Cardiac-Specific Overexpression of Human Stem Cell Factor Promotes Epicardial Activation and Arteriogenesis After Myocardial Infarction

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Background—The adult epicardium is a potential source of cardiac progenitors after myocardial infarction (MI). We tested the hypothesis that cardiomyocyte-specific overexpression of membrane-associated human stem cell factor (hSCF) enhances epicardial activation, epicardium-derived cells (EPDCs) production, and myocardial arteriogenesis post MI.

Methods and Results—Wild-type and the inducible cardiac-specific hSCF transgenic (hSCF/tetracycline transactivator) mice were subjected to MI. Wilms tumor-1 (Wt1)-positive epicardial cells were higher in hSCF/tetracycline transactivator compared with wild-type mice 3 days post MI. Arteriole density was significantly higher in the peri-infarct area of hSCF/tetracycline transactivator mice compared with wild-type mice 5 days post MI. In cultured EPDCs, adenoviral hSCF treatment significantly increased cell proliferation and growth factor expression. Furthermore, adenoviral hSCF treatment in wild-type cardiomyocytes significantly increased EPDC migration. These effects of hSCF overexpression on EPDC proliferation and gene expression were abrogated by ACK2, a neutralizing antibody against c-kit. Finally, lineage tracing using ROSA
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Key Words: heart failure ■ myocardial infarction ■ stem cell factor ■ stem cells ■ regeneration

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Cardiovascular disease remains a leading cause of death worldwide.1 In the past decade, adult stem cell therapy has emerged as a promising new strategy for ischemic heart disease.2 To this end, both cardiac and extracardiac progenitor cells have been demonstrated to improve cardiac function after myocardial infarction (MI).2 The epicardium-derived cells (EPDCs) are cardiac progenitors that contribute to coronary artery formation during embryonic heart development.3-5 Transplantation of adult EPDCs into the ischemic myocardium in mice preserves cardiac function and attenuates ventricular remodeling via enhancement of neovascularization, suggesting their therapeutic potential in cardiac repair post MI.8

Stem cell factor (SCF) binds to c-kit and promotes survival, proliferation, mobilization, and adhesion of all c-kit–expressing cells.18 Alternative splicing leads to 2 isoforms of SCF, the soluble and the membrane-associated isoforms, which differ in the absence or presence of a proteolytic cleavage site encoded by exon 6.19 The membrane-associated SCF is the dominant isoform in vivo and induces more persistent and long-term support of target cell survival. We have shown that cardiac-specific overexpression of membrane-associated human SCF (hSCF) improves animal survival and cardiac arteries with contributions from the endocardium and sinus venosus.6-11 Wilms tumor-1 (Wt1) is expressed in the EPDCs and promotes EMT,12 whereas its expression is turned off in fully differentiated EPDCs.13-15 The adult epicardium is inactive under normal physiological conditions, but can be reactivated and produces EPDCs after MI. Some of the EPDCs also express c-kit.16,17 However, the number of endogenously generated EPDCs is not sufficient for adequate cardiac repair post MI. Measures that promote epicardial activation and EPDC production may enhance cardiac repair post MI.
function post MI. However, the underlying cellular mechanisms require further investigation. In the present study, we hypothesized that inducible cardiomyocyte-specific overexpression of hSCF promotes epicardial activation and EPDC production post MI. To test this hypothesis, mice with cardiomyocyte-specific overexpression of hSCF were subjected to coronary artery ligation to induce MI. Epicardial Wt1 expression, myocardial growth factor levels, and arteriole density were studied. Furthermore, cell fate of Wt1 expressing epicardial cells post MI was determined using lineage tracing. Our results demonstrated that cardiomyocyte-specific overexpression of hSCF enhances epicardial activation and arteriogenesis post MI.

Methods

Experimental Animals
Animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). The conditional cardiac-specific overexpression of hSCF and tetracycline transactivator (tTA) double-transgenic (hSCF/tTA) mice were generated as described previously. The hSCF was expressed on the sarcolemma of cardiomyocytes in hSCF/tTA mice (Figure IA in the Data Supplement). Furthermore, soluble hSCF was not detectable in the serum of hSCF/tTA mice (Figure IB in the Data Supplement). In the doxycycline group, mice were treated with doxycycline (0.2 mg/mL in drinking water) to turn off hSCF overexpression post MI.21 In total, 68 wild-type (WT), 68 hSCF/tTA, and 24 Ad-hSCF or Ad-LacZ mice were generated as described previously.20 The hSCF was expressed on the sarcolemma of cardiomyocytes in hSCF/tTA mice (Figure IA in the Data Supplement). Furthermore, soluble hSCF was not detectable in the serum of hSCF/tTA mice (Figure IB in the Data Supplement). In the doxycycline group, mice were treated with doxycycline (0.2 mg/mL in drinking water) to turn off hSCF overexpression post MI.21,22 In total, 68 wild-type (WT), 68 hSCF/tTA, and 24 ROSA<sup>tm1Gor;Wt1<sup>c<sup>Cre<sub>ER</sub></sup></sup> mice (Stock No. 010912) were purchased from Jackson Laboratory and bred to generate ROSA<sup>tm1Gor;Wt1<sup>c<sup>Cre<sub>ER</sub></sup></sup> mice. These reporter mice were treated with tamoxifen (40 mg/kg, IP) for 5 days before surgery to trace the lineage of Wt1<sup>c</sup> cells in the myocardium post MI. To inhibit c-kit function, mice were injected intraperitoneally with 100 µg c-kit-neutralizing antibody ACK2 (a gift from Dr Shin-Ichi Nishikawa, Kyoto University, Kyoto, Japan) per day post MI.21 In total, 68 wild-type (WT), 68 hSCF/tTA, and 24 ROSA<sup>tm1Gor;Wt1<sup>c<sup>Cre<sub>ER</sub></sup></sup> mice were used for in vivo experiments. Each EPDC culture was from 12 to 16 hearts of postnatal day 0 (P0) or embryonic day 13.5 (E13.5) mice.

