
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<td>6.1±0.9</td>
<td>4.2±0.8</td>
<td>2.6±0.4</td>
<td>6.5±1.4</td>
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<td>LVPWd, mm</td>
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<td>LVPWs, mm</td>
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<td>46±8</td>
<td>62±4</td>
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**Echocardiographic measurements. All values represent mean±SD.**

SCF indicates stem cell factor; Ad.SCF, adenoviruses expressing SCF membrane-bound form and green fluorescent protein; Ad.β-gal, adenoviruses containing β-gal and green fluorescent protein; IVSd, interventricular septal thickness at diastole; IVSs, interventricular septal thickness at systole; LVIDd, left ventricular internal dimension at diastole; LVIDs, left ventricular internal dimension at systole; LVPWd, left ventricular posterior wall at diastole; LVPWs, left ventricular posterior wall at systole; FS, fraction of shortening; EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction.
To assess the impact of cardiomyocyte proliferation on LV hypertrophy because of SCF therapy, we measured LV weight (LVW) and heart weight (HW) to body weight (BW) ratio at 2 weeks post-MI. After measuring the ratio of LVW/BW and HW/BW after MI and SCF administration, we found a trend toward a decrease in LVW/BW after MI (0.20%±0.04% Ad.β-gal versus 0.23%±0.01% sham, P=NS) and HW/BW (0.25%±0.05% Ad.β-gal versus 0.30%±0.02% sham), which was reversed after SCF administration in LVW/BW (0.23%±0.04% Ad.SCF versus 0.23%±0.01% sham; P=NS) and in HW/BW (0.28%±0.05% Ad.SCF versus 0.30%±0.02% sham). We next assessed the transverse diameter of cardiomyocytes, by analyzing the minimum cross-sectional diameter at the nuclear level, to minimize the effects of orientation on cardiomyocyte size measurements. Significant smaller myocyte cross-sectional diameters detected by vinculin staining were found after SCF treatment (34.05±0.27 μm Ad.SCF versus 41.65±0.40 μm Ad.β-gal; P<0.0001). Echo analysis of cardiac wall thickness also documented reduction in cardiac hypertrophy in SCF-treated rats (Table; Online Figure VII).

Figure 3. Cardiac fibrosis is decreased after stem cell factor (SCF) overexpression. A, Representative Masson trichrome–stained myocardial sections from control and SCF-treated rats 1 month post-myocardial infarction (MI). Blue, scar tissue; red, viable myocardium. B–E, Intramyocardial (B) and perivascular fibrosis (C) analyzed by Picrosirius Red and Masson Trichrome staining 1 month post-MI. D and E, Effect of SCF overexpression in intramyocardial (D) (5.1%±0.2% in adenoviruses containing β-gal and green fluorescent protein [Ad.β-gal] vs 2.0%±0.1% in adenoviruses expressing SCF membrane-bound form and green fluorescent protein [Ad.SCF]; P<0.0001) and perivascular fibrosis (E) (19.3%±1.2% in Ad.β-gal vs 6.4%±0.7% in Ad.SCF; P<0.001). F, Analysis of collagen I, III, and transforming growth factor-β (TGF-β) expression by WB in cardiac lysates from remote (R) and infarcted (I) regions 3 months post-MI.
These results showing decreased LV mass decrease along with reduced cardiomyocyte size are all concordant with decreased LV hypertrophy detected after SCF administration. Together, these data suggest that SCF overexpression promotes cardiac regeneration by inducing newly dividing cardiomyocytes and preventing hypertrophy and apoptosis of existing cardiac cells in the ischemic heart.
Wnt/β-Catenin Pathway Is Activated Upon SCF Overexpression