Myocardial Infarction
MI was induced by occlusion of the left main coronary artery. Briefly, mice were anesthetized using a ketamine (20 mg/kg) and xylazine (12.5 mg/kg) cocktail intramuscularly, intubated, and artificially ventilated. The adequacy of anesthesia was monitored by absence of withdrawal reflex to tail pinch. Subsequently, a left intercostal thoracotomy was performed and the left coronary artery was surgically ligated. To achieve in vivo adenosine infection, 10<sup>4</sup> transducing units of adenoviral vector – expressing recombinant hSCF (Ad-hSCF) or LacZ (Ad-LacZ) was injected directly into the peri-infarct myocardium immediately after coronary artery ligation. The infection efficiency was confirmed by Western blot analysis.

Epicardium-Derived Cell Culture
EPDCs were cultured as previously described.23 Hearts were removed from P0 or E13.5 mice, cut into pieces, and plated onto 1% gelatin-coated culture dish (epicardial side facing down) in Dulbecco's modified eagle medium containing 15% fetal calf serum for 6 to 9 days. EPDCs migrated from the heart explants were used for further experiments. Passage 1 EPDCs were used to assess cell proliferation. Each well of a 24-well plate was seeded with 10<sup>5</sup> EPDCs. Ad-hSCF or Ad-green fluorescent protein (Ad-GFP) was added at a concentration of 10 multiplicity of infection. After 24 hours, cells were trypsinized and counted by NucleoCounter (New Brunswick Scientific, Enfield, CT).

Neonatal Cardiomyocyte Culture
P0 mouse hearts were isolated and digested using Liberase (Roche, Canada). Cardiomyocytes were obtained and cultured in 24-well plates according to the methods described previously.24,25 To isolate c-kit cardiomyocytes, c-kit<sup>−</sup> cells were eliminated by c-kit antibody (Santa Cruz, CA)– conjugated magnetic beads (Invitrogen, CA). Overexpression of hSCF in WT neonatal cardiomyocytes was accomplished through the adenosivirional infection of Ad-hSCF. Viral vectors containing LacZ served as the controls. Cells were infected with adenoviruses at 10 multiplicity of infection for 4 hours and incubated for an additional 48 hours before further experiments.

Scratch Migration Assay
A scratch migration assay was performed using a modified protocol as previously described. Briefly, enhanced green fluorescence protein positive (EGFP<sup>+</sup>) EPDCs were passed into the wells (10<sup>4</sup> per well) containing cultured c-kit negative neonatal cardiomyocytes infected with Ad-hSCF or Ad-LacZ. After overnight seeding, a scratch was created with a p100 pipette tip. Cell debris was removed and culture medium was changed to serum-free Dulbecco's modified eagle medium. After 24 hours, fluorescent images were taken using a fluorescence microscope (Observer D1, Carl Zeiss, Germany), and percentage of scratch closure was quantified.

Transwell Migration Assay
A transwell system was used to investigate EPDC migration. Neonatal cardiomyocytes infected with Ad-hSCF or Ad-LacZ were cultured in the lower compartment. EGFP<sup>+</sup> EPDCs were isolated from EGFP transgenic mice (Stock No. 004353, Jackson Laboratory) at P0, and passage 1 cells (5x10<sup>5</sup>) were subcultured into the transwell cell culture inserts (8-µm diameter pores). After 24 hours of incubation in Dulbecco's modified eagle medium containing 1% fetal calf serum, cells on upper layer of the insert membrane were completely removed by a cotton swab. EGFP<sup>+</sup> EPDCs migrated to the other side of membrane were imaged using a fluorescence microscope (Observer D1, Carl Zeiss, Germany) and quantified.

Real-Time Reverse Transcription-Polymerase Chain Reaction
Total mRNA was isolated with TRIzol reagent (Invitrogen, Grand Island, NY) as previously described.26 cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers. Real-time polymerase chain reaction was conducted using SYBR Green PCR Master Mix (Eurogentec, San Diego, CA). 28S rRNA was used as a loading control. The oligonucleotide primer sequences were as follows: transforming growth factor (TGF)-β1, forward, CAC TGC TTC CCG AAT CTC GT, reverse, GCC CGA AGC GGA CTA CTA TG; soluble mouse SCF with exon 6, forward, GAT TGA GAC CCT GGT GGA CAT C, reverse, TCT TTC TGT T; basic fibroblast growth factor, forward, GAT TGA GAC CCT GGT GGA CAT C, reverse, TCT CCT ATG TGC TGG CTT TGG T; vascular endothelial growth factor A (VEGF-A), forward, GTT CGT TTC CTG GAG CTA CT, reverse, TGA AAT TCT TCT TCT TGT T; vascular endothelial growth factor B (VEGF-B), forward, TGC TGG GAG CTA AAA GGA CAT C, reverse, TCT CCT ATG TGC TGG CTT TGG T; basic fibroblast growth factor, forward, CAA GGG AGT GTG TGG TCG CAA CC, reverse, TGC CCA GTT CGT TTC AGT GC. Samples were amplified for 35 cycles using Eppendorf Mastercycler Real-Time PCR machine (Eppendorf, Hamburg, Germany). The mRNA levels in relation to 28S rRNA were determined using the comparative Ct method.

Immunohistochemistry
Paraffin sections of the heart were deparaffinized, hydrated, and rinsed in PBS. Antigen retrieval was achieved by microwaving the slides in 10 mmol/L sodium citrate buffer (pH 6.0) at 94°C for 12 minutes. After normal serum blocking, slides were incubated with primary antibodies followed by biotinylated secondary antibody. Color was developed using an ABC kit (Vector Laboratory, Burlingame, CA). Nuclei were counterstained with hematoxylin. Quantification of epicardial Wt1<sup>c</sup> cells was presented by epicardial Wt1<sup>c</sup> cell number divided by epicardial length. The nuclear size of epicardial cell
was measured by the shortest distance of nuclei. Arteriole number per millimeter squared and length density (mm/mm³) in the entire peri-infarct area were evaluated. Wheat germ agglutinin conjugated with Texas red (10 μg/mL, 30 minutes room temperature; Sigma, St Louis, MO) was used to stain cell membrane and epicardial cell surface area was quantified.

Frozen tissue sections and cells cultured on cover glass were fixed in 4% paraformaldehyde. Endogenous fluorescence was bleached by incubation in 0.3% H₂O₂ at room temperature overnight in a UV box. Primary antibodies were applied followed by fluorescent second antibodies. Nuclei were stained with Hoechst 33342. Images were taken using a fluorescence microscope (Observer D1, Carl Zeiss, Germany). Primary antibody used were Wt1 (1:300; Calbiochem, San Diego, CA); phosphorylated Smad2 (1:200; Cell Signaling Technology, Danvers, MA); smooth muscle actin (SMA, 1:1000; Sigma, St Louis, MO); GFP (1:2000) and hSCF (1:200) from Abcam, Cambridge, MA); epicardin (1:300), c-kit (1:300), and TGFβ receptor 2 (1:200) from Santa Cruz Biotechnology, Santa Cruz, CA.

Western Blot Analysis

Myocardial expression of hSCF in ROSA<sup>Wt1CreER</sup> mice after Ad-hSCF adenoviral infection was measured by Western blot analysis. Briefly, 40 μg of protein was separated by 12% SDS-PAGE gel and transferred to nitrocellulose membranes, and the blots were probed with antibodies against hSCF (1:500; Abcam, Cambridge, MA), TGFβ1 (1:500; Cell Signaling, Danvers, MA), or α-actinin (1:2500; Sigma, St Louis, MO). Blots were then washed and probed with horseradish peroxidase–conjugated secondary antibodies (1:3000; Bio-Rad, Hercules, CA), and detected by using an enhanced chemiluminescence detection method.

VEGF-A ELISA

VEGF-A protein expression in P0 EPDCs was determined by using VEGF-A ELISA kit (RAB0510, Sigma). Briefly, passage 1 EPDCs were infected with either Ad-hSCF or Ad-GFP for 48 hours. Cell lysate were harvested, and equal amount of proteins was loaded to an ELISA plate according to the manufacturer’s instructions. Color was developed using 3,3′,5,5′-tetramethylbenzidine, and absorbance was measured at 450 nm.

Statistical Analysis

Data were expressed as mean±SEM and analyzed using GraphPad Prism (version 3.0, La Jolla, CA). For data with multiple groups,
2-way ANOVA was performed followed by Bonferroni corrections, which were set for all possible pairwise comparisons. Wilcoxon rank-sum test was used to confirm findings from ANOVA. Results were not presented when they were agreeable with those from ANOVA. Unpaired Student t test was used for 2 group comparisons. A 2-tailed P value <0.05 was considered to be statistically significant.

Results

Epicardial Activation 3 Days Post MI

WT and hSCF/tTA mice were subjected to coronary artery ligation to induce MI. Three days after surgery, animals were euthanized and heart sections were immune-stained for Wt1, a specific marker for epicardial activation (Figure 1A). The number of Wt1+ cells in the epicardial layer was barely detectable in sham surgeries in WT and hSCF/tTA mice (0.36±0.05 versus 0.33±0.04 cells/mm; n=6 per group; P=ns). Three days post MI, the number of Wt1+ cells was increased to 5 to 10 cells/mm epicardium in WT mice (Figure 1A–1C). Furthermore, the population of Wt1+ cells in hSCF/tTA mice was almost doubled (18 cells/mm) in the epicardium of the infarct area (including the peri-infarct area) compared with WT (P<0.05; Figure 1B). Treatment with either doxycycline to turn off hSCF expression or ACK2 to block the SCF/c-kit signaling abrogated this response (P<0.05; Figure 1B). The number of Wt1+ cells was similar in the noninfarct area between hSCF/tTA and WT mice (Figure 1C). Also, few Wt1+ cells were observed in the myocardium post MI (Figure 1A) as Wt1 expression is turned off after EMT.13–15,31 Notably, the nuclear size of epicardial cells was significantly increased in the infarct area post MI compared with sham groups in both WT and hSCF/tTA mice (P<0.05; Figure II in the Data Supplement); however, no significant difference was found in their nuclear or cell size between WT and hSCF/tTA MI mice (Figure II in the Data Supplement). C-kit+ epicardial cells have been shown to have stem cell potential.16 Thus, we performed c-kit staining to investigate the c-kit+ epicardial cell population. Expression of SCF receptor c-kit was found in epicardial cells (Figure 1D). Overlapping signals between c-kit and a membrane marker wheat germ agglutinin suggest that c-kit receptors are expressed on the epicardial cell membrane (Figure III in the Data Supplement). The total number of epicardial c-kit+ cells per heart section was significantly increased post MI (P<0.05; Figure 1E) and was equal to ≈1% of epicardial cells (Figure 1F), which is consistent with previous studies.16 However, there was no significant difference between WT and hSCF/tTA mice (Figure 1E and 1F).