We next examined the molecular and cellular mechanisms responsible for cardiac progenitor and stem cell expansion in the ischemic myocardium after Ad.SCF overexpression. Wnt signaling has been shown to promote the self-renewal and differentiation of undifferentiated cells including c-kit+ hematopoietic stem cell and Isl1+ cardiovascular progenitor cells, enhancing also the differentiation of c-kit+ adipose-derived murine stromal vascular cells into cardiac myocytes. The accumulation of c-kit+ cells, together with the proliferation of cardiomyocytes after SCF treatment and MI, led us to study Wnt/β-catenin signaling in the context of cardiac regeneration. The hallmark of canonical Wnt pathway activation is β-catenin accumulation in the cytoplasm and its translocation to the nucleus. An increase in β-catenin stabilization was observed after SCF overexpression in remote and infarcted regions (Figure 6A). We next determined whether Wnt signaling might be regulating genes involved in c-kit+ stem cell self-renewal and proliferation, such as Notch1, HoxB4, and cyclin D1. By using real-time polymerase chain reaction analysis on cardiac extracts on either SCF-treated or control hearts, we found that Notch1, HoxB4, and cyclin D1 were upregulated an average of 2, 6, and 7-fold, respectively, in the remote area and 1.2-, 2.2-, and 2-fold, respectively, in the infarcted area upon SCF administration (Figure 6B; Online Table I).

Furthermore, Wnt pathway activation was also detected because of SCF overexpression in isolated cardiomyocytes (Online Figure VIII) through glycogen synthase kinase 3β phosphorylation and β-catenin accumulation in the cytoplasm (Figure 6C). We hypothesize that SCF administration may be inducing Wnt signaling activation in either CPCs by promoting their recruitment and self-renewal and also in cardiomyocytes by inducing their reentry into cell-cycle after MI (Figure 6D).

Discussion

Recent studies have shown that stem cells may be able to replace damaged tissue and potentially treat cancer, Type 1 diabetes mellitus, Parkinson disease, Huntington disease, and cardiac failure along with many other diseases. Clinical experience from the first ≈1000 patients who have received stem cell therapy for acute MI or chronic heart failure indicates a favorable safety profile with modest improvement in cardiac function and structural remodeling. However, cell therapy challenges remain formidable and include isolation of the appropriate cell, low efficiency of engraftment, expansion of the cells ex vivo, and ethical and regulatory hurdles. In the

Figure 5. Stem cell factor (SCF) therapy induces cardiomyocyte cell-cycle activation 1 week post-MI. A, Cardiomyocytes cell-cycle reentry (arrows) analyzed by costaining with α-sarcomeric actin (α-SA) and phosphohistone 3 (P-H3) or Ki67 proliferative markers. DNA synthesis is detected by bromodeoxyuridine (BrdU) incorporation. B, An increase of BrdU+ cells (10.1±1.4% adenoviruses expressing SCF membrane-bound form and green fluorescent protein [Ad.SCF] vs 3.7±0.3% adenoviruses containing β-gal and green fluorescent protein [Ad.β-gal]; *P<0.001; left) and BrdU+α-SA+ cells (CM) (3.8±0.3% Ad.SCF vs 1.8±0.2% Ad.β-gal, *P<0.001; right) is shown in the border area after SCF therapy. C, Cyclin D1, proliferating cell nuclear Ag (PCNA), and P-H3 proliferation expression markers in lysates from remote (R) and infarcted (I) regions. D-F, SCF prevent apoptosis in the infarcted myocardium. Apoptotic cells detection by terminal deoxynucleotidyl transferase dUTP nick end labeling staining (red) in the border area (D). Apoptotic index (%) analysis by TUNEL (E) (7.0%±0.5% Ad.β-gal vs 3.5%±2.1% Ad.SCF; *P<0.0001). Caspase 3 activity analysis by western blot (WB) (F).
last decade, gene therapy has reemerged with novel vectors that are safer and more targeted to various diseased states. In fact clinical gene therapy for cardiovascular diseases may be able to fulfill an unmet need for effective therapies in heart failure.41

The identification of the resident c-kit+ CSC population in the adult heart has demonstrated the regenerative potential of the adult heart.42–44 The need to activate in situ the intrinsic cardiac regenerative capacity suggests the use of growth factors, cytokines, and drugs to promote cardiac repair. Alternatively, BM progenitor cells may migrate to the myocardium leading to the formation of new myocyte progeny.45 Several studies have investigated the increased cardiac progenitor recruitment after SCF soluble protein administration,11 overexpression in transplanted cells12 and in transgenic mice.12

These experimental results were recently tested in a clinical trial, whereby intracoronary infusion of autologous c-kit+ cells in patients with ischemic cardiomyopathy resulted in significant improvement in systolic function and a reduction in infarct size.14 These encouraging clinical results emphasize the importance of recruiting c-kit+ cells in the setting of ischemic cardiomyopathy.