TGFβ1 is a key regulator of the EMT process. To assess its expression, TGFβ1 mRNA and protein levels in the epicardium and subepicardium of the peri-infarcted area were determined. Consistent with previous studies,32,33 we found that expression of TGFβ1 mRNA and protein were significantly higher post MI compared with sham groups (P<0.05; Figure 2A and 2B). Our data further showed that TGFβ1 expression in the epicardial and subepicardial tissues was

Figure 2. Myocardial transforming growth factor (TGF)-β expression and signaling in the epicardium 3 days post myocardial infarction (MI). A, There was significantly higher TGFβ1 mRNA expression in human stem cell factor/tetracycline transactivator (hSCF/tTA) mice compared with wild-type (WT) post MI. This difference was abrogated by doxycycline (DOX) and ACK2 treatment. B, There was also significantly higher TGFβ1 protein expression in hSCF/tTA mice compared with WT post MI. C, Representative images of Wilms tumor-1 (Wt1)/phosphor-Smad2 (P-Smad2) and Wt1/TGFβR2 staining in the epicardium 3 days post MI. P-Smad2 and TGFβR2 expression was observed in all epicardial Wt1+ cells. n=4 to 6 per group. *P<0.05 vs respective Sham, †P<0.05 vs WT MI.
significantly higher in the peri-infarct area of hSCF/tTA compared with WT mice \((P<0.05; \text{Figure 2A} \text{ and 2B})\), and the response was abolished by either doxycycline or ACK2 treatment \((P<0.05; \text{Figure 2A})\). As expected, Wt1+ epicardial cells were also positive for TGFβ receptor 2 \((\text{TGFβR2})\) and nuclear phosphor-Smad2 3 days post MI \((\text{Figure 2C})\), indicating activation of TGFβ1 signaling.

**Arteriole Density in Peri-Infarct Area 5 Days Post MI**

EPDCs are essential for coronary artery formation during embryonic development. To investigate the effects of enhanced epicardial activation on arteriogenesis post MI, SMA staining was used to stain smooth muscle cells, and the number of arterioles \((\text{size between 15 and 150} \ \mu\text{m})\) was quantified in the whole peri-infarct area. Length density of arterioles was also calculated to evaluate the arteriolar bed independent of vessel orientations. Representative photomicrographs of SMA staining are presented in Figure 3A. Each arrow is pointing to an arteriole. Arteriole density was similar 5 days after sham surgery between WT and hSCF/tTA mice \((\text{Figure 3B})\). However, 5 days post MI, arteriole number and length density in the peri-infarct area were significantly higher in hSCF/tTA compared with WT mice \((P<0.05; \text{Figure 3B} \text{ and 3C})\). Furthermore, the response was abrogated by doxycycline or ACK2 treatment \((P<0.05; \text{Figure 3B})\), suggesting SCF/c-kit signaling promotes myocardial arteriogenesis post MI.

**Outgrowth of Cultured EPDCs**

EPDCs can outgrow from P0 heart explants culture and form a monolayer of cobblestone-shaped cells \((\text{Figure IV A in the Data Supplement})\). The purity of EPDCs was confirmed by immunostaining of epicardin and Wt1, which are EPDC-specific markers \((\text{Figure IVB in the Data Supplement})\). Moreover, \(\approx3\%\) to \(5\%\) of the EPDCs were also positive for c-kit \((\text{Figure IVB in the Data Supplement})\). To study the effects of SCF on outgrowth of EPDCs, heart tissue sections from P0 WT mice were cultured and randomly divided into 2 treatment groups: control or recombinant mouse SCF \((100 \ \text{ng/mL})\). Six days after culturing, outgrowth of EPDCs from the heart tissue was observed \((\text{Figure 4A})\). Treatment with recombinant mouse SCF resulted in a significant higher EPDC outgrowth compared with controls \((P<0.05; \text{Figure 4B})\).

**Proliferation and Proangiogenic Growth Factor Expression of Cultured EPDCs**

Cultured EPDCs isolated from P0 hearts of WT mice were treated with Ad-hSCF or Ad-GFP. The expression of hSCF protein after Ad-hSCF infection was confirmed by Western blot analysis \((\text{data not shown})\). After 24 hours of treatment, EPDC cell numbers were significantly increased in the Ad-hSCF group compared with Ad-GFP control group \((P<0.05; \text{Figure 4C})\). Treatment with ACK2 \((10 \ \mu\text{g/mL})\) significantly decreased EPDC cell numbers in both Ad-hSCF and Ad-GFP groups \((P<0.05; \text{Figure 4C})\). To investigate proangiogenic growth factor expression, cultured EPDCs from P0 hearts
of WT mice were treated with Ad-GFP or Ad-hSCF for 24 hours. VEGF-A and basic fibroblast growth factor mRNA levels were significantly increased in the Ad-hSCF group compared with Ad-GFP control group (P<0.05; Figure 4D and 4E). Treatment with ACK2 (10 μg/mL) abrogated this effect (Figure 4D and 4E). Furthermore, overexpression of hSCF significantly increased VEGF-A mRNA expression in EPDCs as determined by ELISA (P<0.05; Figure 4F). Interestingly, significantly more c-kit+ EPDCs were observed after the overexpression of hSCF, which may contribute to the enhanced EPDC proliferation and proangiogenic growth factor expression (P<0.05; Figure V in the Data Supplement).

Migration of Cultured EPDCs

To study the direct effect of hSCF overexpression in cardiomyocytes on EPDC migration, a scratch migration assay was performed in cardiomyocyte and EPDC cocultures. EGFP+ EPDCs were cocultured overnight with c-kit neonatal cardiomyocytes infected with Ad-LacZ or Ad-hSCF. A scratch was then induced (Figure 5A). Twenty-four hours later, a significantly higher scratch closure ratio by EGFP+ EPDCs was observed after the overexpression of hSCF in cardiomyocytes directly promotes EPDC migration in the coculture.

A transwell assay was also used to study the effect of hSCF overexpression on EPDC migration.27 EGFP+ EPDCs were seeded on the upper inserts with 8-μm pores. Neonatal cardiomyocytes infected with either Ad-LacZ or Ad-hSCF were seeded on the lower chamber. Cells were allowed to migrate for 24 hours, and the number of EGFP+ EPDCs, which had migrated through the insert membrane, was detected under fluorescent microscopy. As shown in Figure VI in the Data Supplement, the green cells are EGFP+ EPDCs, which had migrated through the membrane. Quantitative analysis showed that Ad-hSCF significantly increased EPDC migration compared with Ad-LacZ (P<0.05; Figure 5C). Furthermore, inhibition of c-kit signaling by ACK2 (10 μg/mL) significantly attenuated EPDC migration in both Ad-LacZ and Ad-hSCF treatment groups (P<0.05; Figure 5C). The data indicate that EPDC migration is enhanced by chemokine release from the Ad-hSCF–treated neonatal cardiomyocytes in the lower chamber.

The c-kit+ progenitors exist in the neonatal heart.34,35 Activation of progenitor cells leads to release of multiple cytokines, growth factors, and chemokines, including soluble SCF.36 Thus, we measured soluble mouse SCF in neonatal cardiomyocytes treated with Ad-hSCF or Ad-LacZ. As expected, soluble mouse SCF expression was significantly higher in hSCF overexpressing cardiomyocytes compared with Ad-LacZ group (P<0.05; Figure 5D), which was abrogated by ACK2 treatment (P<0.05; Figure 5D).

Lineage Tracing of Wt1+ EPDCs

EPDC activation and migration into the myocardium post MI was further investigated by lineage tracing of Wt1+ EPDCs.
Cre activation was induced by tamoxifen injection for 5 days before MI in ROSA<sup>cmTmG</sup>;Wt1<sup>CreER</sup> mice (Figure 6A). Five days post MI, 70% to 80% of the Wt1<sup>+</sup> cells expressed EGFP, whereas all EGFP<sup>+</sup> cells in the epicardium were Wt1<sup>+</sup> (data not shown). Myocardial overexpression of hSCF in ROSA<sup>cmTmG</sup>;Wt1<sup>CreER</sup> mice was induced by intramyocardial injection of Ad-hSCF in the peri-infarct area immediately after coronary artery ligation. The expression of hSCF protein in the peri-infarct area 5 days post MI was detected by Western blot analysis (Figure VII in the Data Supplement). EGFP<sup>+</sup> cells representing EPDCs derived from Wt1-expressing cells were found in the epicardium 5 days post MI (Figure 6B), which is consistent with our findings in Figure 1A and 1B. Importantly, EGFP<sup>+</sup> cells were also observed in the infarcted myocardium (Figure 6C), providing direct evidence that Wt1<sup>+</sup> EPDCs are activated and migrated into the infarct area. Furthermore, treatment with Ad-hSCF significantly increased the number of EGFP<sup>+</sup> cells in the epicardium and infarcted myocardium compared with Ad-LacZ (P<0.05; Figure 6D and 6E), which was abrogated by ACK2. Additionally, Ad-hSCF increased epicardial expression of TGFβ, a key factor in promoting EMT, and the effect was also abrogated by ACK2 treatment (Figure 6F).