However, we sought to develop a clinically relevant local expression system for the membrane-bound SCF isoform to optimally stimulate cardiac repair after experimental MI. Furthermore, the administration of SCF membrane-bound form, as opposed to the soluble form, limited the expansion of mast cells in BM and blood, an attribute which has severely limited the clinical use of the soluble form of the molecule (Stemgen; ancestim). Our results show that SCF gene transfer stimulates a regenerative response after MI that appears to restore the injured heart and function.

In the present study, we used intramyocardial SCF adenoviral gene transfer to enhance cardiac repair after LAD ligation by recruiting and expanding resident c-kit+ cardiac progenitor/stem cells. Our studies have shown several significant findings. Using transient SCF expression, we observed an increase in cardiac c-kit+ cell recruitment after MI. Increased c-kit+ CPC population in SCF hearts is demonstrated by 2 independent techniques: cell counting by histology and sorting flow cytometry. These CPCs are a mixed population containing not only CSCs and precursors but also cells committed to other lineages. Although the majority population of c-kit+ cells are presumably endogenous,26 we do observe a high enrichment of c-kit+CD45+ fraction post-MI,26,30 suggesting a BM origin for many of the CPCs isolated. This conclusion is also consistent with the studies reporting that BM progenitor cells are mobilized after MI, homing to injured areas promoting...
cardiac repair. Regardless of cell origin, the increase in c-kit+ cells in SCF-treated hearts, whether CSC, BM cells, or a combination, does mediate a beneficial effect on survival, cardiac structure, and ventricular function after MI.

Previous studies show that cardiomyocytes self-renew though proliferation of endogenous c-kit+ CPCs, maintaining the cardiomyocyte turnover in the adult heart after cell loss. However, BM progenitor cells can also populate the heart, adopting a cardiomyogenic phenotype as a source of cardiomyocyte self-renewal. Our results show that SCF stimulates the regenerative potential of the heart tissue by inducing cardiomyocyte cell-cycle activation, undergoing DNA synthesis 1 week post-MI. It is possible that the significant increase of P-H3-, Ki67-, and Aurora B kinase synthesis 1 week post-MI may drive cardiomyocyte cell-cycle reentry after MI; and (4) the effects of SCF overexpression are still measurable 2 months after its administration, suggesting the existence of a feedback loop triggered by external stimuli, such as Wnt signaling, which could explain the long duration of the regenerative cardiac response after MI.

In summary, these results provide the proof that SCF gene therapy promotes cardiac regeneration in the injured myocardium and may offer a novel strategy for patients with ischemic cardiomyopathy, as well as other diseases in which c-kit+ stem cells have demonstrated use. The ability to induce cardiac repair with essential signaling molecules allows for a strategy to both dissect CPC niches effects and selectively induce cardiomyocyte cell-cycle reentry useful in myocardial regeneration treatments.

Acknowledgments

This paper is dedicated to Mikel Yaniz-Galende who taught us to be persistent and patient in pursuing our goals and who also inspired this study.

Sources of Funding

This work was supported by the National Heart, Lung, and Blood Institute as a Program of Excellence in Nanotechnology (PEN) Award, Contract #HHSN268201000045C; by Leducq Foundation through the network (Dr Hajjar) and by grants from the US National Institutes of Health: r01 HL093183, HL088434, HL071753, HL080498, HL083156, and P20HL100396 (Dr Hajjar).

Disclosures

None.

References


What Known?
• Stem cell factor (SCF), a c-kit receptor ligand, is involved in the proliferation and recruitment of several stem cells populations.
• Cardiac progenitor cells traffic to the injured myocardium after myocardial infarction (MI).
• Gene transfer and stem cell–based therapies have been successfully used in clinical trials, enhancing cardiac function after heart failure.

What New Information Does This Article Contribute?
• SCF therapy enhanced survival, left ventricle function, and cardiac regeneration after MI and was associated with a smaller infarct size, remodeling, and apoptosis.
• SCF administration promotes cardiac progenitor cell recruitment and cardiomyocyte cell-cycle reentry after MI through Wnt signaling activation.

Novelty and Significance
• Gene transfer and stem cell–based combination therapy have successfully induced cardiac regeneration in rats and may have the potential to promote tissue restoration in patients with heart failure.

Gen transfer and stem cell–based therapies have been developed to replace the loss of cardiac mass after heart failure. The current study demonstrates that SCF gene transfer has the potential to promote cardiac repair after MI through cardiac progenitor cells recruitment and cardiomyocyte cell-cycle reentry. Our results also provide evidence that local SCF gene transfer induces cardiac regeneration by enhancing survival and left ventricle function after MI, and by decreasing infarct size, hypertrophy, fibrosis, and apoptosis of existing cardiac cells, in the ischemic heart. These findings introduce a novel therapeutic approach for heart failure, through a combination of gene transfer and stem cell–based therapy.
Stem Cell Factor Gene Transfer Promotes Cardiac Repair After Myocardial Infarction via In Situ Recruitment and Expansion of c-kit⁺ Cells

Elisa Yaniz-Galende, Jiqiu Chen, Elie Chemaly, Lifan Liang, Jean-Sebastien Hulot, LaTronya McCollum, Teresa Arias, Valentin Fuster, Krisztina M. Zsebo and Roger J. Hajjar

_Circ Res._ 2012;111:1434-1445; originally published online August 29, 2012; doi: 10.1161/CIRCRESAHA.111.263830

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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METHODS

All procedures and animal care were approved by our institutional research committee and conformed the animal care guideline in Mount Sinai School of Medicine.

Generation of Recombinant Adenovirus Ad.SCF.

The Ad.SCF recombinant adenovirus was generated using the AdEasy XL Adenoviral Vector System (Stratagene). The full length of the SCF membrane isoform cDNA was cloned into the pShuttle-IRES-hrGFP2 vector. The linearized shuttle vector was recombined in Escherichia coli strain BJ5183 with AdEasy, a serotype 5 first-generation adenoviral backbone. The recombinant viral backbones were transformed into AD-293 cells and grown at large scale. Adenoviruses were purified by standard CsCl ultracentrifugation and desalting. Ad-EGFP-β-gal (Ad.β-gal) adenovirus was used as a control. Cardiac myocytes were infected with recombinant adenoviruses for 8 hours at a multiplicity of infection 50 for Ad.β-gal and 100 for Ad.SCF for an additional 24 to 48 hours to ensure transgene expression.

Gene transfer.

Male Sprague-Dawley rats were anesthetized with pentobarbital (60 mg/kg) by intraperitoneal injection and placed on a ventilator. The chest was accessed from the left side through the fourth intercostal space and the pericardium was incised. The left anterior descending coronary artery (LAD) was identified and encircled with a 7-0 silk suture at the apex of the left ventricle. The suture was briefly snared to confirm the ligation by blanching the arterial region. Ad.β-gal and Ad.SCF adenoviruses (200 µl at a concentration of ~5x10^11 pfu/ml) were injected into the peri-infarct zone after permanent LAD ligation.

Quantitative RT-PCR.

Total RNA (2 µg) was isolated with Trizol (Sigma-Aldrich) and transcribed to cDNA (Applied Biosystems). Gene expression was analyzed by quantitative PCR performed in an ABI PRISM Sequence Detector System 7500 (Applied Biosystems) with SYBR Green (Bio-Rad Laboratories) as fluorescent and ROX (Bio-Rad Laboratories) as reference dyes. Fragments were amplified for 40 cycles with the following specific primers:

- Notch1 forward: GTTTGTGCAAGGATGGTGTG
- Notch1 reverse: CCTTGAGGCATAAGCAGAGG
- Hox B4 forward: GCACGCTAACCCTCAATTA
- Hox B4 reverse: GGCAACTTGTGGTCTTTTTT
- Cyclin D1 forward: TGAGTCTGGCAGTCTTTG
- Cyclin D1 reverse: TCCTGGAGTCATTGGTAGC
**Gene transfer evaluation by X-gal staining.**

One week after LAD ligation and gene transfer, hearts were harvested, embedded in OCT and cryosectioned into 8 µm sections. β-gal/LacZ staining was determined to determine gene transfer expression.