**Wt1<sup>+</sup> EPDC Differentiation**

EPDCs contribute to coronary artery formation during embryonic heart development mainly by differentiating into vascular cells. To demonstrate the ability of EPDCs to differentiate, epicardial cells were cultured from hearts of ROSA<sup>cmTmG</sup>;Wt1<sup>CreER</sup> mice at P0. Treatment with tamoxifen (200 nmol/L) in the cultured ROSA<sup>cmTmG</sup>;Wt1<sup>CreER</sup> EPDCs showed 60% to 70% of Cre activation, resulting in EGFP expression in Wt1<sup>+</sup> EPDCs (Figure VIII A in the Data Supplement). The Wt1<sup>+</sup> EPDCs were also differentiated into SMA<sup>+</sup> cells (Figure VIII B in the Data Supplement). So our next aim was to determine the lineage of Wt1-expressing cells in the infarct area post MI in ROSA<sup>cmTmG</sup>;Wt1<sup>CreER</sup> mice after tamoxifen treatment. As shown in Figure 7A, some of the EGFP<sup>+</sup> cells in the infarct area were also positive for SMA, a marker for smooth muscle cells and myofibroblasts, indicating that Wt1<sup>+</sup> EPDCs are able to differentiate into smooth muscle cells or myofibroblasts. Furthermore, treatment with Ad-hSCF significantly increased the total number of EGFP/SMA double-positive cells (Figure 7B) and total EGFP<sup>+</sup> cells (Figure 7C). However, the percentage of EGFP<sup>+</sup>/SMA<sup>+</sup> cells to total EGFP<sup>+</sup> cells (34%) in the infarcted myocardium was similar between Ad-LacZ– and Ad-hSCF–treated groups (Figure 7D), indicating Ad-hSCF treatment does not affect EPDC differentiation. Additionally, no SMA<sup>+</sup> cells from the Wt1<sup>+</sup> cell lineage were detected in any vessels in the peri-infarct area. Consistent with previous studies, more than half of the EGFP<sup>+</sup> cells in the infarct area were also positive for fibroblast marker FSP1 (Figure IX in the Data Supplement).

**Discussion**

The present study demonstrates for the first time that cardiomyocyte-specific expression of membrane-associated hSCF promotes epicardial activation post MI. The hSCF transgene expression increases epicardial Wt1<sup>+</sup> EPDCs and arteriogenesis post MI. These effects are specific to membrane-associated hSCF because they are abrogated on treatment with doxycycline, which turns off hSCF expression on the membrane of cardiomyocytes. Moreover, through Wt1 lineage tracing, we showed that hSCF overexpression via adenoviral infection enhances EPDC activation and migration into the infarct area post MI through the c-kit signaling pathway. Furthermore, hSCF stimulates EPDCs to express VEGF and basic fibroblast...
growth factor, which are important growth factors in arteriogenesis. These data show that hSCF overexpression enhances epicardial activation, EPDC production, and arteriogenesis post MI. We recently reported that cardiac-specific hSCF overexpression improves cardiac function and survival post MI. The present study provides further insight into the cellular and molecular mechanisms of hSCF to improve cardiac repair post MI.

The adult epicardium can be reactivated and express Wt1 post MI. In the present study, epicardial activation as determined by Wt1 positivity was observed 3 days post MI, which is consistent with previous studies. Our novel finding is that overexpression of hSCF significantly enhanced the epicardial activation post MI, and the response was abrogated by inhibiting c-kit receptor function, suggesting an important role of SCF/c-kit signaling in epicardial activation. TGFβ1, one of the paracrine factors produced by stem/progenitor cells, has been shown to promote EMT in both embryonic and adult EPDCs. A significant increase of TGFβ expression was found in the epicardium of hSCF

Figure 6. Lineage tracing of Wt1+ cell–derived epicardium-derived cells (EPDCs) 5 days post myocardial infarction (MI). A, Experimental outline for genetic fate mapping in the adult heart using ROSA<sup>loxP</sup>Wt1<sup>CreERT</sup>Rosa26-mTmG mice. Representative images of EGFP staining in the epicardium (B) and infarcted myocardium (C) 5 days post MI. Quantification of EGFP<sup>+</sup> cells in the epicardium (D) and infarcted myocardium (E). The number of Wt1<sup>+</sup> cell–derived EPDCs was significantly higher in the epicardium and infarcted myocardium in the adenoviral human stem cell factor (Ad-hSCF)–treated group compared with Ad-LacZ 5 days post MI. ACK2 treatment abolished the difference in EGFP<sup>+</sup> cells in the heart. F, Transforming growth factor (TGFβ) mRNA expression determined by real-time reverse transcription–polymerase chain reaction. In vivo Ad-hSCF treatment in the myocardium significantly increased TGFβ mRNA expression post MI. This increase was abrogated by ACK2. n=6 per group. *P<0.05 vs Ad-LacZ MI, †P<0.05 vs Ad-hSCF MI.
overexpressing mice post MI, and the effect was attenuated by doxycycline treatment to turn off hSCF expression or ACK2 treatment to inhibit c-kit receptor function, which suggests that activation of SCF/c-kit signaling enhances TGFβ expression. Furthermore, TGFβR2 expression and nuclear Smad2 phosphorylation was observed in epicardial Wt1+ cells, suggesting activation of TGFβ signaling in Wt1+ epicardial cells post MI. Expression of TGFβR2 was not observed in c-kit+ epicardial cells through fluorescent double staining (Figure X in the Data Supplement), indicating that TGFβ may not directly act on c-kit+ epicardial cells.

The embryonic epicardium contributes to coronary artery formation.4,41 To assess the effects of enhanced epicardial activation by cardiac-specific overexpression of hSCF on arteriogenesis post MI, myocardial arteriole density was determined. Our data showed that hSCF overexpression significantly increased arteriole density in the peri-infarct area, suggesting a potential contribution of epicardial activation to arteriogenesis post MI. To further investigate the effect of SCF on EPDC activity, overexpression of hSCF was induced in cultured EPDCs. Our data showed that the ability of cultured EPDCs to proliferate and migrate was significantly enhanced by hSCF overexpression via c-kit signaling. Moreover, expression of VEGF-A and basic fibroblast growth factor, 2 well-known proangiogenic growth factors involved in arteriogenesis,42,43 was upregulated through the SCF/c-kit pathway, suggesting an enhanced paracrine effect of EPDCs. Although only 5% of cultured EPDCs and ≈20,000 cells (or 0.65%) in the neonatal heart express c-kit,34,35 activation and expansion of those cells by hSCF may promote activation of many neighboring EPDCs by paracrine factors, which may further initiate signaling pathways to induce cell proliferation and migration. Additionally, the membrane-anchored hSCF on cardiomyocytes infected with Ad-hSCF also directly promotes EPDC migration as shown by a scratch migration assay.