**Histological analysis.**

Frozen heart blocks were cut into 8 µm sections and prepared for hematoxylin and eosin staining and immunohistochemistry. Paraformaldehyde-fixed cryostat heart sections were subjected to confocal microscopy for detection (and merged) images of c-kit and phosphohistone H3 (all from Cell Signaling), Ki67 and Aurora B kinase (all from Abcam) expression in cardiac tissue, using specific antibodies and Zeiss LSM510 META confocal microscope. Masson Trichrome (Sigma) and Picrosirius Red (Sigma) staining, and Sircol Collagen Assay (Biocolor) were conducted according to the manufacturer guidelines.

**Cardiomyocyte isolation.**

Rats were anesthetized with intraperitoneal Ketamine and Xylazine 50/5 mg/Kg IP. The chest was opened and the pericardium removed. The transverse aorta between the carotid arteries was cut and the isolated heart immediately cannulated via the aorta and perfused ex vivo to obtain cardiomyocytes.

**Cell culture.**

Primary cultures of adult cardiomyocytes from 2 month-old Sprague-Dawley rats (Charles River Laboratories) were prepared. After perfusing the heart, ventricular tissue was enzymatically digested with collagenase IV and dissociated. Cell suspensions were plated onto laminin-coated dishes and cultured in cardiac myocyte culture medium.

**Flow cytometry.**

The following antibodies were used for hematopoietic c-kit+ population: anti-c-Kit (2B8), -CD19 (1D3), -CD11b, -CD49b (DX5), -Ter-119, -CD3ε (145-2C11), and -Gr-1(RB6-8C5) (all from BD Pharmingen). Antibodies were directly coupled to phycoerythrin or allophycocyanin. For cardiac c-kit+ population characterization we used the following antibodies: anti-c-kit (2B8), -MEF2, -MEF2C, -Nkx2.5, -GATA4 and -α- sarcomeric actin (all from Abcam), and -CD20, -CD45, -P-GP (BD). c-kit+ population was gated and analyzed in a FACSCalibur cytometer (BD). For isolated cardiomyocytes population characterization we used Troponin T (TnT from abcam).

**Western blot.**

Cardiac tissue was minced and subsequently homogenized in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich). Protein extracts (10 µg) were separated on 10% SDS-PAGE and transferred onto PVDF membrane (Millipore). Antibody binding was visualized by ECL (Immoblilon Western, Chemiluminescent HRP Substrate, Millipore). Monoclonal antibodies used in western blotting included anti-c-kit, -GSK3β, -phospho-GSK3β (P-GSK3β), -β-catenin, -phosphohistone H3 (P-H3) and -CyclinD1 (all from Cell Signaling) and -PCNA, -collagen I, -collagen III and -TGBβ (all from Abcam). Protein loading was controlled by GAPDH (Sigma-Aldrich).
expression. Peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were employed.

**Echocardiography measurements.**

Echocardiography was performed while the rats were under ketamine sedation (60 mg/kg, i.p.). Heart rate was maintained at ≈400 bpm by adjusting ketamine sedation. Short-axis views were obtained using a 14 MHz GE-i1 3L probe as recommended by the American Society of Echocardiography. M-mode and bidimensional images were taken at the midpapillary level of the left ventricle from the parasternal short-axis views. The transducer position was carefully adjusted until the short-axis image of the LV cavity appeared to be circular, indicating perpendicular intersection beam with the long axis of the LV. LV wall thickness and cavity dimensions were measured in systole and diastole with M-mode echocardiography. Additional measurements were made to determine the ejection fraction, LV end-diastolic and LV end-systolic volumes using the area-length method.

**Survival.**

Survival analysis was performed in sham, controls (Ad.b-gal) and SCF-treated groups. During the study period (3 months), rats were inspected daily for deceased animals, examined for MI presence.

**Myocardial apoptosis.**

Frozen heart blocks were cut into 8 µm sections, paraformaldehyde-fixed and prepared for tunel assay (Millipore). Tunel positive-cardiac cell nuclei were counted and normalized per total nuclei. The proportion of apoptotic cells was counted in the peri-border area.

**Cardiac MRI.**

Cardiac MRI scans were performed in the same animals 2 days, 2 weeks and 1 month post-MI. Myocardium segmentation was performed among 7 LV short-axis slices outlining endocardial and epicardial borders in all cardiac frames. The end-diastolic volumes (EDV) and end-systolic volumes (ESV) were considered the largest and smallest areas, respectively, of the LV cavity in each slice. Apical and basal slices without semicircular muscular ring at either end-systole or end-diastole were disregarded and, without including papillary muscle and LV outflow tract. LV function and infarct volume was calculated by Segment software. Infarct area was defined as the brightest area on short-axis images with the largest area of hyperenhancement in both, end-systole and end-diastole frames.