To trace cell fate of the activated epicardium in vivo, a ROSA26R;Wt1CreERT2 mouse was generated, in which Cre
recombinase expression is inducible by tamoxifen treatment and Wt1 expression cells and their progeny will be EGFP+. Myocardial hSCF overexpression was achieved by intramyocardial injection of adenoviral hSCF constructs. Consistent with what we found in the hSCF/tTA mice, overexpression of hSCF by adenoviral infection enhanced epicardial activation post MI in ROSAmTmG;Wt1CreER mice as shown by a significantly higher number of EGFP+ epicardial cells. A hallmark of activated EPDCs is their ability to undergo EMT and migrate into the myocardium.17 Zhou et al37 also investigated the migration of EPDCs using the same lineage tracing system. However, the EGFP+ EPDCs remained in the epicardial region and did not migrate into the myocardium. This is not surprising as their study was specifically designed to trace Wt1+ epicardial cells before the onset of MI. In the present study, we used a tamoxifen treatment protocol to turn on Cre recombinase activity at the peak of epicardial Wt1 expression and were able to see EGFP+ cells in the infarcted myocardium 5 days post MI. More importantly, hSCF overexpression significantly increased the number of EGFP+ cells, which had migrated into the myocardium. A significant increase of TGFβ mRNA expression was also observed post MI in the epicardium of Ad-hSCF–treated ROSAmTmG;Wt1CreER mice. Furthermore, increases in EPDC migration and TGFβ expression were abrogated by the inhibition of c-kit signaling. Together, these data show that activation of SCF/c-kit signaling promotes EMT of Wt1+ EPDCs and their migration into the infarcted myocardium via upregulation of TGFβ.

After EMT and migration into the myocardium during embryonic heart development, EPDCs differentiate into several cellular lineages, including cardiomyocytes, endothelial cells, smooth muscle cells, and interstitial fibroblasts.3,4,31 Although the adult epicardium can be reactivated post MI, cell fate of EPDCs remains to be determined. Through lineage tracing of Wt1+ EPDCs, we showed that the total number of EPDCs and their SMA+ derivatives were significantly increased by hSCF overexpression. However, their commitment to smooth muscle and myofibroblast lineages 5 days post MI was not altered by hSCF overexpression (Figure 7D). The SMA+ cell lineage was mainly found in the infarct area, whereas arteriogenesis was predominantly in the peri-infarct area. The fact that not a single artery is derived from the EPDC lineage suggests that the increased arteriogenesis by hSCF overexpression is mainly caused by paracrine effects. Of interest, lineage commitment of EPDCs post MI may be partially modified. To this end, Smart et al38 successfully induced 0.6% of Wt1+ EPDCs differentiation into structurally coupled cardiomyocytes using thymosin β4. However, this observation was not reproduced by Zhou et al.44 In the present study, we did not observe any effects of hSCF overexpression on cardiomyocyte generation from the EPDCs. Most of the activated EPDCs differentiate into fibroblasts in the infarcted myocardium.

In summary, the present study shows that cardiac-specific hSCF overexpression enhances epicardial activation, EPDC production, growth factor expression, and arteriogenesis post MI (see Figure 8 for the proposed mechanism). These effects may contribute to a significant improvement in cardiac function.
function and survival in hSCF/TA mice post MI.20 Our study provides novel insight in EPDC activation post MI and suggests that cardiac-specific hSCF overexpression may have therapeutic potential in promoting cardiac repair post MI. Further translational studies are required to investigate the effects of hSCF by cardiac-specific gene therapy in large animal models of MI and in patients after acute MI.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

Loss of myocardium and blood vessels after myocardial infarction (MI) results in scar formation and cardiac remodeling, leading to heart failure. Regeneration of the vascular system is critical to cardiac repair post MI. During embryonic heart development, epicardial-derived cells (EPDCs) contribute to coronary artery formation. In the adult heart, the epicardial cells can be reactivated post MI. However, the number of endogenously activated EPDCs is limited to efficiently repair the injured myocardium. We have previously shown that inducible cardiomyocyte-specific overexpression of membrane-associated human stem cell factor (hSCF) improves cardiac function post MI via myocardial angiogenesis. In the present study, we aim to investigate the effects of cardiomyocyte-specific overexpression of hSCF on EPDC activation and arteriogenesis after MI. We demonstrated that cardiomyocyte-specific hSCF overexpression increased EPDC activation and promoted myocardial arteriogenesis after MI. These beneficial effects are likely caused by enhanced activation, proliferation, and migration of EPDCs and release of paracrine factors by hSCF overexpression. This study highlights a therapeutic potential of hSCF in the treatment of MI and heart failure.
Cardiac-Specific Overexpression of Human Stem Cell Factor Promotes Epicardial Activation and Arteriogenesis After Myocardial Infarction
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Supplemental Figure Legends:

**Suppl. Figure 1**: Expression of hSCF protein in hSCF/tTA mice. A. hSCF staining showed the expression of hSCF was located on sarcolemma of cardiomyocytes in hSCF/tTA mice and was turned off by DOX treatment. A WT heart served as a negative control. B. In the serum of hSCF/tTA mice, S- hSCF was not detectable. Heart tissues from hSCF/tTA mice served as positive controls. MA-hSCF, membrane-associated hSCF; S-hSCF, soluble hSCF.