**Statistical analysis.**

The data of this manuscript are expressed as mean ± SEM. Survival analysis was performed by Kaplan-Meier method. Statistical differences were analyzed using one-way ANOVA and pairwise comparisons were determined using Tukey’s honest significance test with P<0.01.
Online Figure I. SCF expression in the myocardium one week post-MI. A) Hematoxylin-eosin staining in the border area from SCF-treated section heart. B) Confocal image of the border area from Ad.SCF-treated section heart confirming SCF gene transfer. Blue, DAPI; green, SCF.
Online Figure II. SCF therapy reduces scar size after myocardium infarction. Representative images of SCF-treated and control hearts. The infarcted area is characterized by LV bleaching.
Online Figure III: SCF over-expression decreases infarct size. A) Short-axis cMRI images at 2 and 4 weeks post-MI. Infarct size was determined by hyperintensity bleached area of the scar tissue. B) Analysis of scar size at 2 weeks post-MI (15.3%±1.4% Ad.β-gal vs 8.6%±1.6% Ad.SCF; P<0.05) and 4 weeks post-MI (17.7%±0.3% Ad.β-gal vs 7.6%±1.2% Ad.SCF; P<0.01). C) Ejection fraction analysis by cMRI at baseline (2 days post-MI) (62.0%±0.5% Ad.β-gal vs 63.5%±1.2% Ad.SCF; P=0.31), at 2 weeks post-MI (48.7%±0.8% Ad.β-gal vs 56.5%±2.9% in Ad.SCF; P=0.06) and at 4 weeks post-MI (48.3%±0.4% in Ad.β-gal vs 58.4%±0.6% in Ad.SCF; P<0.01) post-MI.
Online Figure IV. Cardiac fibrosis is decreased 3 months post-MI after SCF therapy. A, B) Collagen content quantification through Sircol Collagen Assay, in the infarcted (A) (44.8%±4.1% in Ad.β-gal vs 27.8%±4.1% in Ad.SCF; p<0.05) and remote area (B) (26.2%±1.8% in Ad.β-gal vs 18.7%±2.0% in Ad.SCF; p<0.05). C, D) Quantification of TGFβ (C) and collagen III protein levels (D), from representative immunoblots in controls and SCF-treated rats at and 3 months post-MI, in the remote (R) and infarcted (I) area.
Online Figure V. Induction of cardiomyocytes cell cycle activation by SCF therapy. A) Cardiomyocytes co-expressing α-SA and P-H3 and BrdU or BrdU⁺ cells co-stained with vinculin were detected in the border area 1 week post-MI after SCF gene transfer.
Online Figure VI. SCF over-expression stimulates Aurora B expression 1 week post-MI.
Confocal images of representative sections from infarcted hearts at 1 week post-MI in control (Ad.β-gal) and Ad.SCF-treated rats. Blue, DAPI-stained nuclei; Green, Aurora B kinase; Red, α-SA-stained myocardium.
Online Figure VII. SCF over-expression reduces hypertrophy after MI. A) Analysis of left ventricular mass one week post-MI after SCF over-expression. Ratio of LVW/BW (A) and HW/BW (B). LVW, left ventricular weight; BW, body weight; HW, heart weight. C) Confocal images showing vinculin staining of cardiomyocytes cross-sections. Blue, DAPI; green, vinculin. D) Quantitative analysis of cardiomyocytes (CM) diameter in controls and SCF-treated hearts (*P<0.0001 vs Ad.β-gal).
Online Figure VIII. Cardiomyocytes isolation from the myocardium at 1 week post-MI. A) Bright field images from isolated cardiomyocytes at 1 week post-MI. B) Fluorescence images from isolated cardiomyocytes. Blue, DAPI-stained nuclei; green, α-sarcomeric actin-stained myocardium. C, Analysis by FACS of isolated cardiomyocytes stained with troponin T (cTNT).
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Online Table I. qPCR analysis of Notch 1, HoxB4 and cyclin D1 expression at 2 weeks post-MI.