**Suppl. Figure 2**: Cell and nuclear size of epicardial cells. Three days after MI or sham surgery, nuclear size and cell surface area of epicardial cells was quantified. A. Significantly larger epicardial nuclei were observed in the MI compared to sham animals. No significant difference was found between WT and hSCF/tTA mice. Two-way ANOVA followed by Bonferroni corrections: *P<0.05 vs respective sham. B. No significant difference of epicardial cell surface area was found between WT and hSCF/tTA mice. Unpaired Student *t*-test: P=n.s. Data are mean ± SEM, n=3-4 per group.
**Suppl. Figure 3:** Identification of $c$-$kit$ receptor on epicardial cell membrane. Representative images show that $c$-$kit$ (green) receptors are located on the cell membrane (WGA, red) of $c$-$kit^+$ epicardial cells.

**Suppl. Figure 4:** *In vitro* culture of EPDCs. A. 6 days after heart explants culture, cobblestone shaped cell monolayer was observed surrounding the heart explants. B. Cobblestone shaped cells outgrown from the heart explants were 100% positive for epicardin (nuclei: blue, epicardin: red) and Wt1 (nuclei: blue, Wt1: red). Some EPDCs were also positive for $c$-$kit$ (nuclei: blue, $c$-$kit$: red).

**Suppl. Figure 5:** Overexpression of hSCF increases $c$-$kit^+$ EPDCs *in vitro*. A. Representative images of cultured EPDCs 24 hours after Ad-hSCF or Ad-LacZ infection. $C$-$kit$ was stained in green. B. Quantification of $c$-$kit^+$ EPDC percentage. Overexpression of hSCF significantly increased the $c$-$kit^+$ EPDC percentage *in vitro*. Data are mean ± SEM, n=5 per group. Unpaired Student $t$-test: $^*P<0.05$ vs Ad-LacZ.

**Suppl. Figure 6.** Transwell EPDC migration. EGFP$^+$ EPDCs were seeded on the upper inserts with 8 µm pores. Neonatal cardiomyocytes infected with either Ad-LacZ or Ad-hSCF were seeded on the lower chamber. Cells were allowed to migrate for 24 hours in 1% fetal calf serum and the number of EGFP$^+$ EPDCs migrated through the insert membrane was detected under fluorescent microscopy. Shown are representative images of EGFP$^+$ EPDCs migrated through the transwell membrane.
Suppl. Figure 7: Expression of hSCF protein in adenovirus treated hearts. Heart tissues from ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} mice injected with Ad-hSCF or Ad-LacZ were subjected to western blot analysis. Expression of hSCF protein was observed only in Ad-hSCF treated hearts.

Suppl. Figure 8: In vitro lineage tracing of Wt1\textsuperscript{+} cell derived EPDCs. A. Representative images of EPDCs from P0 ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} or ROSA\textsuperscript{mTmG} mice treated with tamoxifen (200 nM). EGFP\textsuperscript{+} cells (green) were observed only in EPDCs from the ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} hearts. B. Representative images of passage 1 EPDCs from ROSA\textsuperscript{mTmG};Wt1\textsuperscript{CreER} hearts stained with EGFP and SMA (nuclei: blue, SMA: red, EGFP: green).

Suppl. Figure 9: Fate mapping of Wt1\textsuperscript{+} cell-derived EPDCs post-MI. A. Representative images of EGFP and FSP1 staining of Wt1-derived EPDCs in the infarcted myocardium. The EGFP\textsuperscript{+} cells are also positive for FSP1. B. Percent FSP1\textsuperscript{+}/EGFP\textsuperscript{+} to total EGFP\textsuperscript{+} cells were similar between Ad-hSCF and Ad-LacZ treatment, both around 50%. Data are mean ± SEM, n=6 per group. Unpaired student t test: \( P=\text{n.s.} \)

Suppl. Figure 10: Representative images of c-kit and TGFβR2 double staining of epicardial cells post-MI. Upper panel: A c-kit\textsuperscript{+} cell (green) is shown in the peri-infarct area of the epicardium. Lower panel: TGFβR2\textsuperscript{+} cells (red) are seen in the epicardium and subepicardium of the infarcted area. No c-kit and TGFβR2 double positive epicardial cells were found in WT or hSCF/tTA mice (n=4 per group). Nuclei, blue.
Suppl. Figure 2

A

Nuclear size of epicardial cells (μm)

WT hSCF/tTA

Sham MI

B

Epicardial cell size (μm²)

WT hSCF/tTA

Suppl. Figure 2
Suppl. Figure 3
Suppl. Figure 4

A

B  Epicardin  Wt1  c-kit

10 µm
Suppl. Figure 5
Suppl. Figure 6
Suppl. Figure 7
Suppl. Figure 8

A

ROSA\(^{mTMG}\)

ROSA\(^{mTMG; Wt1 CreER}\)

B

Hoechst

EGFP

SMA

Merge

20 μm
A

Hoechst  EGFP

Fsp1  Merge

Suppl. Figure 9

20 μm

B

Suppl. Figure 9